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► To cite this version:

Daria D Novikova, Anastasia L Korosteleva, Victoria Mironova, Yvon Jaillais. Meet your MAKR: the membrane-associated kinase regulator protein family in the regulation of plant development. *FEBS Journal*, 2021, 10.1111/febs.16132 . hal-03349749

HAL Id: hal-03349749

<https://hal.science/hal-03349749v1>

Submitted on 20 Sep 2021

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Title: Meet your MAKR: the MEMBRANE ASSOCIATED KINASE REGULATOR protein family in the regulation of plant development

Running title: MAKR function in plants

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Abbreviations.

MAKR1 (AT5G26230), MEMBRANE ASSOCIATED KINASE REGULATOR1; MAKR2 (AT1G64080), MEMBRANE ASSOCIATED KINASE REGULATOR2; MAKR3 (AT2G37380): MEMBRANE ASSOCIATED KINASE REGULATOR3; MAKR4 (AT2G39370): MEMBRANE ASSOCIATED KINASE REGULATOR4; MAKR5 (AT5G52870): MEMBRANE ASSOCIATED KINASE REGULATOR5; MAKR6 (AT5G52900): MEMBRANE ASSOCIATED KINASE REGULATOR6; BKI1 (AT5G42750): BRI1 KINASE INHIBITOR1; BRs: brassinosteroids; BRI1: BRASSINOSTEROID INSENSITIVE 1; BRL: BRI1-LIKE; BAK1: BRI1-ASSOCIATED RECEPTOR KINASE 1; SERK3: SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3; BIP119: BRI1 INTERACTING PROTEIN 119; CLE45: CLAVATA 3/EMBRYO SURROUNDING REGION 45; BRX: BREVIS RADIX; BAM: BARELY ANY MERISTEM; PIN2: PIN-FORMED 2; TMK1: TRANSMEMBRANE KINASE 1; ROP: RHO OF PLANTS; LBD16: LATERAL ORGAN-BOUNDARIES DOMAIN 16; QC: Quiescent Center; TF: Transcription Factors; HCA2: HIGH CAMBIAL ACTIVITY 2; DAP-seq: DNA affinity purification sequencing; OBP3: OBF-BINDING PROTEIN 3; HB33: HOMEBOX PROTEIN 33; BBX31: B-BOX DOMAIN PROTEIN 31; ARF: AUXIN RESPONSE FACTOR; DOF6: DOF TRANSCRIPTION FACTOR6; BIM: BES1-INTERACTING MYC-LIKE PROTEINS; BEH: BES1/BZR1 HOMOLOGS

Keywords. Kinase regulator, MAKR, BKI1, receptor-like kinase, signaling pathway, hormone, brassinosteroids, auxin, BRI1, intrinsically disordered proteins

Conflicts of Interest. The authors declare no conflict of interest.

Abstract

A small family composed of BRI1 KINASE INHIBITOR1 (BKI1) and MEMBRANE ASSOCIATED KINASE REGULATORS (MAKRs) has recently captured the attention of plant biologists, due to their involvement in developmental processes downstream of hormones and Receptor-Like Kinases (RLK) signaling. BKI1/MAKRs are intrinsically disordered proteins (so-called unstructured proteins) and as such lack specific domains. Instead, they are defined by the presence of two conserved linear motifs involved in the interaction with lipids and proteins, respectively. Here, we first relate the discovery of the *MAKR* gene family. Then, we review the individual function of characterized family members and discuss their shared and specific modes of action. Finally, we explore and summarize the structural, comparative, and functional genomics data available on this gene family. Together, this review aims at building a comprehensive reference about BKI1/MAKR protein function in plants.

Introduction

The MEMBRANE ASSOCIATED KINASE REGULATOR (MAKR) family is comprised of seven members in *Arabidopsis thaliana* (*Arabidopsis*), including BRI1 KINASE INHIBITOR 1 (BKI1) and MAKR1 to MAKR6 [1]. The common unifying similarity between all the proteins from the MAKR family across all plant species is that they are intrinsically disordered (also known as unstructured). This means that they do not have any predicted folded domains over their entire length. Unstructured proteins (or unstructured regions in proteins) often rely on linear motifs for function. These linear motifs are short stretches of amino acids that are involved in molecular interactions, for example with other proteins, metabolites, or lipids. They are linear in the sense that the particular three-dimensional organization of the protein is not required to make these motifs functional. They can themselves be completely disordered, but the motif can also have local secondary structures (e.g. alpha helix) or the protein may fold upon specific interaction with additional molecules. Fully unstructured proteins are often rapidly evolving, since they are made of few conserved linear motifs buried in additional sequences with little functional relevance and thus low complexity and poor conservation.

What allows to classify MAKR proteins as belonging to the same family, is the conserved presence of two linear motifs within their sequence. The founding member of the family is BKI1. It is a plant specific protein, which was originally thought to be a unique gene in plant genomes [2]. However, structure-function analyses uncovered important functional motifs within BKI1, which turned out to be present in additional *Arabidopsis* proteins and led to the

discovery of the MAKR family (**Figure 1A**). Below, we review how BKI1 was historically identified and how its functional characterization led to the identification of the MAKR family.

Main sections

BKI1 and Brassinosteroids Signalling

BKI1, the founding member of the MAKR family, serves as a negative regulator of the brassinosteroids' (BRs) signal transduction pathway [2,3]. BRs are a class of steroid hormones mediating developmental responses to environmental cues. BRs are perceived at the plasma membrane by a receptor complex, which consists of a pair of RLK composed of two small gene families: BRASSINOSTEROID INSENSITIVE 1 (BRI1)/BRI1-LIKE (BRL1 and BRL3) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1)/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (SERK3) and related SERK family members [3]. BKI1 was originally found in a phage display assay as BRI1 INTERACTING PROTEIN119 (BIP119), a Rice protein that could bind to and be phosphorylated by the isolated kinase domain of the Rice BRI1 [4]. Wang and Chory (2006) later found an Arabidopsis BIP119 ortholog in a yeast two-hybrid screen against AtBRI1 kinase domain, which they coined BRI1 KINASE INHIBITOR1 [2]. They confirmed that the Arabidopsis BKI1 interacts with BRI1 *in vitro* and *in vivo* and, like BIP119 in rice, could also be phosphorylated by the BRI1 kinase *in vitro* [2]. At the resting state (i.e. in the absence of brassinosteroid treatment), an overexpressed BKI1-YFP fusion localizes at the plasma membrane (**Figure 1B, C**) and induces a constitutive inhibition of the brassinosteroid pathway. However, following brassinosteroid treatment, BKI1-YFP is displaced from the plasma membrane into the cytosol (**Figure 1B, C**). This dynamics is vital to BKI1 function, as keeping BKI1 constitutively anchored at the cell surface leads to a strong and long lasting inhibition of the brassinosteroid pathway [2].

As stated above, BKI1 was an unknown protein with no functional signature in its sequence. The work from Wang and Chory thus raised several questions on how BKI1 could regulate brassinosteroid at the molecular level, including: 1) how is BKI1 interacting with BRI1? 2) How is it inhibiting brassinosteroid signaling, 3) How is it targeted to the plasma membrane? and 4) how is membrane interaction dynamically regulated upon brassinosteroid signaling?

The answers to these questions lie, at least in part, in the linear motifs present within BKI1. Indeed, three main linear motifs were identified within BKI1. The first linear motif is present in roughly the middle of the protein. It is a membrane targeting motif that was coined the "membrane hook" and that consists of a repetition of four doublets of arginine and lysine

residues ([KR][KR]-repeat) (**Figure 1A**) [1]. Upon mutation of individual [KR][KR] doublets into alanine, the targeting of BKI1 to the plasma membrane is impacted, with BKI1 being present at the plasma membrane but also in intracellular compartments. Mutation of more than two [KR][KR] doublets leads to a full relocalization of the mutant protein into the cytosol (**Figure 1C**). The second linear motif is a ~20-residue long alpha helix near BKI1 C-terminus called the C-terminal tail (**Figure 1A**) [1,5]. This peptide is necessary and sufficient for interaction with BRI1 kinase domain *in vitro* and *in vivo* [1]. The third linear motif consists of 14-3-3 binding sites located between the membrane hook and the C-terminal tail (**Figure 1A**) [6]. Linear motifs are often the target for post-translational modifications, including phosphorylation, and BKI1 is no exception. Indeed, both the membrane hook and the 14-3-3 binding sites are phosphorylated by BRI1, on residues Y211 and S270/S274, respectively (**Figure 1A**) [1,6]. In particular, BKI1 is phosphorylated on a conserved tyrosine (Y211) residue within the membrane hook, which triggers membrane dissociation (**Figure 1A-C**) [1].

Although it was renamed BRI1 KINASE INHIBITOR1, BKI1 is not a kinase inhibitor *sensu stricto*. Indeed, *in vitro* experiments showed that BRI1 is a perfectly active kinase in the presence of BKI1, which does not bind to the active site or compete with ATP [1,5]. This would explain how BRI1 is able to phosphorylate BKI1 when the two proteins are interacting. Instead, BKI1 C-terminal tail binds at the back of BRI1 kinase domain, on its C-lobe, away from the active site [1,3,5]. However, BKI1 inhibits the formation of a stable heterodimer between BRI1 and its co-receptor BAK1 (**Figure 1B**) [1,2]. One possible mechanistic explanation, which has remained so far untested, is that the BAK1 kinase interacts with the BRI1 kinase on its C-lobe [1,3]. In such a scenario, BKI1 would prevent the two kinases to stably interact by competing for the same binding interface on BRI1. Binding of BR by BRI1 triggers the interaction between the extracellular domains of the two receptors, bringing their kinase domains in close proximity [7]. BAK1 may then outcompete BKI1 for BRI1 binding, leading to the transphosphorylation of their kinase domain, phosphorylation of BKI1 on Y211 and its subsequent relocalization in the cytosol for full and durable activation of the receptor complex [1,3,8]. This activation mechanism was dubbed a “double lock” activation system [8], since it requires binding of BRI1 with BAK1 from both the outside and inside of the cell, in a BR- and BKI1-regulated manner, respectively (**Figure 1B**).

It was initially proposed that BKI1 function is to keep the BRI1 receptor inactive in the absence of ligands. This model is compatible with the phenotype of BKI1 loss- and gain-of-function

mutants. Indeed, in both Arabidopsis and rice, BKI1 null-mutants have an enhanced BR response, while BKI1 overexpressing plants possess strong BR-related dwarfism phenotypes [1,2,9]. However, these phenotypes are also compatible with an alternative hypothesis in which BKI1 can downregulate an active receptor complex. Because the function of BKI1 has been mainly studied from an overexpression point of view, the endogenous physiological function of BKI1 on BR signaling remains largely enigmatic. However, loss-of-function analysis showed that it operates at the base of a feedback response between brassinosteroid and iron [10]. This feedback loop is dependent on BR signaling but independent from the ligand (BR) itself. In this case, BR signaling is not modulated by variations in BR production but by a regulation in BKI1 translation [10]. This model highlights the potential relevance of the “double lock” mechanism [3,8], showing that BRI1 activation is not restricted to BR binding in the apoplast, but also relies on cytosolic regulation. It also raised the question whether other RLK could be regulated by similar mechanisms, perhaps using BKI1-like proteins.

Discovery of the MAKR family and general properties of the different members.

Although BKI1 was thought to be a single gene in Arabidopsis, the characterization of BKI1 functional regions prompted a search for similar motifs in additional plant proteins. This led to the discovery of six additional genes that fulfilled the following three criteria: i) they are predicted to be fully unstructured proteins according to ExPASy [11], ii) they have a conserved C-terminal tail and iii) they possess a KR-rich motif near the middle of their protein [1]. Based on the function of these two linear motifs, these six genes were coined MEMBRANE ASSOCIATED KINASE REGULATORS pointing toward their hypothetical molecular function. Additionally, MAKRs have several other conserved motifs over 10-20 aa in length, but that are characteristics of only some of the proteins (**Figure 2A**).

The first prediction –i.e. membrane association– was subsequently experimentally verified for MAKR1 to MAKR5 [9, 12-14]. Indeed, the membrane hook, corresponding to repetition of [KR][KR] doublets, initially characterized in BKI1, turned out to be a polybasic motif involved in electrostatic interactions with the anionic plasma membrane [12]. The plant plasma membrane is highly electronegative by comparison to other endomembrane compartments, owing to its enrichment in anionic lipids such as phosphatidylinositol 4-phosphate (PI4P), phosphatidylserine (PS), and phosphatidic acid (PA) (**Figure 1B**) [12, 15-17]. This allows to specifically recruit protein with a polycationic region to the plasma membrane, despite the

presence of anionic lipids at the surface of other compartments [12, 18]. BKI1 as well as MAKR1 to MAKR4 were shown to directly bind anionic phospholipids *in vitro* and to rely on plasma membrane electrostatics when heterologously expressed in yeast [12]. All of them are localized at least in part to the plasma membrane, and their N-terminal half, which contains the KR-rich membrane hook, is sufficient for this localization [12,13].

Importantly, specific plasma membrane targeting is achieved only above a certain threshold of positive charges, and proteins with a polybasic region of intermediate charges localize to both the plasma membrane and intracellular compartments such as the trans-Golgi network [12,19]. Interestingly, this is exactly what was observed when only one out of the four KR-doublets was mutated in BKI1 [1]. Mechanistically, it is likely that phosphorylation of Y211 within the BKI1 cationic membrane hook triggers an electrostatic switch by locally changing the charges of the hook and thereby displacing BKI1 out of the membrane (**Figure 1B**) [20]. Accordingly, mutation of Y211 in the non-phosphorylatable phenylalanine (Y211F) keeps BKI1 constitutively at the plasma membrane, which is associated with strong BR-related phenotypes [1]. By contrast, mutation of Y211 into an aspartic acid (Y211D), which is an overall poor mimic of tyrosine phosphorylation but adds a negative charge to the membrane hook, leads to a constitutive localization of BKI1 in the cytoplasm and no associated overexpression phenotype (**Figure 1C**). This experiment suggests that the charge carried by residue 211 within the BKI1 membrane hook is critical for membrane and lipid interaction.

The second prediction –i.e. kinase regulator– is based on the presence of a conserved C-terminal tail, which in BKI1 interacts with the kinase domain of BRI1. *In vitro* experiments showed that only BKI1 and MAKR1 directly bind to BRI1 kinase, while other MAKRs did not [1]. Thus, all MAKRs are not involved in BR signaling and might bind to and regulate other kinases. While this prediction has not been verified for all family members to date, we will detail some examples below that substantiate to some degree this hypothesis.

MAKR5 is a positive regulator of CLE45-BAM3 signaling in root protophloem

MAKR5 is a positive regulator of the CLAVATA 3/EMBRYO SURROUNDING REGION 45 (CLE45) signal transduction pathway [13]. It is expressed in developing protophloem, a specialized vascular tissue in growing plant organs, such as root meristems (**Figure 1D**). Protophloem development is largely dependent on BR and CLE45 signalling [13]. CLE45 inhibits protophloem differentiation in Arabidopsis roots [21] mimicking the root meristem phenotype of *brx* (*BREVIS RADIX*) mutant [22,23]. BRX encodes a plant-specific plasma

membrane-associated protein expressed in the developing protophloem and polarly localized at the rootward side of the cells [17, 24]. MAKR5 accumulates on the plasma membrane and is stabilized upon CLE45 perception in developing sieve elements [13]. CLE45 interacts with the RLK BARELY ANY MERISTEM 3 (BAM3) in the developing protophloem [22]. CLE45 and BAM3 form a signalling module which prevents differentiation of the phloem [21,23]. While, MAKR5 acts as a positive regulator downstream of BAM3, no direct interaction between MAKR5 and BAM3 were detected, suggesting that BKI1 and MAKR5 may have different modes of action at the molecular level [13].

As was described above, all MAKRs share a KR-rich membrane hook [1]. Mutational analyses revealed that MAKR5 membrane hook is required for function [13]. However, by contrast to BKI1, MAKR5 membrane association is promoted rather than decreased upon ligand (i.e. CLE45) perception. Interestingly, MAKR5 N terminal domain might contain destabilizing motifs responsible for the decrease of MAKR5 abundance in non-phloem pole cell files [13]. Thus, MAKR5 is required but not sufficient for the CLE45 signal transduction acting as a positive regulator and amplifier of the signal [13].

MAKR2 regulates root gravitropic bending and TMK-dependent rapid auxin signaling

Similar to MAKR5, MAKR2 has a very specific expression pattern as it is expressed in the epidermis and cortex in the root meristem as well as the Quiescent Center (QC) and surrounding initials (**Figure 1D**) [25]. MAKR2 regulates the dynamic of the root gravitropic response, with loss-of-function mutants responding faster than wild-type to a gravistimulus and gain-of-function mutants responding much more slowly than the control. Interestingly, MAKR2 is coexpressed in the epidermis and cortex with PIN2, an auxin efflux carrier involved in root gravitropism [26]. Furthermore, in MAKR2 loss- and gain-of-function mutants, PIN2 dynamics at the plasma membrane is affected, suggesting that the gravitropic phenotype of MAKR2 is linked with its effect on the PIN-FORMED2 (PIN2) protein [25].

The effect of MAKR2 on PIN2 dynamics is likely not direct but rather via its regulation of the LRR-RLK TRANSMEMBRANE KINASE 1 (TMK1) [25]. This receptor is involved in rapid auxin responses [26-28]. It is part of a small multigenic family of four members that are partially redundant and have pleiotropic developmental function related to auxin [29]. For example TMK1 is involved in apical hook formation in seedlings [28], lateral root development [29,30] or pavement cell morphogenesis [27,31]. In pavement cells, TMK1 acts upstream of the Rho GTPases of Plant ROP2 and ROP6 to regulate cytoskeleton dynamics and the jigsaw puzzle shape of these cells [27,31]. Auxin treatment triggers the rapid (i.e. within minutes)

activation of these Rho GTPases [27], and their nanoclustering at the plasma membrane [31], which is required for downstream signaling [16, 31-33]. Interestingly, ROP6 is also involved in root gravitropism and PIN2 dynamics [26,34,35], suggesting that TMK1 could regulate root gravitropism. Indeed, *tmk1tmk4* mutant has a reduced gravitropic response and TMK1 overexpression has the opposite phenotype, with a fast bending of the root following gravistimulation [25].

TMK1 and MAKR2 gain- and loss-of-functions have the opposite gravitropic phenotypes, suggesting that MAKR2 may act as a negative regulator of TMK1 signaling [1, 25]. Genetics and biochemical analyses confirmed that hypothesis. Indeed, MAKR2 binds to TMK1 *in vitro* and *in vivo*, and in turn, TMK1 phosphorylates MAKR2 likely to regulate its plasma membrane targeting [25]. MAKR2 relocates rapidly from the plasma membrane into the cytosol following auxin treatment, in a TMK1-dependent manner. Like for BKI1, this dynamic membrane association is required for MAKR2 function. Indeed, a MAKR2 variant mutated on its KR-rich membrane hook is cytoplasmic and its overexpression fails to induce any phenotype. By contrast, keeping MAKR2 constitutively at the plasma membrane through lipid anchoring induces a very strong gravitropic phenotype even when these variants are expressed at a low level [25]. Thus, it appears that BKI1 and MAKR2 have a similar mode of action as i) they are both negative regulators of a LRR-RLK, ii) they directly bind to the kinase domain of their cognate receptor, and iii) their plasma membrane localization is antagonized by the activation of their receptor upon ligand perception, which allows full receptor activation [1,25].

MAKR4 acts in auxin-mediated lateral root initiation process

Another member of the family, MAKR4, was found associated with the earliest steps of lateral root formation in Arabidopsis [14]. The sequential initiation of lateral roots occurs via auxin signalling oscillations at the distance of the root tip, the prebranch sites, that contains competent cells visualized by the auxin response marker DR5 [36]. The competent cells can later originate a lateral root primordia from the protoxylem-pole pericycle cells. MAKR4 is specifically expressed in the protoxylem pole of the meristem (**Figure 1D**), it has very low expression at the sites of DR5 oscillation, but becomes much stronger in newly formed prebranch sites [14]. MAKR4 is expressed in the lateral root founder cells before their nuclear migration, and is required for the establishment and maintenance of lateral root founder cells with the auxin response maximum. Accumulation of MAKR4 on the pericycle cells plasma membrane induces the processes of lateral root initiation and control of regular distance between lateral

roots formation sites. Knocking down *MAKR4* has no impact on prebranch sites numbers but produced significantly fewer lateral root primordia.

Almost nothing is known about the regulation of *MAKR4* during the lateral root initiation. It was suggested that *MAKR4* is a downstream target of the transcription factor LATERAL ORGAN-BOUNDARIES DOMAIN 16 (LBD16) and its related LBD family members that are involved in lateral root initiation [37]. The expression patterns of *MAKR4* and LBD16 are coordinated in the primary root and they are both required to begin the initiation events [14,37]. It is currently unknown whether *MAKR4*, like *BKI1*, *MAKR5* and *MAKR2* also act downstream of a RLK module. Hopefully, the study of *MAKR4* interactors among the RLKs expressed during the lateral root formation will clarify the mechanisms of auxin-mediated lateral root development.

Shared and specific features of MAKR family members

Altogether, the examples described above on characterized family members suggest some common features and specificities on how *MAKR* proteins function. Indeed, all characterized members are found at the plasma membrane, a process that is dependent on the KR-rich plasma membrane hook [1,12-14,27]. In the case of *BKI1*, *MAKR2* and *MAKR5*, it has been experimentally determined that this membrane hook is not only required for plasma membrane localization but also for their function [1,13,25]. These results strongly suggest that these three *MAKR* proteins are active when localized at the plasma membrane. Although this remains to be experimentally determined, we can predict that plasma membrane association might be a common prerequisite for the function of other *MAKR* proteins. The association of *BKI1*, *MAKR2* and *MAKR5* proteins with the plasma membrane also appears to be highly dynamic [1,2,13,25]. Transient and regulated plasma membrane localization might thus also be a common feature of *MAKR* protein function. In the case of *BKI1* and *MAKR2*, this dynamics was actually shown to be critical by experimentally locking their interaction with the plasma membrane [1,2,25]. However, there are also differences that can be noted in the dynamics of association between *MAKR* proteins and the plasma membrane. Indeed, *BKI1* and *MAKR2* rapidly dissociate from the plasma membrane upon hormonal treatment (brassinosteroid and auxin, respectively), while *MAKR5* is instead recruited to this compartment upon CLE45 signaling [1,2,13,25]. This difference perhaps stems in the fact that *BKI1* and *MAKR2* are negative RLK regulators, while *MAKR5* is a positive RLK regulator. In addition, *MAKR5* does not appear to interact with *BAM3*, while *BKI1* and *MAKR2* directly interact with the kinase domain of *BRI1* and *TMK1*, respectively [1,2,13,25]. Last but not least, a common

feature of MAKR2, MAKR4 and MAKR5 are their high cell type-specific expressions in the root meristem. For these three MAKR proteins, their expression patterns are largely non-overlapping, with MAKR2 being expressed in the epidermis, cortex, QC and surrounding initials; MAKR4 being expressed in the protoxylem pole of the meristem and MAKR5 in protophloem sieve elements and surrounding cells (metaphloem, companion cells and phloem pole pericycle cells) (**Figure 1D**) [13,14,25]. Whether the shared and specific features of MAKR expression and function can be extended to the whole family remains to be determined. In the next section of this review, we use publicly available genomic data to mine for the possible conservation of some of the above mentioned general concepts on MAKR protein function and expression.

Evolution of the MAKR family

Currently MAKR3/6 functions have not been analyzed in plants. Moreover, the studies for other MAKRs were focused on specific developmental processes leaving open questions about their roles in other tissues. In the next section, we analyze the structural, comparative, and functional genomics data available on MAKR family.

We analyzed the MAKR protein sequences to reveal their features and signatures which might imply their potential functions. In Arabidopsis MAKR proteins are ~300 aa in length in average (the longest one is MAKR2 length of 411 aa and the shortest - MAKR6 length of 235 aa) (**Figure 2A**). As explained above, BKI1/MAKR proteins are defined by the presence of two functional linear motifs: i) a lysine-arginine rich membrane hook characterized by the repetition of doublets of dibasic residues, and ii) a conserved C-terminal tail that might represent a new class of kinase interacting peptides [1] (**Figure 2A, B**). We searched for MAKRs' homologs throughout the plant kingdom using BLAST [38], PLAZA [39], 1KP (The 1000 plant transcriptomes initiative) [40], and PhyloGenes [41] resources. Being unstructured fast-evolving proteins MAKRs possess very low conservation out of angiosperms. Thus, due to high sequence heterogeneity of MAKRs, we considered only homological sequences possessing a conserved C-terminal tail (**Figure 2B**), which in BKI1 interacts with the kinase domain of BRI1 [1]. We were able to detect MAKR family members in mosses with no homologous sequences detected in charophytes and other algae (**Figure 2C**). Four MAKRs were detected in *Physcomitrella patens* which evolved independently from the common ancestor of MAKRs. Three homologs were detected in *Selaginella moellendorffii* and one in *Pinus radiata*, however different gymnosperm species can have from one to three MAKR homologs. The expansion of MAKR family genes occurred in angiosperms via a series of

duplications. The core phylogenetic tree of MAKR family in flowering plants has two clades: (1) MAKR2/5/6 and (2) BKII with MAKR1/3/4 (**Figure 2D**). The first one started specializing earlier in evolution with separate branches of MAKR2, MAKR5 and MAKR6 detected from the basal angiosperm *Amborella trichopoda* and higher. The number of MAKR5 and MAKR6 homologues increased in monocots evolution. The second clade has one common ancestor in *Amborella* which started duplicating and specializing later in evolution. MAKR1 and BKII branches diverged first, and MAKR3/4 later. The tree is coherent with the functional annotation of MAKRs, where MAKR1 and BKII, but not other MAKRs showed a common function as BRI1 interactors [1].

To sum up, MAKR proteins have spread widely among terrestrial plants and contemporary members evolved as a result of several duplications, nowadays they could be divided into two groups according to the observed clades.

MAKRs expression and transcriptional responses in Arabidopsis

While MAKRs expression patterns have not been systematically described and compared, a clear picture emerging from the functional studies of MAKR2, MAKR4 and MAKR5 is that these genes tend to have very specific expression patterns (**Figure 1C**). We thus extensively analyzed the expression pattern of each *MAKR* gene based on available whole-genome expression profiles in *Arabidopsis* (**Figure 3**).

MAKRs demonstrate specific expression patterns in particular cell types and tissues in different organs (**Figure 3A**). Namely, *BKII* and *MAKR1* are highly expressed in leaves, flowers, and siliques. Although their expression patterns significantly overlap, *BKII* expression level is overall higher than that of *MAKR1*, except in the petiole and hypocotyl. This co-expression strengthens the idea that these two genes are functionally redundant, at least to some extent. *MAKR5* and *MAKR6* tend to have higher expression levels than other members of the family. For instance, *MAKR6* is highly expressed in different types of leaves. *MAKR4* has a more narrow and specific expression pattern, being expressed in hypocotyl, petiole, pollen and roots. *MAKR3* level is rather low in plant tissues, but it becomes highly expressed in callus (**Figure 3A**).

We next focused on MAKRs' levels and expression patterns in the *Arabidopsis* roots. Data from two separate single-cell studies [42,43] were merged and depicted in **Figure 3B**. This analysis showed a specific expression pattern of *MAKRs* suggesting their dynamic activation/repression along different developmental trajectories. While most *MAKRs* are expressed in the stem cell niche (*BKII*, *MAKR1,2,5*), and the steller (*BKII*, *MAKR3,4,5,6*), only

MAKR5 and *6* are detected in the columella, *BK11* and *MAKR6* in the endodermis, *MAKR1* and *4* in the epidermis, and lateral root cap (**Figure 3B**). As described above, *BK11* and *MAKR1* cell-type specific patterns are also partially overlapping in roots.

Another question is whether plant hormones may influence *MAKR*s expression. We thus analyzed if *MAKR*s transcription in seedlings is affected by plant hormones such as auxin, brassinosteroids, ACC (i.e. precursor of ethylene), zeatin, gibberellic, abscisic and jasmonic acids (**Figure 3C**) in a time dependent manner. Note that these results are based on available ATH1 microarray data, in which the probe for *MAKR2* is absent [48]. Interestingly, *MAKR4* was found to be regulated by most hormones; its expression level was decreased by more than two fold in early response to brassinolide, zeatin, ACC and abscisic acid treatment and was highly up-regulated by auxin at both the early and late time point (**Figure 3C**). This result is in accordance with the function of *MAKR4* in lateral root formation and its reported expression pattern [14]. *MAKR3* was more than two times down-regulated in both temporal periods after zeatin and brassinolide treatments. *BK11* and *MAKR1* were up-regulated by IAA and gibberellic acid treatment, but not by BR itself.

The results of this expression analysis confirm that *MAKR*s have tissue- and condition-specific expression, which highlight their tight regulation and specific developmental functions.

Mining for potential upstream regulators of MAKR genes expression pattern

Considering the highly dynamic and tissue-specific expression pattern of *MAKR*s, we wondered which transcription factors (TF) guide their transcription. For this, we scanned *MAKR*s promoters for the presence of experimentally shown TF binding regions in DAP-Seq experiments [49]. This search returned many potential upstream regulators that were able to bind to *MAKR*s promoter (**Figure 3D**). HIGH CAMBIAL ACTIVITY2 (HCA2) binds to all six promoters (**Figure 3D**). HCA2 is a known regulator of interfascicular cambium development without any targets confirmed in fine-scale experiments so far [50]. According to recently published Arabidopsis inflorescence stem atlas [51], HCA2 and *MAKR*s are co-expressed in the cambium, so the functional genomics data predicts that they might play a role in interfascicular cambium development too. Overall, the transcriptional regulatory network reconstructed on the basis of DNA affinity purification sequencing (DAP-Seq) data is enriched with vasculature development regulators, which is consistent with the single-cell data on *MAKR*s expression in the root stele (**Figure 3B**). Namely, vasculature regulators OBF-BINDING PROTEIN3 (OBP3) [52], HOMEBOX PROTEIN33 (HB33) [53], B-BOX DOMAIN PROTEIN31 (BBX31) [54], AUXIN RESPONSE FACTOR2 (ARF2) [53]/5

[55,56], DOF TRANSCRIPTION FACTOR6 (DOF6) [57] TFs bind the promoters of almost all MAKRs. Auxin response regulator ARF5 has DAP-Seq binding regions in the promoters of *MAKR1-4,6*, ARF2 can bind the promoters of *BKI1*, *MAKR1,6*, implying a major role of auxin in regulation of tissue specific MAKRs expression. BR-related TF regulators (BES1-interacting Myc-like proteins (BIM2, BIM1) and BES1/BZR1 homologs (BEH2,4), were also detected among potential upstream regulators of *BKI1* and *MAKR2, 3* and *4*.

Presence of DAP-Seq peak does not guarantee that the transcription factor directly regulates the gene expression *in vivo*, as it shows transcription factor binding to the naked DNA. Direct regulation will depend on the accessibility of the chromatin in the tissue where the transcription factor is expressed. But such data may be valuable when having specific insights on the phenotype associated with MAKRs gain- or loss-of-function mutants. For example, the fact that *MAKR2, 4* not only participate in auxin-mediated pathways in the root [1,14,25], but also possess DAP-Seq binding regions of ARF5 (**Figure 3D**), the major regulator of auxin signalling that is specifically important for root development, adds an additional weight to the functional genomics data and persuades scientists to verify the direct regulation experimentally.

Conclusions

MAKR family genes probably evolved together with land plants, first detected in the mosses and lycophytes (representing the *BKI1*, *MAKR1, 3, 4* clade). Also depending on the species, few *MAKR* homologs were detected in gymnosperms being closer to the *BKI1*, *MAKR1, 3, 4* clade as well. The extended *MAKR* family genes are the result of duplication series in Angiosperm. These proteins are widely spread among terrestrial plants such as mosses, lycopsids, gymnosperms, and angiosperms both in dicots and monocots. The latter uncovers their potentially important role in the plant kingdom in general and in *Arabidopsis thaliana* in particular. Overall both paralogs and orthologs are sharing a C-terminal tail and KR-rich membrane hook in their protein sequences [1]. However, *MAKR* genes are poorly conserved and are fastly evolving with some sequence of low complexity. A certain degree of conservation was observed between orthologs, but not paralogs, with the exception of the C-terminal tail. Yet, *MAKR*s might share common function or molecular modes of action, which may be closer among orthologs and more divergent among paralogs. *BKI1*, *MAKR1/2* were shown to directly bind RLKs [1,25], while *MAKR5* function downstream of the RLK *BAM3* likely without directly binding to its kinase domain. Whether kinase interaction is a common feature of all *MAKR*s remains to be determined. Another common property of *MAKR*s is their

high specificity of expression which is already evident in transcriptomic data and highlighted by the in depth study of reporters in planta. Transcriptomic and genomic data also suggest that they could be highly sensitive to various hormones, and that they possess putative hormone responsive motifs in their promoters. Taken together, these findings suggest that MAKR family genes might play important roles in plant development downstream of small molecule and peptide hormones signaling pathways. Functional analyses of additional family members are now required to test these predictions and uncover the potential RLK regulated by MAKR proteins.

Author contribution. D.D.N. performed the phylogenetic and bioinformatic analyses, D.D.N., A.L.K., V.M. and Y.J. wrote the manuscript.

Acknowledgments. We thank Nadya Omelyanchuk, Alexey Doroshkov, Mar Marquès and Laia Armengot for fruitful discussions as well as all the members of the SiCE group. We thank Wei Xuan and Tom Beeckman for providing the MAKR4prom::GFP-MAKR4 micrograph, as well as Yeon Hee Kang and Christian Hardtke for the MAKR5prom::MAKR5-GFP micrograph.

Funding sources and disclosure of conflict of interest.

Promoter analysis was funded by RFBR and Novosibirsk region according to the research project № 19-44-543006; phylogenetic analysis was funded by the Budget Project 0259-2021-0009. Research on MAKRs in Y.J lab was founded by ERC grant 3363360-APPL under FP/2007–2013 and an ANR-supported ERA-CAPS project (SICOPIID: ANR-17-CAPS- 0003-01). The authors declare no conflict of interest.

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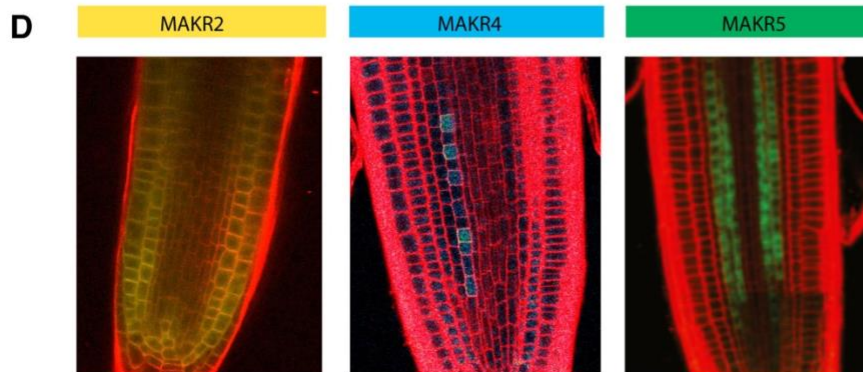
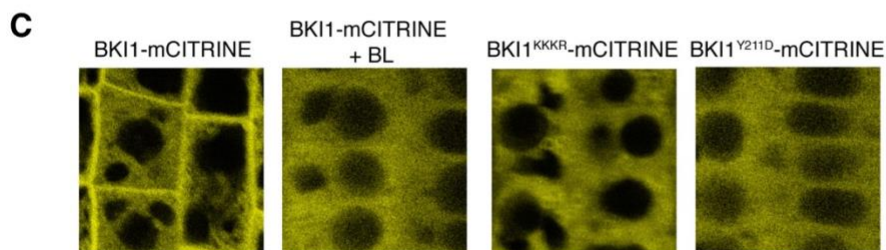
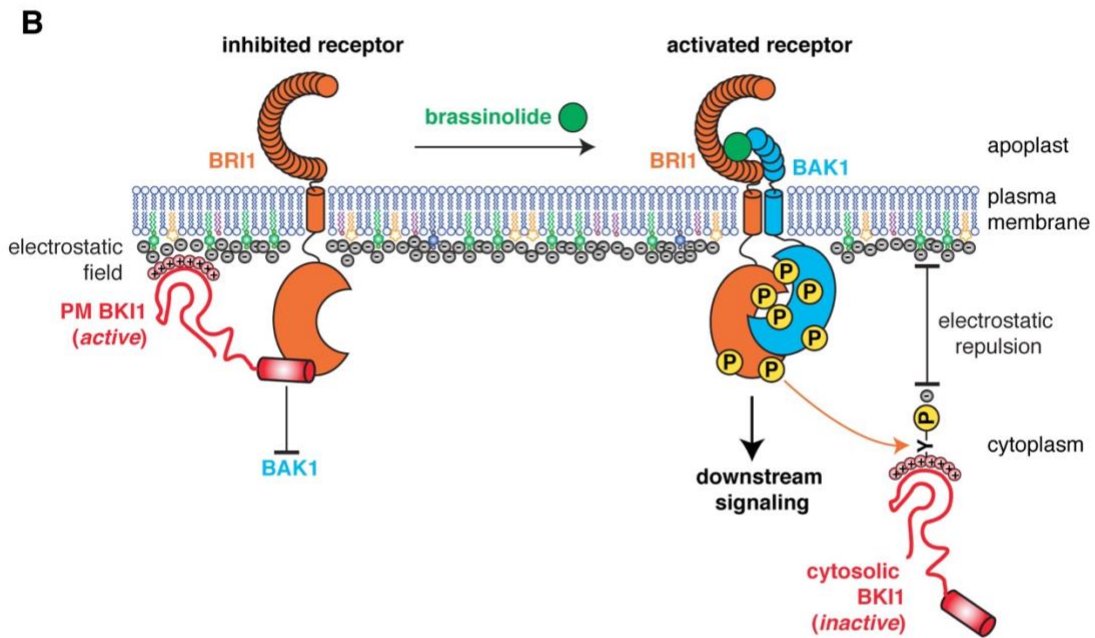
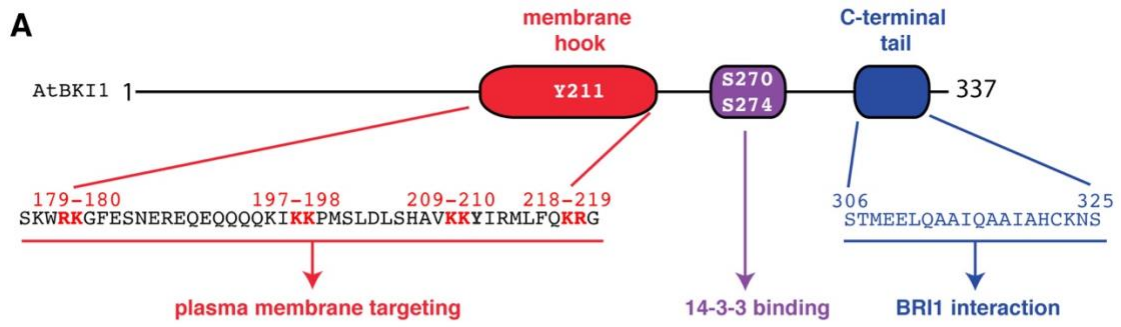


Figure 1: Mutual regulation between BRI1 and BKI1 during brassinosteroid signaling. A) Schematic representation of BKI1 organisation with its respective functional linear motifs shown. Phosphorylation sites are labelled in white, the membrane hook in red, the 14-3-3 binding site in purple and the C-terminal tail in blue. B) Model showing how BKI1 is i) recruited to the plasma membrane by its cationic membrane hook and interaction with anionic lipids (in green PI4P, in yellow PS, in purple PA and in blue structural lipids) and ii) inhibits the formation of the BRI1/BAK1 heterodimer through its C-terminal tail. Upon brassinolide perception, BRI1 and BAK1 extracellular domains dimerize, BRI1 phosphorylates BKI1 on a tyrosine within its membrane hook, which triggers BKI1 membrane release through an electrostatic switch. Once BKI1 is in the cytosol it is inactive, allowing BRI1 and BAK1 kinase domains to stably interact and to initiate downstream signaling. C) Confocal pictures showing the subcellular localization of BKI1-mCITRINE in the absence or presence of brassinosteroid treatment, as well as the localization of BKI1^{KKKR}-mCITRINE (mutation of two [KR][KR] doublets within BKI1 membrane hook into alanine) and BKI1^{Y211D}-mCITRINE (mutation of Y211 into aspartic acid). Images reproduced with permission from Jaillais et al., G&D, (2011). D) Example of expression profile obtained from translational reporters (MAKR2prom::MAKR2-tdYFP, MAKR4prom::GFP-MAKR4 and MAKR5prom::MAKR5-GFP). A schematic summary of MAKR2 (yellow), MAKR4 (blue), and MAKR5 (green) expression in the root is depicted on the right. Note that the expression patterns of the three MAKR proteins are largely non-overlapping in the Arabidopsis root meristem. GFP-MAKR4 photo courtesy of Wei Xuan and Tom Beeckman. The image of MAKR5-GFP was reproduced with permission from Christian Hardtke and taken from Kang et al., EMBO reports, 2016.

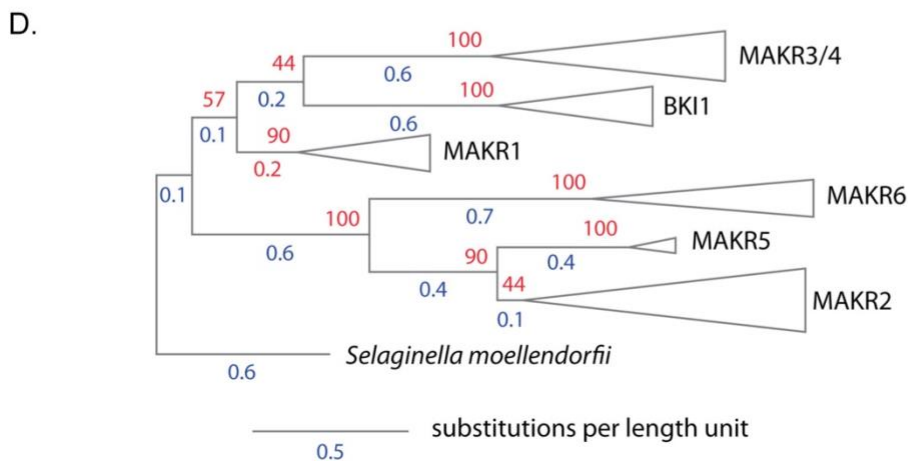
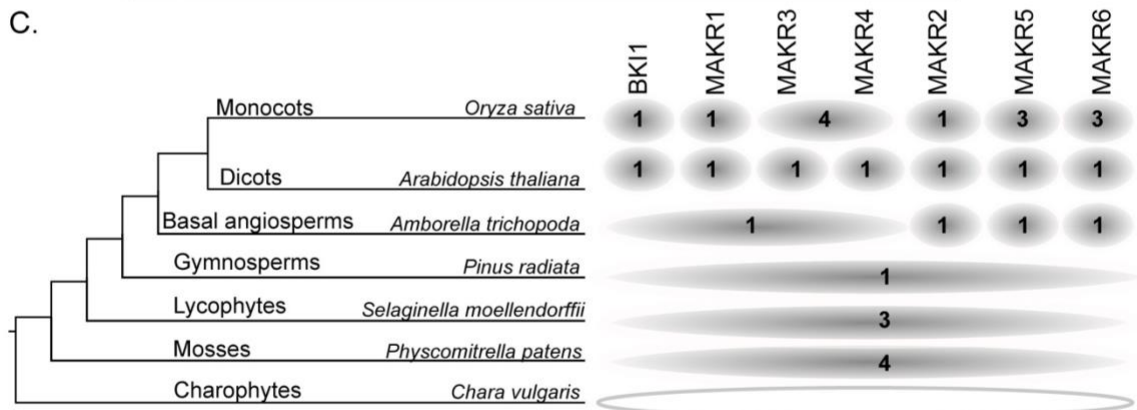
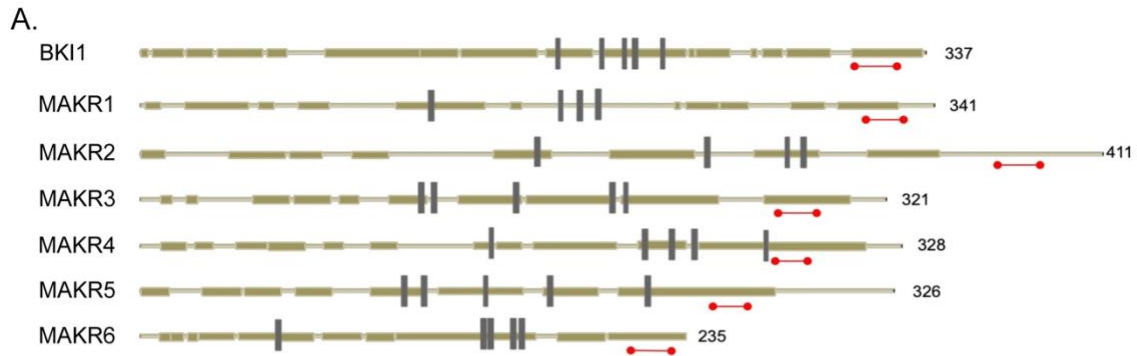


Figure 2. Insight into structure and evolution of MAKR family genes. A) Schematic representation of the MAKR family proteins. Conserved regions are presented as brown boxes, the C-terminal tails are highlighted in red, and lysine-arginine repeats in gray. B) Alignment of

MAKR family genes C-terminal tail motif of *Arabidopsis thaliana* and their orthologs in *Physcomitrella*, *Selaginella*, *Picea* and *Amborella*. Alignment was constructed in MEGA 6.06 with MUSCLE method. * - entire conservation of position; . - average conservation of position; : - low conservation of position. The box highlights the HCK motif, which is invariant in all MAKR proteins. C) Distribution of MAKR proteins over plant taxons. White and gray circles indicate the absence and presence of MAKR proteins in the particular species, respectively. Amounts of MAKR homologs in different species are shown with numbers in the circles. D) Core phylogenetic tree of MAKR family genes molecular evolution in angiosperms. The phylogenetic tree was constructed in MEGA 6.06 with Maximum Likelihood method, *Selaginella moellendorffii* was taken as outgroup, bootstrap adjustment (red) is shown for each node. To conduct the phylogenetic analysis, the evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [58]. The trees with the highest log likelihood were shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with a superior log likelihood value. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 amino acid sequences of MAKR family genes in angiosperms for the core molecular evolution phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 91 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [59].

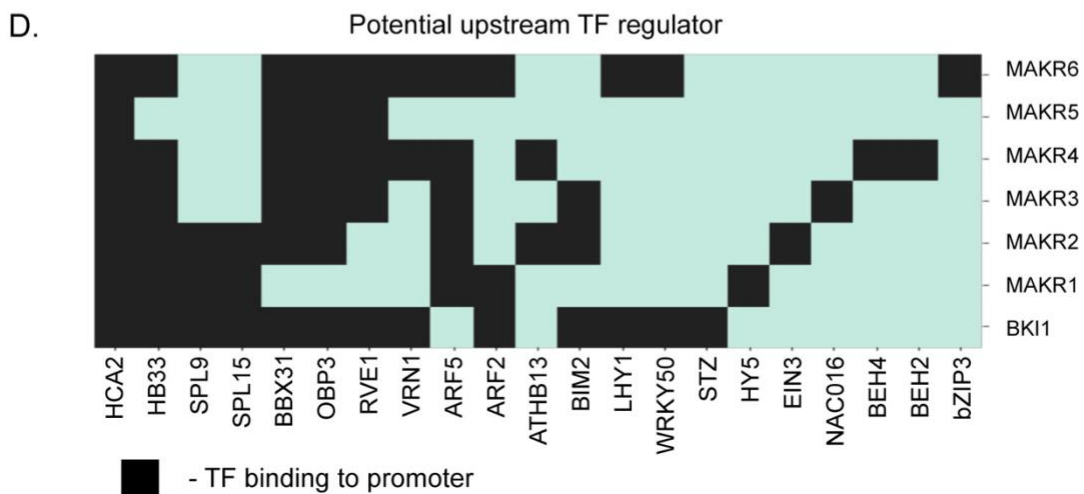
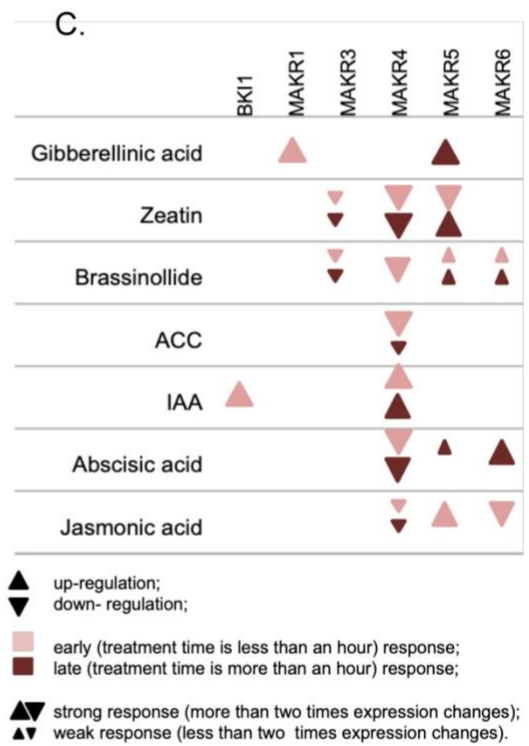
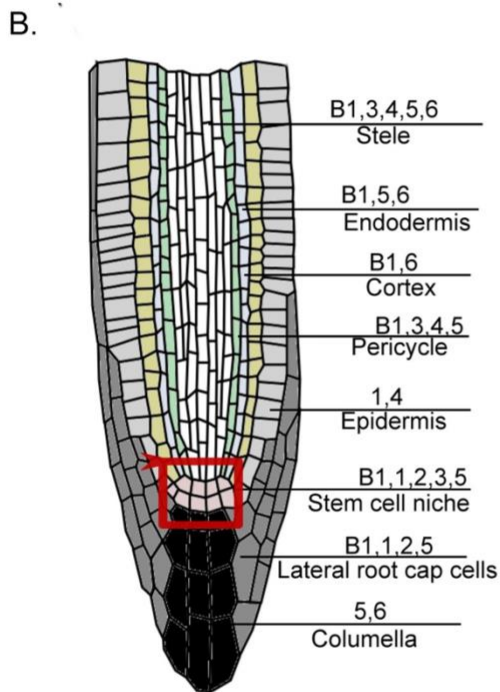
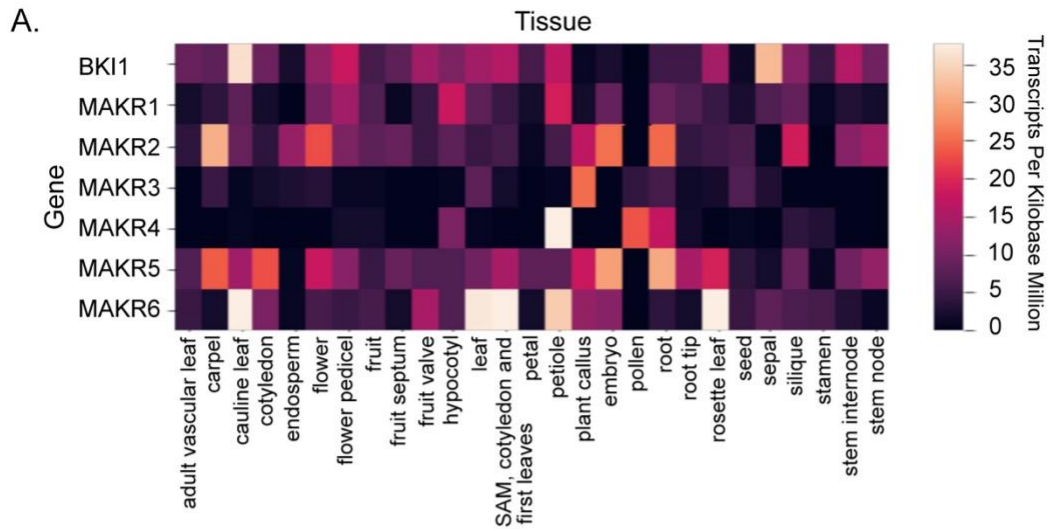


Figure 3. Expression profiles and regulation of *BKI1* and *MAKR*s. A) Expression of *MAKR* family genes in various tissues of wild-type *Arabidopsis*; data were obtained from scRNA-seq experiments [42,45-47]. Expression profiles of *MAKR*s were described using transcriptomics (microarray and RNA-Seq) data on tissue- [42,45-47] and condition- [48] specific gene expression, single-cell RNA-Seq data in the root meristem [43,44] accessed via Expression Atlas (<https://www.ebi.ac.uk/>) and eFPbrowser [49]. For the *MAKR* expression in different tissues, average means over several experiments (when applicable) were taken as final ones for the description and discussion. *MAKR*s expression in root tips considered to be present if detected in at least one of used in the analysis transcriptomics experiments. B) Cell-specific expression of *BKI1/MAKR*s in *Arabidopsis* roots. Data were obtained from scRNA-seq and microarray experiments [42-44]. B1 -*BKI1*, 1-6 - *MAKR1-MAKR6*. C) Differential expression of *MAKR* family genes in response to hormone treatments. Data were obtained from microarray experiments [48], *MAKR2* is absent since it does not have a probe on ATH1 microarray. Triangles in the table demonstrate whether *MAKR*s are up- or down- regulated in early or late response and whether the last one is weak or strong. D) Heatmap of TF binding in upstream regions of *MAKR*s for selected TFs. Binding of TFs to upstream regions of *MAKR* genes [-1500; 5'UTR] was shown with DAP-Seq profiling in [49]. Detected binding events are depicted in black, absence of binding - blue. To determine if *MAKR* genes can be directly regulated by a certain transcription factor (TF), we checked if their upstream regions [-1500; +5'UTR] overlap with experimentally verified DNA-binding profiles. Namely, we used the experimental data on 568 genome-wide DAP-Seq profiles for *Arabidopsis* TFs (the Plant Cistrome Database) [49].