

Comparative Insights into the ERECTA-YODA Pathway in *Arabidopsis thaliana*

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
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Tübingen
2022

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der
Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:

24.11.2022

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Abbreviations

ABA	Abscisic acid
ABI	ABA insensitive
ACTL	ACT-like
BASL	Breaking of asymmetry in the stomatal lineage
BES1	BR1-EMS-suppressor 1
bHLH	basic helix-loop-helix
BIN2	BR-insensitive 2
BR	Brassinosteroid
BRI1	Brassinosteroid insensitive 1
BSK	Brassinosteroid-signaling kinase
BSL	BRI1 suppressor 1-like
BSU1	BRI1 suppressor 1
BZR1	Brassinosteroid-resistant 1
CBF	C-repeat binding factor
CLE8	CLAVATA3/ESR-related 8
CoIP-MS	Co-immunoprecipitation Mass Spectrometry
EFR	Elongation factor-Tu receptor
EF-TU	Elongation factor-Tu
EPF	Epidermal patterning factor
EPFL	Epidermal patterning factor-like
ER	ERECTA
ERf	ERECTA family
ERL1/2	ERECTA-like 1/2
ESF1	Embryo surrounding factor 1
flg22	Flagellin
FLS2	Flagellin-sensitive 2
GC	Guard cell
GMC	Guard mother cell
GRD/RKD4	GROUNDED/RWP-RK domain-containing 4
GSO1/2	GASSHO1/2
HDG11/12	Homeodomain glabrous 11/12
HOS1	High expression of osmotically responsive genes 1
JA	Jasmonic acid
JAZ	Jasmonate ZIM-domain protein
LRR-RK	Leucine-rich repeat receptor kinase
MAPK/MPK	Mitogen-activated protein kinase
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MMC	Meristemoid mother cells
MYB15	MYB domain protein 15

OST1	Open stomata 1
PAMP	Pathogen associated molecular patterns
PEPR	Pep1 receptor
PI	Propidium iodide
POLAR	Polar localization during asymmetric division and redistribution
PRR	Pattern recognition receptors
PTI	Pattern-triggered immunity
RLCK	Receptor-like cytoplasmic kinase
RLK902	Receptor-like kinase 902
ROS	Reactive oxygen species
SCRM/ICE1	SCREAM/Inducer of CBF expression 1
SERK	Somatic embryogenesis receptor kinase
SIZ1	SAP and MIZ1 domain-containing ligase 1
SLGC	Stomatal lineage ground cell
SPCH	SPEECHLESS
SSP	Short suspensor
TMM	Too many mouth
TPR	Tetratricopeptide repeat
WOX8	WUSCHEL related homoeobox 8
YDA	YODA
yda-CA	YODA-constitutively active
ZOU	ZHOUP1

Summary

Mitogen-activated protein kinase (MAPK) pathways are highly conserved signaling cascades in eukaryotes and play a key role in plant growth, development, reproduction and stress responses. In *Arabidopsis*, its role in cell differentiation has drawn close attention in the past two decades, with a body of research focusing on the ERECTA-YODA (ER-YDA) pathway in embryogenesis and stomata development. ER-YDA signaling essentially comprises three transduction steps: 1) extracellular ligands are recognized by the upstream ER family (ERf) receptor complex which contains two receptor-like cytoplasmic kinases BRASSINOSTEROID-SIGNALING KINASE 1/2 (BSK1/2) at the membrane; 2) signals from the ERf receptor complex through BSK1/2 activate the YODA-MKK4/5-MPK3/6 phosphorylation cascade; 3) the MAPK phosphorylation activates the downstream transcription factor WRKY2 in the embryo or represses the heterodimer complex of two basic helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH) and SCREAM (SCRM) in stomata. Previous publications revealed details of the ER-YDA pathway in these two developmental contexts and indicated conserved upstream receptor components and distinct downstream targets. However, former genetic analysis is yet to demonstrate any functional difference in BSK1 and BSK2 as vital upstream signal relays connecting the ER receptor complex and the YDA cascade, and little is known about the conserved mechanism in the regulation of downstream regulators during cell proliferation.

This project aims to fill the gaps mentioned above and to gain a better understanding of the ER-YDA pathway from a comparative point of view. To accomplish this, we generated the new loss-of-function mutants of BSK1 and BSK2 using CRISPR/Cas9 and conducted detailed phenotypic analyses which showed that BSK1 contributes mainly to the cell polarity in the embryo and stomata, while BSK2 plays a prominent role in the growth of rosette leaves. Confocal microscopy and co-immunoprecipitation coupled with mass spectrometry (CoIP-MS) assays in YPet-tagged BSK1/2 transgenic lines further identified the differences in expression patterns, new phosphorylation sites and interacting proteins for BSK1 and BSK2 *in vivo*. These functional differences between BSK1 and BSK2 suggest an evolutionary divergence and support us to propose a collaborative working model. Furthermore, we disclose that SCREAM (SCRM), a well-known target of the ER-YDA pathway in stomata, functions as a regulator of embryo development. However, the regulation of SCRM by the ER-YDA pathway switches from phosphorylation-dependent degradation in stomata to phosphorylation-dependent activation in the embryo. Further studies verified that SCRM works in synergy with WRKY2 to upregulate WUSCHEL RELATED HOMEODOMAIN 8 (WOX8) in the apical-basal axis formation during embryogenesis. Finally, we conducted a phenotypic screen and found bHLH35, a paralogue of SCRM subfamily, is a potential interacting partner of SCRM in early embryos.

This work provides a comparative insight into the ER-YDA pathway, enabling us to have a more comprehensive understanding of the MAPK cascade. Two aspects of this thesis, the overlapping but differential functions of BSK1/2 and the altered regulation of the conserved target SCRM, together exhibit the conservation and diversity of the ER-YDA pathway, further expanding the knowledge on how the MAPK pathway delicately controls the signal for cell differentiation and proliferation.

Zusammenfassung

MITOGEN-ACTIVATED PROTEIN (MAP) Kinase Signalwege sind konservierte Signalkaskaden in Eukaryoten, die eine fundamentale Rolle in Pflanzenwachstum, Reproduktion und Stressantwort spielen. In der Modellpflanze *Arabidopsis thaliana* hat sich die MAP Kinase Forschung in den letzten zwei Dekaden unter anderem auf die Rolle des ERECTA-YODA Signalwegs bei der Zelldifferenzierung während der Embryogenese und Stomata Entwicklung konzentriert. Die Signaltransduktion in diesem Signalweg beinhaltet drei prinzipielle Schritte: 1) Extrazelluläre Liganden binden und aktivieren einen Rezeptorkinase Komplex aus Rezeptorkinasen der ERECTA Familie (ERf) und SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) Familie. Die membranständigen Proteine BRASSINOSTEROID SIGNALING KINASE1 (BSK1) und BSK2 sind zytoplasmatische Bestandteile dieses Komplexes und werden von ERf Kinasen phosphoryliert. 2) Aktivierte BSK1 und BSK2 Proteine rekrutieren die MAPKK Kinase YODA (YDA) zum Rezeptorkomplex und führen zur Aktivierung der YODA-MKK4/5-MPK3/6 Phosphorylierungskaskade. 3) Phosphorylierung durch MPK3/6 aktiviert den Transkriptionsfaktor WRKY2 im Embryo bzw. inaktiviert den heterodimeren Komplex der bHLH Transkriptionsfaktoren SPEECHLESS (SPCH) und SCREAM (SCRM) während der Stomata Entwicklung.

Während die Komponenten dieses Signalwegs oberhalb der MAP Kinasen MPK3 und MPK6 im Kontext der Embryogenese und der Stomata Entwicklung weitestgehend identisch sind, scheinen sich die Zielproteine kontextabhängig zu unterscheiden.

Diese Arbeit hat zum Ziel, in einem vergleichenden Ansatz zwischen Stomata Entwicklung und Embryogenese die Gemeinsamkeiten und Unterschiede in der molekularen Zusammensetzung des ERECTA-YODA Signalwegs herauszuarbeiten. Dabei liegen die Schwerpunkte vor allem bei den BSK Proteinen BSK1 und BSK2 und dem Transkriptionsfaktor SCRM, den wir als weiteres Zielprotein des embryonalen ERECTA-YODA Signalwegs identifizieren konnten. Dabei scheint die MPK6-abhängige Phosphorylierung bei der Embryogenese eine aktivierende Rolle zu spielen, während sie bei der Stomata Entwicklung die Aktivität des SCRM/SPCH Transkriptionsfaktorkomplexes hemmt.

Diese Arbeit liefert tiefergehenden Einblick in die kontextabhängige Funktion des ERECTA-YODA MAP Kinase Signalweg während verschiedener Aspekte der Pflanzenentwicklung.

Introduction

Mitogen-activated protein kinase cascades in plants

Mitogen-activated protein kinases (MAPKs), which belong to the serine/threonine-specific protein kinase family, function as vital signaling modules in eukaryotic systems to link the perceptions of extracellular stimuli and intracellular biological responses to regulate the normal growth and development of organisms (Widmann *et al.*, 1999; Ichimura *et al.*, 2002). A canonical MAPK cascade is composed of at least one MAPK kinase kinase (MKKK or MEKK), one MAPK kinase (MKK or MEK), and one MAPK (MPK) and they are associated with the transduction of phosphorylation signal. When induced by certain developmental or environmental cues, the uppermost MKKKs can be activated and phosphorylated, and then activate MKKs by phosphorylation. The MKKs transmit the phosphorylation signal to the MPKs which completes the sequential activation of the MAPK cascades. For each layer of the MAPK cascade, functionally redundant members of the MAPK family could cooperate to regulate signal transduction. Finally, activated MPKs would phosphorylate downstream targets including transcription factors, structural proteins, metabolic enzymes and protein kinases to elicit the corresponding cellular processes (Ichimura *et al.*, 2002; Rodriguez *et al.*, 2010; Xu & Zhang, 2015; Zhang & Zhang, 2022).

MAPK cascades participate in various biological processes involving in the cell differentiation, immune response, abiotic stress and hormonal synthesis in plants (Rodriguez *et al.*, 2010; Xu & Zhang, 2015; Zhang & Zhang, 2022). In Arabidopsis, there are around 80 MKKKs, 10 MKKs and 20 MPKs from genome data (Ichimura *et al.*, 2002; Jonak *et al.*, 2002; Cvetkovska *et al.*, 2005; Colcombet & Hirt, 2008). MKKK family comprises of three subfamilies containing 21 MEKK1-like (MKKK1-21), 11 ZIK-like (ZIK1-11) and 48 Raf-like (Raf1-48) MAPKKK members (Jonak *et al.*, 2002; Colcombet & Hirt, 2008). Genetic and biochemical evidences of MEKK1-like MKKKs basically confirmed their upstream position in typical MAPK cascades such as the MKKK4 (YODA or YDA)-MKK4/5-MPK3/6 cascade in stomata development and MEKK1-MKK1/2-MPK4 signaling in plant immunity, while Raf-like MKKKs are still unclear (Wang *et al.*, 2007; Gao *et al.*, 2008). For example, Raf2 was found to negatively regulate MKK4/5-MPK3/6, which contradicts the sequential activation model of the canonical MAPK cascade (Zhao *et al.*, 2014).

Plant MKKs are highly conserved in sequences and easily identified from genomic data. The consensus sequence S/T-XXXXX-S/T in the activation loop is recognized by MKKKs and the N-terminal sequence K/R-K/R-K/R-X₁₋₆-L-X-L/V/I acts as a docking site for MPKs (Jonak *et al.*, 2002). Arabidopsis MKK family has only 10 members which could be categorized into 4 subfamilies based on sequence similarities: Group A (MKK1/2/6), Group

B (MKK3), Group C (MKK4/5) and Group D (MKK7/8/9/10). Group A members are mainly involved in stress responses including cold, salt and pathogens in the upstream of MPK4 (Teige *et al.*, 2004; Gao *et al.*, 2008; Kong *et al.*, 2012). MKK3, the only member in group B, plays a role in the regulation of pathogen resistance, jasmonic acid (JA) and abscisic acid (ABA) signaling (Dóczy *et al.*, 2007; Takahashi *et al.*, 2007; Verma *et al.*, 2020). MKK4 and MKK5 in Group C, together with the downstream MPK3 and MPK6, are involved in regulating development and defense in Arabidopsis (Asai *et al.*, 2002; Ren *et al.*, 2002; Wang *et al.*, 2007; Zhang, M *et al.*, 2017; Bi *et al.*, 2018; Sopeña-Torres *et al.*, 2018; Sun *et al.*, 2018; Shao *et al.*, 2020). In Group D, constitutively activated MKK7 and MKK9 could replace MKK4/5 to activate MPK3/6 in stress resistance and stomatal differentiation (Zhang *et al.*, 2007; Xu *et al.*, 2008; Lampard *et al.*, 2014). MKK10 activates MPK6 to mediate red light response in cotyledons (Xin *et al.*, 2018). Based on the limited number of MKKs and high redundancy in the subfamily, it is possible that MKKs might have multiple functions as signaling relays between MKKKs and MPKs in different cascades.

MPKs with the TXY activation motif are activated by MKKs and could be classified by the middle amino acid in the TXY motif into TEY subfamily and TDY subfamily (Ichimura *et al.*, 2002; Jonak *et al.*, 2002). The TEY subfamily could be further categorized into three groups. Group A includes MPK3/6/10, and MPK3/6 are the most extensively studied members in MAPK cascades. They were first identified as core modules in responses to biotic and abiotic stresses and then found to be irreplaceable components in growth and development (reviewed in Zhang & Zhang, 2022). MPK Group B consists of MPK4, MPK5, MPK11, MPK12 and MPK13 and the research focus is mainly on MPK4. MPK4 regularly appears in multiple biological contexts and its functions cover immune responses and cold tolerance (Teige *et al.*, 2004; Gao *et al.*, 2008; Kong *et al.*, 2012; Thulasi Devendrakumar *et al.*, 2018). There are only a few reports on members of Group C, which showed that MPK1/2/7 are associated with wounding responses (Sözen *et al.*, 2020), MKK4/5-MPK1/2 regulates the salicylic acid (SA) in leaf senescence (Zhang *et al.*, 2020), and MPK14 regulates auxin responses in root development (Lv *et al.*, 2020; Lv *et al.*, 2021). For the TDY subfamily or Group D MPKs including MPK8, MPK9, MPK15, MPK16, MPK17, MPK18, MPK19, and MPK20, they have the plant-specific TDY motif and lack the C-terminal common docking domain as the interaction site for MKKs (Dóczy *et al.*, 2012). Hence, TDY-subfamily members might not act as canonical MPKs.

Taken together, MAPK cascades play diverse roles *in planta*. A highly conserved cascade MKK4/5-MPK3/6 is repeatedly captured in Arabidopsis and it receives signals from distinct upstream MKKKs (such as YDA and MKKK3/5) or other signal components in different biological processes (Asai *et al.*, 2002; Ren *et al.*, 2002; Wang *et al.*, 2007; Zhang, M *et*

et al., 2017; Bi *et al.*, 2018; Sopeña-Torres *et al.*, 2018; Sun *et al.*, 2018; Huang *et al.*, 2019). Remarkably, YDA seems to hold a relatively conserved upstream position of MKK4/5-MPK3/6. The YDA-MKK4/5-MPK3/6 cascade attracted enormous attention in the last 20 years due to its conservation and pleiotropy in cell fate determination, especially in the contexts of embryos and stomata (Bergmann *et al.*, 2004; Lukowitz *et al.*, 2004; Wang *et al.*, 2007; Zhang, M *et al.*, 2017).

ERECTA-YODA pathway contributes to zygotic polarity

Multicellular organisms rely on well-ordered cell differentiation to develop specialized cells or tissues to carry out life activities. And the first division in the unicellular zygote appears to initiate and guide the delicate and complicated differentiation steps. In most angiosperms, the first asymmetric division in the zygote accompanied by the establishment of cell polarity enables cells to adopt distinct developmental fates (Lindsey & Topping, 1993). After fertilization, Arabidopsis zygote elongates and then divides asymmetrically to yield a smaller apical cell with embryo fate and a larger basal cell primarily contributing mostly to the extraembryonic suspensor (Fig.1A), which establishes an apical-basal axis for further embryonic and body axis formation (Mansfield & Briarty, 1991).

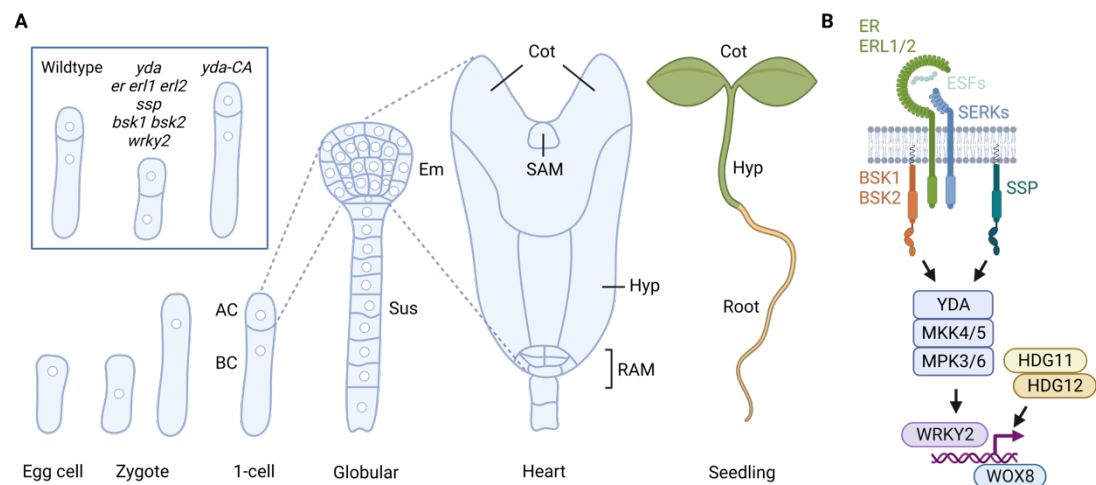


Fig.1 ERECTA-YODA pathway in embryogenesis (created with BioRender).

- (A) Zygotic embryogenesis in Arabidopsis. The fertilized egg cell first loses the polarized structure to have a central-localization nucleus, and then experiences a repolarization process with the nucleus at the apex during the elongation of zygote. When elongation is complete, the zygote reaches its maximum size and divides asymmetrically into an apical cell (AC) and a basal cell (BC). The apical cell contributes to embryo proper (EM) and differentiates into all tissue precursors at heart stage with cotyledons (Cot), shoot apical meristem (SAM), hypocotyls (Hyp) and part of root apical meristem (RAM). The basal cell forms a transient structure called suspensor (Sus) to support embryo proper, and the uppermost suspensor cells integrate into RAM.
- (B) ERECTA-YODA signaling pattern in the embryo. Receptors ERECTA (ER) or ER LIKE 1/2 (ERL1/2), cooperated with the co-receptor SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs), recognize the potential ligands EMBRYO SURROUNDING FACTORS (ESFs) to initiate the signal. BRASSINOSTEROID SIGNALING KINASE 1/2 (BSK1/2) receive signals from the ER receptor complex and trigger the YDA-MKK4/5-MPK3/6 cascade to activate the transcription factor WRKY2 to regulate the WUSCHEL RELATED HOMEODOMAIN 8 (WOX8). SHORT SUSPENSOR (SSP) acts as an independent signal input with a paternal effect to activate the YDA cascade, and HOMEODOMAIN GLABROUS 11/12 (HDG11/12) collaborate with WRKY2 to activate WOX8.

The first asymmetric division and zygotic polarity in the Arabidopsis embryo is controlled by the aforementioned YDA-MKK4/5-MPK3/6 cascade and the upstream signal has been shown to come from the Leucine-rich repeat receptor kinases (LRR-RKs) called ERECTA (ER) and its close paralogues ER-LIKE 1/2 (ERL1/2) (Lukowitz *et al.*, 2004; Zhang, M *et al.*, 2017; Wang *et al.*, 2021). Here, we refer the whole signaling as the ER-YDA pathway to include the main signal transduction components and simplify the following descriptions. The embryonic ER-YDA pathway follows a typical MAPK signaling pattern (Fig.1B), which comprises extracellular ligands, membrane receptor complex, intracellular MAPK cascade

and final substrates in the cytoplasm or nucleus. EMBRYO SURROUNDING FACTOR 1 (ESF1) family proteins are proposed to be the ligands of embryonic ER-YDA pathway (Costa *et al.*, 2014), and they are derived from the central cell and probably recognized by the ER family (ERf) receptors (Wang *et al.*, 2021) with the potential co-receptor SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family members in the early embryos. Another possible candidate is the small peptide CLAVATA3/ESRRELATED 8 (Fiume & Fletcher, 2012), which is exclusively expressed in young embryos and endosperm. Although these previously mentioned molecules influence the development of suspensor, there is still a lack of direct evidence that these two ligands could activate the ER-YDA pathway. When ligand perception occurs in the ER receptor complex, BRASSINOSTEROID SIGNALING KINASE 1/2 (BSK1/2) would function as the signal relays to transmit the phosphorylation signal to YDA (Neu *et al.*, 2019). Besides, SHORT SUSPENSOR (SSP), a close paralogue of BSK1, activates YDA in a phosphorylation-independent manner in parallel (Bayer *et al.*, 2009; Neu *et al.*, 2019). These two signal inputs are integrated into the YDA-MKK4/5-MPK3/6 cascade, and MPK6 phosphorylates the transcription factor WRKY2 to mediate the expression of the patterning protein termed WUSCHEL RELATED HOMEODOMAIN 8 (WOX8) (Ueda *et al.*, 2011; Ueda *et al.*, 2017). HOMEODOMAIN GLABROUS 11/12 (HDG11/12) function together with WRKY2 to maintain the WOX8 expression (Ueda *et al.*, 2017). In addition to transcription factor complexes related to WRKY2 and HDG11/12, RWP-RK domain-containing (RKD) protein GROUNDED (GRD or RKD4) might be the downstream target of YDA, while its functions seem not be associated with the phosphorylation of MAPK cascade (Jeong *et al.*, 2011). In light of other RKD members, GRD might play a role in setting up the transcriptional networks in the transition of egg-to-zygote, ensuring the normal operation of the YDA pathway (Waki *et al.*, 2011; Rövekamp *et al.*, 2016).

An artificial construct *yda-CA* with a deletion at the N-terminus of YDA gives rise to a constitutively active variant that has a longer zygote (Fig.1A). By contrast, defects in zygotic elongation and polarity are commonly detected in the loss-of-function mutants of the ER-YDA pathway such as *yda*, *er erl1 erl2*, *ssp*, *bsk1 bsk2*, and *wrky2* (Fig.1A). And it is intriguing to see the parental effects of different components in the ER-YDA pathway with these mutants. ERf receptors, BSK1/2, MKK4/5 and MPK3/6 are all under maternal control, while SSP functions as an independent and constitutively active signal input with paternal effect (Bayer *et al.*, 2009; Zhang, M *et al.*, 2017; Wang *et al.*, 2021). Together, signal inputs from ER-BSK1/2 and SSP form a Y-shaped pattern to show the independent parental contributions to zygote polarization (Wang *et al.*, 2021).

ERECTA-YODA pathway regulates stomatal polarity

Stomata are specialized epidermal structures in the aerial parts of land plants, regulating gas and water vapor exchange between plants and the environment. Proper density and distribution of stomata is needed to ensure the health and fitness of the seedlings. In *Arabidopsis*, epidermal cells including pavement cells, trichomes and meristemoid mother cells (MMCs) are all derived from the protodermal cells in the embryo. The initiation of the stomatal lineage begins with the cell polarity establishment and the first asymmetric division of the MMC, which generates a small and triangle-shaped meristemoid and a stomatal lineage ground cell (SLGC) (Fig.2A). The meristemoid can undergo several asymmetric divisions to produce new smaller meristemoids, or lose its stem cell-like property to become a round guard mother cell (GMC). The following symmetric division in the GMC finally gives rise to a pair of kidney-shaped guard cells (GCs) with a stomatal pore in between (Fig.2A). The surrounding SLGCs remain the differentiation potential to produce new pairs of meristemoid and SLGC through additional asymmetric spacing divisions. Alternatively, SLGCs can also directly differentiate into pavement cells (Nadeau & Sack, 2002; Bergmann & Sack, 2007; Pillitteri & Torii, 2012; Herrmann & Torii, 2021).

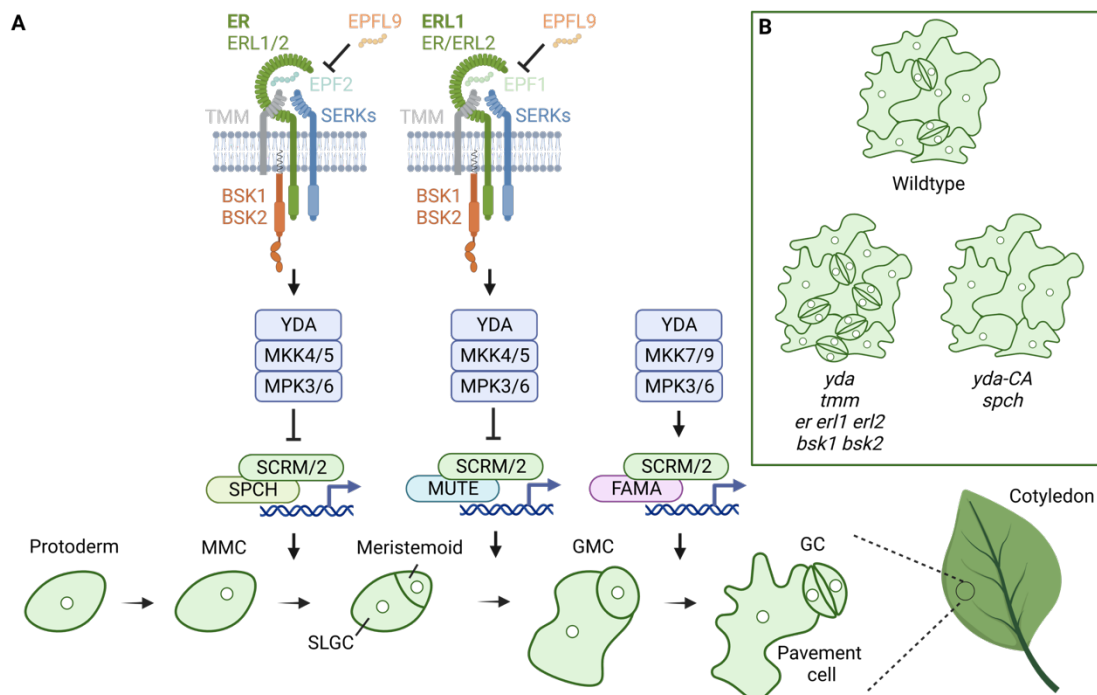


Fig.2 ERECTA-YODA pathway in stomata development (created with BioRender).

(A) Overview of *Arabidopsis* stomata initiation and corresponding ERECTA-YODA pathway. ER/ERL1/ERL2 function together with a receptor-like protein TOO MANY MOUTHS (TMM) and co-receptor SERKs in the recognition of ligands from EPIDERMAL PATTERNING FACTOR (EPF) family. EPF2 triggers the ER-YODA pathway to repress the transcription factor complex of SPEECHLESS (SPCH) and SCREAM/2 (SCRM/2) in the first asymmetric division of meristemoid mother cells (MMCs), and EPF1 induces the signaling cascade to negatively regulate MUTE-SCRM/2 complex in the transition from meristemoid to guard mother cell (GMC). EPF-LIKE 9 (EPFL9 or STOMAGEN) competes for ER receptor complexes with EPF1/2. The final differentiation step from GMC to mature guard cell (GC) is mediated by YDA-MKK7/9-MPK3/6 with an activation effect on FAMA-SCRM/2 complex.

(B) Representative stomatal phenotypes in mutants.

Cell polarization formation and the first asymmetric division in the entry pattern of the

stomata lineage is strikingly similar to that in embryo development and the ER-YDA pathway is recapitulated in the underlying mechanism of the stomata with slight differences in the signaling molecules (Fig.2A). In comparison to embryos, stomata development provides a relatively easier model to dissect the signaling cascade in detail, and nowadays the stomatal ER-YDA pathway has been analyzed in depth (reviewed in Herrmann & Torii, 2021).

During the progression of stomata lineage, several EPIDERMAL PATTERNING FACTOR (EPF)/EPF-LIKE (EPFL) members from a secreted cysteine-rich peptide family have been identified as ligands in the upstream of stomata ER-YDA pathway (Hara *et al.*, 2007; Hara *et al.*, 2009; Hunt & Gray, 2009; Hunt *et al.*, 2010; Kondo *et al.*, 2010; Sugano *et al.*, 2010). The stomatal precursor cell-derived EPF2 peptides are perceived by the cell-surface receptor complex including ERf receptors (ER plays the major role), a receptor-like protein TOO MANY MOUTHS (TMM) and the potential SERK co-receptors. The receptor complex then recruits and phosphorylates BSK1 and BSK2 (Shpak *et al.*, 2005; Lin *et al.*, 2017; Neu *et al.*, 2019). Phosphorylated BSK1 and BSK2 communicate with YDA, which activates the YDA-MKK4/5-MPK3/6 cascade to phosphorylate and destabilize the basic helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH) and SCREAM/2 (SCRM/2) (Kanaoka *et al.*, 2008; Lampard *et al.*, 2009; Putarjuna *et al.*, 2019). The phosphorylation-dependent degradation in SPCH and SCRM interferes the formation of SPCH-SCRM heterodimer and further represses the stomatal polarity and the first asymmetric division in MMC (Putarjuna *et al.*, 2019). Therefore, EPF2 provides an inhibitory influence during the early stomatal development.

When proceeding to the transition from meristemoids to GMCs, the EPF1-directed ER-YDA pathway takes over. In principal, EPF1 is expressed in middle-stage stomata cells (late meristemoid, GMCs and young GCs) and is proposed to maintain the “one-cell-spacing” rule (Hara *et al.*, 2007), namely that two stomata could not be in contact and must be separated by at least one nonstomatal cell. The ER-YDA pathway induced by EPF1 is prominently controlled by ERL1, and the final phosphorylation leads to the degradation in another bHLH heterodimer MUTE- SCRM (Qi *et al.*, 2017). The downstream MUTE-SCRM transcription factor complex, similar to SPCH-SCRM, plays a positive role in stomatal differentiation (MacAlister *et al.*, 2007). In contrast, EPFL9/STOMAGEN derived from mesophyll cells promotes the stomatal proliferation in a paracrine manner, and biochemical evidence indicates that EPFL9 competes with EPF1/2 for ER receptor complexes to inhibit the ER-YDA pathway (Hunt *et al.*, 2010; Kondo *et al.*, 2010; Sugano *et al.*, 2010; Lee *et al.*, 2015; Lin *et al.*, 2017).

The last step of the stomata lineage (from GMCs to mature GCs) is driven by the third downstream bHLH heterodimer FAMA-SCRM/2 (Ohashi-Ito & Bergmann, 2006; Kanaoka *et al.*, 2008). Although the FAMA-SCRM/2 complex is under the control of a MAPK cascade YDA-MKK7/9-MPK3/6 (Lampard *et al.*, 2009; Lampard *et al.*, 2014), phosphorylation of the FAMA-SCRM/2 complex results in an activation effect, which is opposite to the degradation mechanism in the SPCH-SCRM/2 and MUTE-SCRM/2 complexes. Furthermore, it is still unknown about the ligand-receptor pair working upstream of YDA during the symmetric division in GMCs to generate GCs.

In general, ER-YDA pathway prominently provides an inhibitory effect on the initiation of stomata lineage. The loss-of-function mutants in the ER-YDA signaling molecules such as *yda*, *tmm*, *er*, *erl1*, *erl2* and *bsk1*, *bsk2* (Fig.2B) show the increased stomata density and clustered stomata in the cotyledons (Yang & Sack, 1995; Bergmann *et al.*, 2004; Shpak *et al.*, 2005; Neu *et al.*, 2019). On the contrary, the constitutively active YDA version *yda-CA* could strongly inhibit the positive regulator SPCH during the early stage and results in an epidermal structure without any mature GC. A similar no-stomata phenotype is also observed in *spch* (Bergmann *et al.*, 2004; Lampard *et al.*, 2008).

Although the aforementioned signaling components are critical for normal stomata development starting from the first asymmetric division to mature GCs, polarity formation, namely how MMC builds up an axis to give rise to an asymmetric position of the nucleus prior to division, requires some extra polarity cues. The plant-specific protein designated BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) provides a polarity cue through a dynamic subcellular localization, which is first expressed in the nucleus of protodermal cells and then partially accumulates with a crescent shape on the membrane at the cell periphery distal to the migrated nucleus before division (Dong *et al.*, 2009). The polarized accumulation at the cell periphery of BASL is dependent on MPK3/6 phosphorylation (phosphorylated BASL is prone to relocate in the cell periphery), and BASL directly recruits YDA into the cell cortex, thus forming a feedback loop to reinforce the polarity in premitotic MMCs (Zhang *et al.*, 2015; Zhang, Y *et al.*, 2016). Therefore, polarized BASL functions as a scaffold to polarize YDA signaling. POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION (POLAR) is also included as a scaffold protein with BASL to contribute to cell polarity during asymmetric division in stomata. The polarized localization of POLAR is dependent on BASL and follows patterns similar to polarized BASL on the membrane at the cortical sites away from the follow-up division plane in the MMCs (Pillitteri *et al.*, 2011). POLAR recruits GLYCOGEN SYNTHASE KINASE3/BR-INSENSITIVE2 (BIN2) kinase to phosphorylate YDA to inhibit the signal cascade (Khan *et al.*, 2013; Houbaert *et al.*, 2018), which ensures the abundant

SPCH to promote the asymmetric division in MMCs. After the asymmetric division, BIN2 partitions into the nucleus to directly phosphorylate SPCH and relieves suppression of the YDA pathway, thus inhibiting the stomatal differentiation of SLGC (Gudesblat *et al.*, 2012; Houbaert *et al.*, 2018).

Previous genetic evidence suggested that there is a polarity complex comprising of BASL, YDA, POLAR and BIN2 during the stomata lineage, and successful stomatal asymmetric division necessitates the proper spatial distribution of these polarity complex molecules. BRI1 SUPPRESSOR1-LIKE (BSL) proteins from the serine-threonine protein phosphatase family are identified as interacting partners of BASL *in vivo*. Intriguingly, different BSL proteins seem to have opposing effects on stomata development. Membrane-localized BSL1 activates YDA by dephosphorylation and associates with BIN2 to promote BIN2 partitioning to the nucleus at the cell cortex, thereby negatively regulating stomatal initiation (Guo *et al.*, 2021). In contrast, BSL2/BSL3/BSU1 deactivate MPK6 in the nucleus, leading to stabilized SPCH-SCRM complex to promote the stomatal differentiation (Guo *et al.*, 2021; Guo *et al.*, 2022), which indicates a complicated crosstalk between BSLs and the YDA cascade. Taken together, several key components from BASL-centric polarity complex have been characterized, yet the spatiotemporal molecular patterns between different components and potential regulatory feedback loops still need more careful and detailed investigations.

BSKs: signal relays for multiple biological processes

Comparison of signaling components in the ER-YDA pathways between the embryo and stomata indicates that the upstream receptor complexes and MAPK members are highly conserved, and it is clear that BSK1 and BSK2 function as signal relays between the ERF receptor complex and the YDA-MKK4/5-MPK3/6 cascade (Fig.1B and Fig.2A). When we reviewed pathway patterns in plant innate immunity and brassinosteroid (BR) signaling, BSK1 is recapitulated in FLAGELLIN-SENSITIVE 2 (FLS2) receptor complex and BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor complex (Fig.3A).

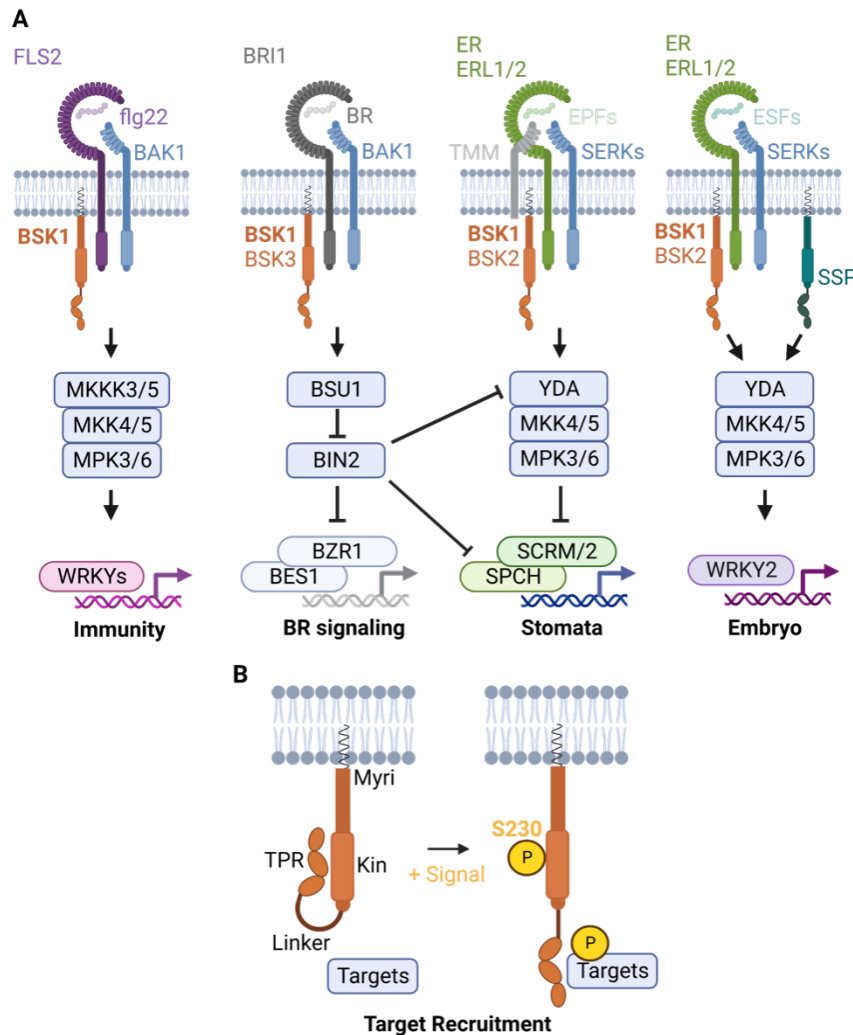


Fig.3 Brassinosteroid-signaling kinases (BSKs) participate in multiple biological signalings (created with BioRender).

(A) Overview of BSKs-directed signaling pathways in Arabidopsis. In plant immunity, BSK1 receives the signal from FLAGELLIN-SENSITIVE 2 (FLS2) receptor kinase and its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE (BAK1/SERK3) to activate MKKK3/5-MKK4/5-MPK3/6 to phosphorylate a group of WRKY transcription factors in response to pathogen infections. BR signaling requires BSK1/3 to transduce the signal from BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor complex with BAK1 to a protein phosphatase termed BRI1 SUPPRESSOR1(BSU1), which further dephosphorylates GLYCOGEN SYNTHASE KINASE3/BR-INSENSITIVE2 (BIN2) to alleviate the phosphorylation-dependent repression on two bHLH transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1(BES1/BZR2). Signaling patterns of the ER-YODA pathways in embryo and stomata displayed here are similar to Fig.1 and Fig.2.

(B) Putative working model of BSKs. N-terminal myristoylation (Myri) directs BSKs to the plasma membrane, and non-phosphorylated BSKs maintain an auto-inhibitory conformation by the intramolecular interaction between the kinase (Kin) domain and the tetratricopeptide repeats (TPR) domain. When upstream signal transduction occurs, the conserved phosphorylation site in the activation loop of the kinase domain would be phosphorylated (for example, Serine 230 in BSK1),

and then the auto-inhibitory would be relieved because of conformation changes. The TPR domain could recruit downstream substrates to transduce the signal.

BSK1 bridges the FLS2 receptor complex and MKKK3/5-MKK4/5-MPK3/6 to trigger pathogen responses by activating WRKY transcription factors and other resistance genes (Shi *et al.*, 2013; Yan *et al.*, 2018). In the BR signaling, BSK1 communicates between the BRI1 receptor complex and the protein phosphatase BRI1 SUPPRESSOR 1 (BSU1) to inhibit the activity of GLYCOGEN SYNTHASE KINASE3/BR-INSENSITIVE2 (BIN2), thus removing the BIN2-directed repression of two downstream bHLH transcription factors BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1) (Tang *et al.*, 2008; Kim *et al.*, 2009). The formerly listed signaling pathways seem to have a conserved choice in applying the BSK members for relay communications, raising the questions about the molecular functions and signaling specificity of BSKs in the diverse biological processes.

The Brassinosteroid-Signaling Kinase family belongs to the receptor-like cytoplasmic kinase (RLCK)-XII subfamily (Liang & Zhou, 2018), and a typical BSK protein comprises a myristoylation site at the N-terminus, a kinase catalytic domain and a tetratricopeptide repeat (TPR) domain at the C-terminus (Tang *et al.*, 2008). BSKs are localized at the plasma membrane due to N-terminal myristoylation, and this subcellular localization is crucial for its biological functions *in vivo* (Zhang, B *et al.*, 2016; Majhi *et al.*, 2019; Neu *et al.*, 2019; Ren *et al.*, 2019; Majhi *et al.*, 2021). 12 BSKs were identified from the Arabidopsis genome, and functions of reported BSK members are principally involved in the BR-responsive pathway and innate immune responses. Arabidopsis BSK1, BSK2 and BSK3 are the first three members characterized. BSK1 and BSK3 are confirmed to associate with BRI1 to perceive the phosphorylation signal. BSK1 serine 230 (S230), localizing at the activation loop of kinase domain, is identified as the main phosphorylation site with BRI1, and phosphorylated BSK1 is supposed to release from BRI1 receptor complex to interact with BSU1 to trigger the downstream signaling (Kim *et al.*, 2009). Overexpression of BSK1, BSK3 and BSK5 could suppress the dwarf phenotypes in *bri1-5* mutant, while phenotypic analyses on the single loss-of-function mutants only detected reduced BR sensitivity in *bsk3-1* (Tang *et al.*, 2008). The genetic results of multiple mutants demonstrated the redundant function of BSKs in BR signaling (Sreeramulu *et al.*, 2013), and BSK3 could play a more predominant role in BR-related growth and development (Ren *et al.*, 2019).

In plant innate immunity, pattern recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs), such as the 22-amino acid epitope from bacterial flagellin (flg22), ELONGATION FACTOR-TU (EF-Tu) peptide (elf18) and fungal cell wall chitin molecules, which is referred as pattern-triggered immunity (PTI) (Couto & Zipfel, 2016; DeFalco & Zipfel, 2021). The best-known immune receptor FLS2 is in charge of

perceiving flg22 with the co-receptor BAK1 to elicit the immune responses including calcium influx, reactive oxygen species (ROS) burst, and activation of MAPKs (Gómez-Gómez & Boller, 2000; Chinchilla *et al.*, 2007). The FLS2 receptor complex requires BSK1 to transduce signals, while FLS2-phosphorylation makes BSK1 dissociated from the FLS2 receptor complex (Shi *et al.*, 2013). The dissociation enables BSK1 to phosphorylate MKKK5 at Serine 289 to trigger the MAPK cascade (Yan *et al.*, 2018). BSK1 also interacts with RECEPTOR-LIKE KINASE 902 (RLK902) on the plasma membrane, and its S230 again acts as the crucial phosphorylation site for the RLK902-mediated defense pathway (Zhao *et al.*, 2019). In addition to BSK1, BSK5 is associated with multiple RLKs in a yeast two-hybrid screen and further assays confirmed the interaction between BSK5 and the immune receptors ELONGATION FACTOR-TU RECEPTOR (EFR) and PEP1 RECEPTOR1 (PEPR1), but not with FLS2 (Majhi *et al.*, 2019). On the contrary, BSK7 and BSK8 are merely related to FLS2-induced PTI, not mediating the immune responses with EFR and PEPR1 (Majhi *et al.*, 2021). It is worth noting that pathogen responses in *bsk5*, *bsk7*, *bsk8*, and *bsk7 bsk8* are only altered in the ROS production, cell wall callose deposition, and expression level of the defense-related gene PATHOGENESIS-RELATED GENE 1 (PR1), whereas the activation of MAPK cascade and pathogen-responsive WRKY genes is not influenced (Majhi *et al.*, 2019; Majhi *et al.*, 2021). These observations could be explained by the redundancy in the BSK family, similar to the situation in BR signaling, although BSKs could display preference for the different immune PRRs.

The redundancy of gene functions in BR signaling and innate immunity could come from the potential dimerization process between BSKs. The tandem TPR domain containing a 34-amino acid structural motif is generally regarded as a scaffold in mediating protein-protein interaction and higher order protein complex formation (Allan & Ratajczak, 2011). Our group uncovered the essential functions of the TPR domains in BSK1 and SSP/BSK12 during the suspensor development (Bayer *et al.*, 2009; Neu *et al.*, 2019), and BSK3 requires the tandem TPRs to fully rescue the defects in BR signaling (Ren *et al.*, 2019). Interestingly, BSK3 can interact with itself and BSK1, and deletion of TPR domains abolishes interactions in BSK3-BSK3 and BSK1-BSK3 (Ren *et al.*, 2019), which suggests the possible homodimer or heterodimer formation between BSKs via TPR motifs. Meanwhile, the interaction between sibling BSKs is common and the loss-of-function effects from the single *bsk* mutant might be quickly complemented by the homodimer from another close orthologue or the heterodimer from two sibling BSKs. Conversely, the TPR motif of rice OsBSK3 negatively regulates its BR-related functions, contradicting the functional necessity of the TPR domain in Arabidopsis BSK orthologues (Zhang, B *et al.*, 2016). This observation could be explained by the putative working model that the TPR motif associates with kinase domain to form an auto-inhibitory state, and certain

conformation changes caused by the protein-protein interactions or post-translational modifications might release the intramolecular interaction between TPR and kinase domains, which makes BSKs free to recruit downstream targets (Fig.3B). Therefore, the proceeding of BSK biological functions is dependent on the synchronous rearrangements of the TPR domain and the kinase domain, raising the question about the kinase activity of BSKs. According to structural analyses of the BSK8 kinase domain (Grutter *et al.*, 2013), BSKs are considered as pseudokinases, and *in vitro* kinase assays for BSK1, BSK3, BSK7, BSK8 and SSP supported the notion (Neu *et al.*, 2019; Ren *et al.*, 2019; Majhi *et al.*, 2021). But the possibility of the potential kinase activity of BSKs *in vivo* could not be excluded in light of the kinase activity of BSK1 in the context of MKKK5 (Yan *et al.*, 2018).

The protein-protein interaction and accompanying protein modifications, especially in BSKs-receptors or BSKs-MAPKs, might rearrange the conformation of BSKs and switch to a catalytically active state *in planta*. Overall, further structural analysis on the differential recruitment mechanism between BSKs and interacting partners, such as sibling BSKs, upstream receptors and downstream substrates, would provide new insights into the signal transduction and specificity determination regulated by BSKs.

A shout-out to SCREAM: an indispensable bHLH transcription factor

The ER-YDA pathway uses the highly conserved MAPK cascade (YDA-MKK4/5-MPK3/6) to transduce the signal in Arabidopsis, whereas it regulates different kinds of transcription factors in the embryo (WRKY2) and stomata (bHLHs) (Fig.3A). It is rational to speculate that there might be a potential bHLH transcription factor complex working in the embryo development due to the high conservation of the ER-YDA pathway. Based on transcriptomic data from isolated embryos, the broadly expressed *bHLH* gene *INDUCER OF CBF EXPRESSION 1 (ICE1)* or *SCREAM (SCRM)* could be a potential target of the ER-YDA pathway during embryogenesis.

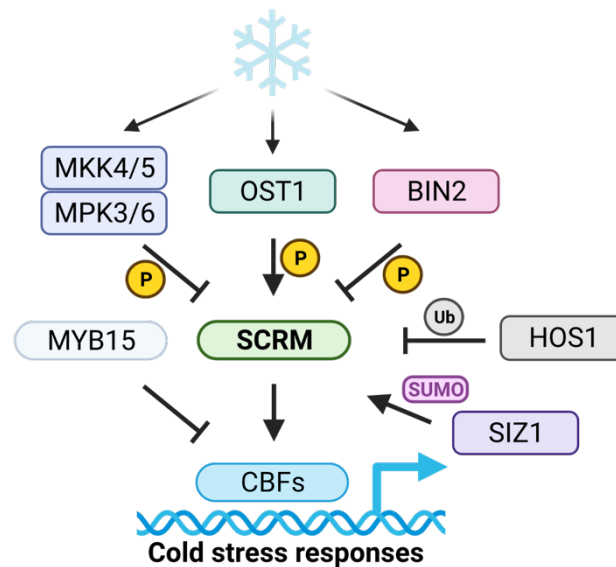


Fig.4 SCRM-centric regulatory networks under cold stress (created with BioRender).

SCRM in freezing tolerance

SCRM or ICE1, was initially identified as a positive regulator for cold-responsive genes *C-REPEAT BINDING FACTOR (CBF)* in freezing tolerance from a genetic forward screen analyzed by the expression strength of *pCBF3-LUC* (Chinnusamy *et al.*, 2003). The genetic screen found a EMS mutant designated *ice1-1D* with a single amino acid substitution (arginine to histidine) at the position 236 (R236H) in the SCRM protein sequence. *ice1-1D* exhibits impaired responses to chilling or freezing stress and loses induction of *CBF* genes in the cold, whereas overexpressing SCRM strongly promotes the expression of CBFs and increases the cold tolerance of the transgenic plants. SCRM is a ubiquitously expressed bHLH transcription factor and recognizes the E box (CANNTG) or G box (CACGTG) motifs to transcriptionally activate downstream genes. The close paralogue SCRM2 or ICE2, shares redundant functions in positively regulating CBF1 expression under cold stress (Fursova *et al.*, 2009).

SCRM has a constitutive expression pattern in contrast to cold-induced CBF expression,

suggesting that its activation effects on CBFs could be mediated by protein modifications. Several regulators involved in the post-translational modifications of SCRM in the cold response have been characterized (Fig.4). HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) is an ubiquitin E3 ligase with a RING finger motif, and interacts with SCRM to mediate the ubiquitination of SCRM (Dong *et al.*, 2006). Overexpression of HOS1 leads to degradation of SCRM and further repression on the downstream cold-responsive genes. In particular, a recent study showed that BIN2 interacts directly with and phosphorylates SCRM, which facilitates the interaction between HOS1 and SCRM, therefore, promoting the HOS1-mediated degradation of SCRM (Ye *et al.*, 2019). Therefore, BIN2 and HOS1 function sequentially as negative regulators of SCRM in the cold responses. Apart from the mentioned BIN2 and HOS1, SAP AND MIZ1 DOMAIN- CONTAINING LIGASE1 (SIZ1) and OPEN STOMATA 1(OST1) are described as positive regulators in enhancing the stability of the SCRM protein. *SIZ1* encodes a small ubiquitin-like modifier (SUMO) E3 ligase and facilitate conjugation of SUMO to SCRM at Lysine in position 393, which prevents the polyubiquitination and degradation of SCRM, thereby enhancing the freezing tolerance in plants (Miura *et al.*, 2007). Additionally, OST1 belongs to SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2 (SnRK2) family and it phosphorylates SCRM at Serine 278 under cold stress to increase the stability and transcriptional activity of SCRM. OST1 associates with HOS1 to interfere the interaction between SCRM and HOS1, which suppresses the HOS1-directed ubiquitination and accompanying degradation of SCRM under cold stress (Ding *et al.*, 2015).

Previously, two independent research groups reported that SCRM is a phosphorylation target of MPK3 and MPK6 in response to cold stress (Li *et al.*, 2017; Zhao *et al.*, 2017). Genetic evidence revealed that MKK4/5-MPK3/6 cascade functions in the upstream of SCRM and the MAPK phosphorylation on SCRM would induce ubiquitination-mediated degradation. Mutagenesis analyses of the SCRM detected phosphorylation sites confirmed the negative regulation by MPK3 and MPK6. By contrast, OsHHLH002 or OsICE1, a SCRM paralogue in rice, has been shown to be phosphorylated by OsMPK3 in response to freezing stress (Zhang, Z *et al.*, 2017). However, phosphorylated OsICE1 could escape from the ubiquitination of OsHOS1 and accumulate *in vivo*, thus enhancing the chilling tolerance in rice. The regulatory outputs of the homologous components (AtMPK3/6-AtSCRM in Arabidopsis and OsMPK3-OsICE1 in rice) are the opposite under the chilling stress, suggesting that the regulatory mode at the same target of MAPKs could change. It also raises the question that whether there is a phosphorylation-dependent activation on SCRM by MAPK cascade before the ubiquitination and degradation steps. SCRM is also in association with some transcriptionally negative regulators in cold stress. MYB DOMAIN PROTEIN 15 (MYB15), a R2R3 type MYB transcription factor, competes

for the CBF3 promoter with SCRM to inhibit cold responses (Agarwal *et al.*, 2006). It is noteworthy that MPK6 recognizes MYB15 as a phosphorylation substrate as well, and the physical association between SCRM-MYB15 makes it possible that there might be a regulatory network between MPK6, SCRM and MYB15 (Kim *et al.*, 2017). Competition for CBF3 promoter binding sites with SCRM to bring up negative effects is also detected in two close bHLH orthologues MYC67 and MYC70 (Ohta *et al.*, 2018). Besides, JA signaling repressor proteins JASMONATE-ZIM-DOMAIN PROTEINS (JAZs) are identified as interacting partners of SCRM (Hu *et al.*, 2013). JAZ1 and JAZ4 repress the transcriptional activity of SCRM to attenuate the induction of CBFs. Further studies showed JAZ proteins could establish direct connections with many bHLH and MYB transcription factors (Fernández-Calvo *et al.*, 2011; Qi *et al.*, 2011), which implies the existence of a high-order complex of transcription factors under cold stress.

SCRM in plant development

Detailed analyses in the field of cold stress demonstrate that SCRM could cooperate with a plenty of interacting partners to cope with complicated situations, suggesting the multiple functions of SCRM. Reports in plant development revealed and confirmed *SCRM* is a *bona fide* pleiotropic gene, which acts as an indispensable player in stomata initiation and seed development in general.

As previously introduced, the ER-YDA pathway adopts different bHLH complexes in each step of stomata initiation, and the complex normally consists of one stage-specific bHLH (SPCH/MUTE/FAMA) and SCRM or SCRM2 (Fig.2). SPCH triggers the first asymmetric division and initial transition from protodermal cells to meristemoid (MacAlister *et al.*, 2007), therefore the *spch* mutant exhibits a non-stomata phenotype in cotyledons (Fig.5). The mutation in *MUTE* causes aborted meristemoids with more SLGCs, while there is no detectable mature GC (Pillitteri *et al.*, 2007). Although *fama* null mutants could proceed to GMCs, the last symmetric division to form the pair of mature GCs is replaced by several rounds of the symmetric division, eventually resulting in some epidermal “tumors” (Ohashi-Ito & Bergmann, 2006). SPCH, MUTE and FAMA express in a short and specific time window, whereas they exert the essential influence on the transition steps. SCRM and SCRM2, as ubiquitous partners, continue their expression during the entire progression of stomata differentiation (Kanaoka *et al.*, 2008). The single loss-of-function mutant of SCRM has the decreased number of mature GCs and stomatal defects similar to the *mute* and *fama* mutants in cotyledon stomata. The double mutant *scrm scrm2* phenocopies the *spch* mutant or *yda-CA* to have the no-stomata epidermis.

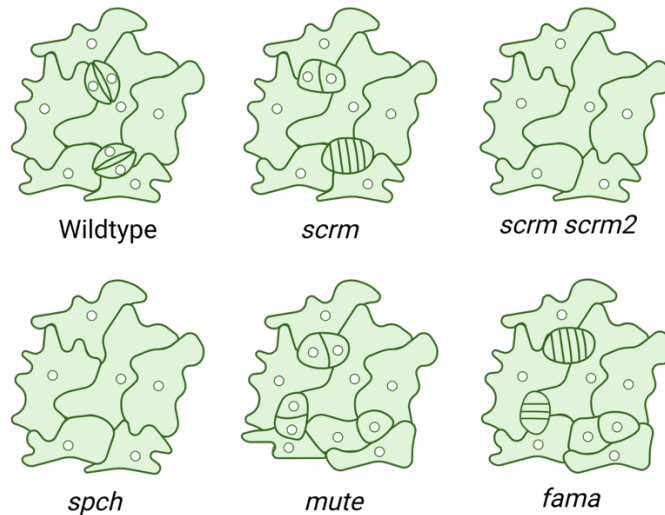


Fig.5 Representative phenotypes in stomata-related *bhlh* mutants (created with BioRender).

Despite weak stomatal defects in the *scrm* single mutant, the association between the ER-YDA pathway and SPCH is dependent on SCRM. Based on the structural analysis, MPK3/6 must first recognize SCRM via its MAPK docking site and KRAAM motif and then phosphorylate the SPCH-SCRM heterodimer (Putarjunan *et al.*, 2019). MPK3/6 could not establish connections with SPCH in the absence of SCRM bridging. Substitutions in the SCRM KRAAM motif would abolish the interaction between SCRM and MPK3/6, further disassociating SPCH from MPK3/6 and eliminating the phosphorylation-directed degradation. Stabilization of SPCH-SCRM promotes excessive stomata in the cotyledon, which is consistent with the stomatal gain-of-function phenotypes of the EMS mutant *scrm-D* with a substitution R236H in the KRAAM motif (Kanaoka *et al.*, 2008). *scrm-D* harbors the same EMS mutation as *ice1-1D*, while the two mutants came from two independent forward screen (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008), emphasizing the importance of the KRAAM motif. Although there are some publications raising questions about SCRM functions in cold stress due to the uncertain genetic background of *ice1-1D* (Kidokoro *et al.*, 2020; Kim *et al.*, 2020), the stomata phenotypes in *scrm-D* and *ice1-1D* are repeatedly confirmed. Recently, another forward genetic screen carried out on *scrm-D* found new mutation sites in the ACT-LIKE (ACTL) domain of SCRM, which attenuated the gain-of-function effects. Further structural studies revealed the ACTL domains between SCRM and SPCH/MUTE/FAMA function as the second interaction surface and determine the specificity of SCRM in switching between bHLH complexes (Seo *et al.*, 2022).

In addition to stomata development, SCRM also participate in several processes with respect to seed development. *SCRM* incorporates with *ZHOUP1* (*ZOU*), an endosperm-specific *bHLH* gene, to regulate the endosperm breakdown (Denay *et al.*,

2014). The *zou* and *scrm* mutant harbors a persistent endosperm, and a precocious endosperm is detected in *scrm-D*, suggesting a synergistic relationship between them. Independent of the functions in endosperm, SCRM-ZOU also determines the depth of seed dormancy, and SCRM *per se* could directly inhibit the ABA-responsive gene ABA INSENSITIVE 3 (ABI3) and ABI5 to promote the seed maturation and germination (Hu *et al.*, 2019; MacGregor *et al.*, 2019). Furthermore, the SCRM-ZOU heterodimer mediates the expression of a subtilisin protease ABNORMAL LEAF-SHAPE 1 (ALE1) in endosperm, which functions upstream of receptor kinases GASSHO1/2 (GSO1/2) to regulate embryonic cuticle formation (Xing *et al.*, 2013; Denay *et al.*, 2014). It is worth mentioning that MPK6 might be the downstream component of GSO1/2 signaling (Creff *et al.*, 2019), indicating a potential feedback loop with SCRM.

Collectively, ubiquitous expression patterns, numerous interacting molecules and diverse modification mechanisms enable SCRM to become a multifunctional gene *in planta*, which suggests its potential to act as a broad and robust regulator in different biological contexts. Therefore, considering the similarity in the ER-YDA pathway between embryo and stomata, it is tempting to speculate that SCRM is a conserved target of the ER-YDA pathway and imposes functions during embryogenesis.

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Objectives and expected outputs

In Arabidopsis, the highly conserved ERECTA-YODA pathway plays an indispensable role in cell differentiation and fate determination with a complicated signaling cascade, whereas it is clear to see the ER-YDA pathway adopts similar but differential signaling molecules to regulate the initiation of embryos and stomata. Based on the observations in the previous publications, this thesis applied a comparative view to revisit the biological functions and associations of certain signaling components to investigate the following topics:

- Signal transduction in the ER-YDA pathway requires the collaboration of BSK1 and BSK2 to bridge the ER receptor complex and YDA cascade, while delicate characterizations of BSK1/2 are missing and the relationships between them are still elusive. To elucidate this, new knock-out mutants generated by CRISPR/Cas9 will be applied for detailed phenotypic analyses in multiple biological processes. The genetic rescue experiments using tagged versions of BSK1/2 and follow-up co-immunoprecipitation mass spectrometry (CoIP-MS) assays would reveal the expression patterns and potential interacting partners *in vivo*, which further sheds light on the underlying mechanism of the synergy between BSK1 and BSK2.

- SCRM, as a phosphorylation target of the ER-YDA pathway in stomata, has high abundance in early embryos, while embryonic functions of SCRM remain unclear. Phenotypic analyses in loss-of-function mutants and expression pattern detection in transgenic plants with fluorescent protein-labelled SCRM will be performed to find out if SCRM participates in embryogenesis. Regulation of SCRM by the ER-YDA pathway would be detected in the stomatal gain-of-function mutant *scrm-D* and phosphor-mutated SCRM versions. We would also study the downstream regulatory networks including the associations with the confirmed regulators WOX8 and WRKY2, and search for potential partners in the SCRM-directed transcription factor complex to provide a comprehensive picture of the embryonic ER-YDA pathway.

Chapter 1: Functional divergences of Brassinosteroid-Signaling Kinase 1/2 in the ERECTA-YODA pathway

Short Summary

BSK1 and BSK2 function as signal relays to transmit phosphorylation signals from the upstream receptor complex to the MAPK cascade in the ERECTA-YODA pathway, although it is not clear whether they have differential functions in the signal transduction and recruitment of the interacting partners. In this chapter, I generated new CRISPR alleles of BSK1 and BSK2 and conducted detailed phenotyping assays which showed the functional divergences in stomata, embryos and rosette leaves. Meanwhile, BSK1/2-YPet transgenic lines showed the differences in expression patterns, and new phosphorylation sites and interacting partners were detected by CoIP-MS assays. Proteomic data indicated that BSK1 prefers membrane receptor components while BSK2 favors cytoplasmic targets, which supports a preliminary model that BSK1 acts as a trigger for the rapid signal transduction and BSK2 behaves as a signal sustainer.

Results

Generation of CRISPR alleles for BSK1 and BSK2

Previous publications used several mutants for BSK1 and BSK2 generated by EMS mutagenesis or transgenic T-DNA insertions, whereas some of them were further identified as non-null alleles (Neu *et al.*, 2019). Our group previously confirmed two loss-of-function mutants *bsk1-2* and *bsk2-2*, and detected the strong defects in stomata and embryos in the double mutant. When considering the absence of phenotypes detected in the single mutant of BSK1 and BSK2 (Neu *et al.*, 2019), it is essential to produce new knock-out mutants with large deletions in the genomic loci to provide additional evidence for phenotypic observations.

To better dissect the differences between BSK1 and BSK2 in biological functions, I generated new knock-out alleles (*bsk1cr* and *bsk2cr*) using an egg-cell-specific expression CRISPR/Cas9 system in the Columbia-0 ecotype. *bsk1cr* harbors a 2764 bp deletion removing the kinase domain and TPR domain, which led to an early stop codon in the final transcripts (Fig.1A and B). The attenuated BSK1 protein only has 35 amino acids (Fig.1C). For the *bsk2cr* mutant, a 3102 bp fragment was removed from the BSK2 genomic locus to completely eliminate the products of the BSK2 gene (Fig.1A). Single mutants were back-crossed twice with Col-0 to get rid of the CRISPR/Cas9 construct and the off-target effect. The double mutant was obtained by crossing with the Cas9-free single mutants. *bsk1cr*

and *bsk2cr* followed the normal segregation ratios in the backcrossing steps, which means that the knocking out worked and produced inheritable alleles in the offspring.

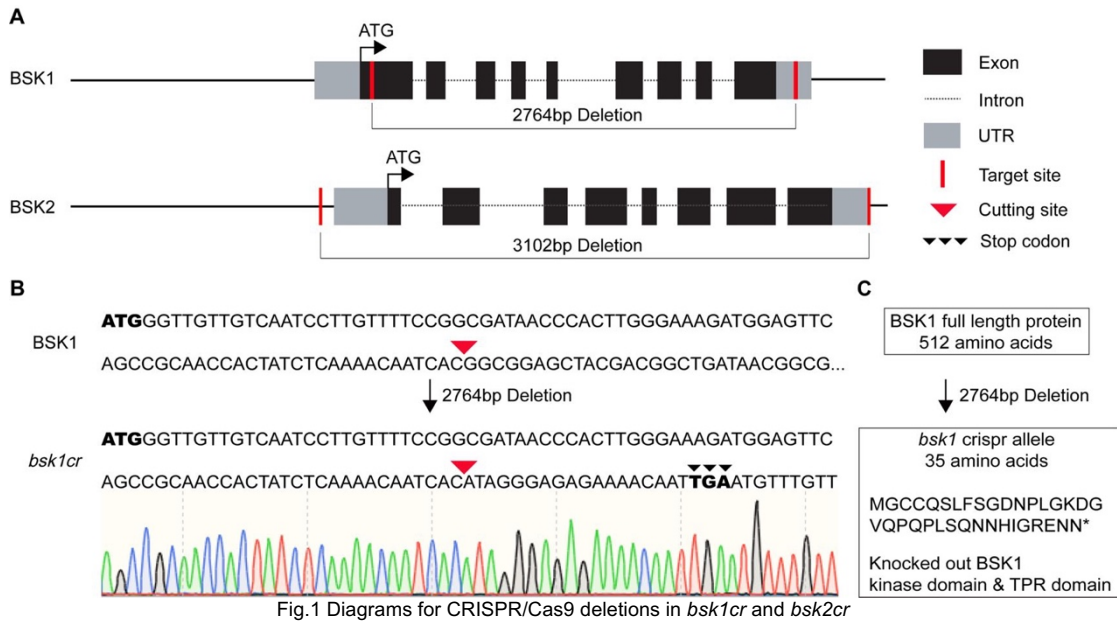


Fig.1 Diagrams for CRISPR/Cas9 deletions in *bsk1cr* and *bsk2cr*

Phenotypic analyses of the new knock-out alleles

To detect the functional differences between BSK1 and BSK2, we took the mutants into phenotypic analyses. Single mutants of *bsk1cr* and *bsk2cr*, similar to the T-DNA mutants (*bsk1-2* and *bsk2-2*), exhibited normal growth and development in the appearance. The double homozygous mutant *bsk1cr bsk2cr* showed similar developmental defects similar to those of *bsk1-2 bsk2-2*, including clustered stomata in cotyledons, small-sized rosette, compact inflorescence, short silique and poor fertility (Fig.2A, B, C, D). Intriguingly, when *bsk1cr +/- bsk2cr* and *bsk1cr bsk2cr +/-* were introduced into the stomata analysis, *bsk1cr* and *bsk1cr bsk2cr +/-* displayed an increase of stomata index in the cotyledons, while *bsk2cr* and *bsk1cr +/- bsk2cr* had no significant difference with Col-0 (Fig.2B). For embryonic phenotypes, *bsk1cr* showed an obvious decrease in zygote elongation compared to Col-0, which is close to the level of the *ssp-6* mutant (Fig.2E, F). There was a weak polarity defect found by counting the ratios of apical cell and basal cell lengths in *bsk1cr* (Fig.2G). Stomatal and embryonic defects observed in *bsk1cr* and *bsk1cr bsk2cr +/-* indicates that the knocking out BSK1 increases Arabidopsis sensitivity during establishment of cell polarity, although both of BSK1 and BSK2 are essential for this process. However, when checking the size of the rosette leaves, we observed a severe reduction in *bsk1cr +/- bsk2cr* and *bsk1cr bsk2cr*. On the contrary, *bsk1cr* and *bsk1cr bsk2cr +/-* appeared to have similar rosette leaves to wildtype (Fig. 2C). Therefore, BSK2 appears to have a more prominent effect on the development of rosette leaves.

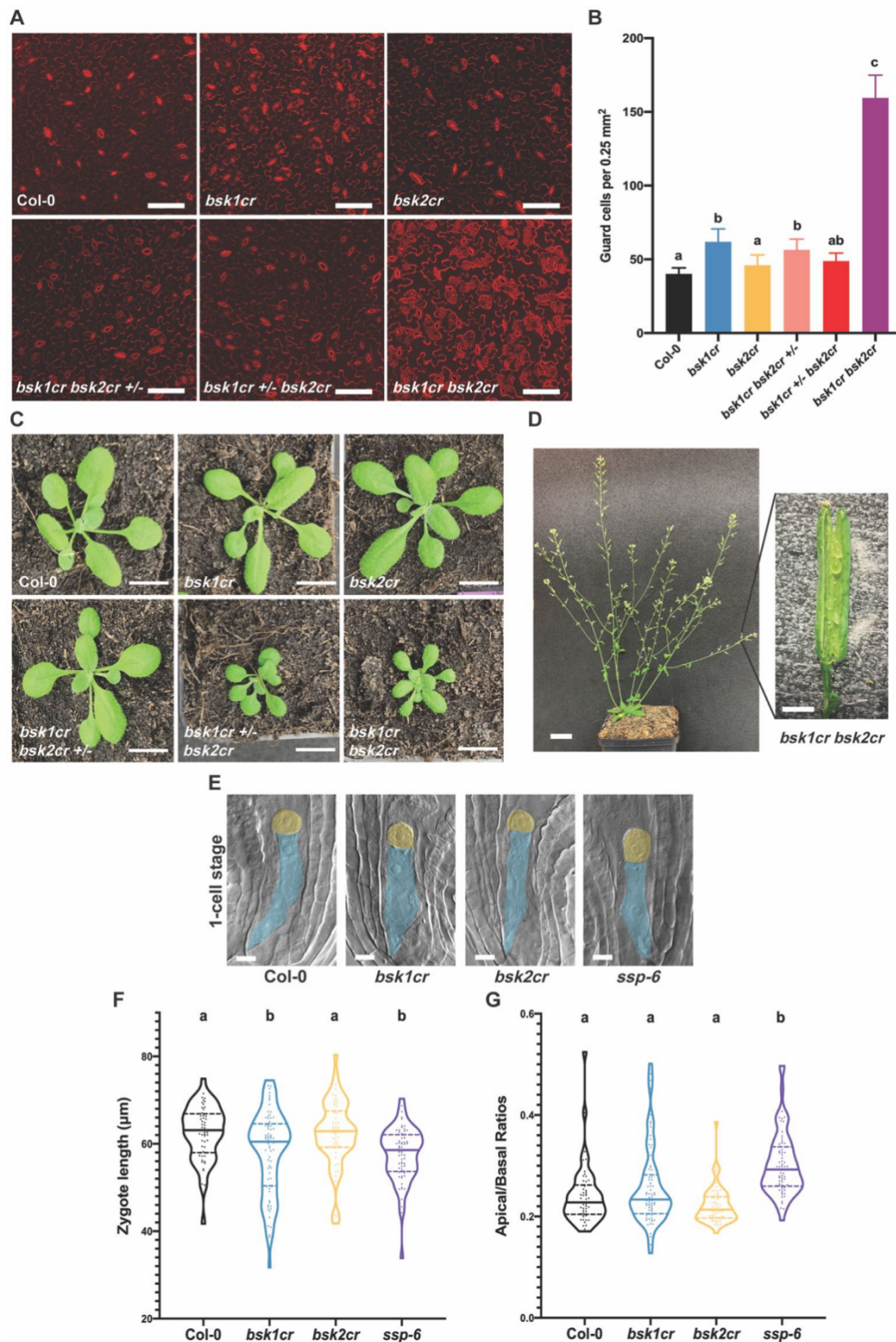


Fig.2 Phenotypic analysis of CRISPR mutants

- (A) Representative confocal images of abaxial cotyledons in 5-dpg (day post germination) seedlings. Scale bar, 100 μm.
- (B) Measurement of guard cells in the cotyledons (the number of guard cells was measured in a 0.25 mm² region, $n \geq 12$ for each genotype).
- (C) Rosette leaves of 15-dpg seedlings. Scale bar, 1 cm.
- (D) Flowering plants of *bsk1cr bsk2cr* (Scale bar, 2 cm) and a typical silique with poor fertility (Scale bar, 100 μm).
- (E) One-cell stage embryos of genotypes as indicated (apical cells colored in yellow and basal cells colored in blue). Scale bars, 10 μm.
- (F) Zygote length (the sum of apical and basal cell lengths at the 1-cell stage, $n \geq 50$ for each genotype).
- (G) Zygotic polarity (ratios of apical and basal cell lengths, $n \geq 50$ for each genotype).

Expression specificity of BSK1 and BSK2

To detect the expression patterns of BSK1 and BSK2, we fused the YPet fluorescence protein sequence to the C-terminus of these genes under the control of their native promoters. These constructs, referred to as BSK1-YPet and BSK2-YPet, respectively, were transformed into the double mutant *bsk1-2 bsk2-2*.

In cotyledons, BSK1-YPet has an even expression in all epidermal cells and there is no detectable polarized localization on the membrane (Fig.3A). BSK2-YPet accumulates more in newly-formed meristemoids or young guard cells compared to pavement cells or mature guard cells (Fig.3A), and BSK2-YPet does not display discernible polarization in our transgenic lines. It is worth noting that BSK2-YPet fully complemented the stomatal defects in *bsk1-2 bsk2-2* mutant, whereas BSK1-YPet only partially rescued the double mutant and still had more mature guard cells in the cotyledons (Fig.3B).

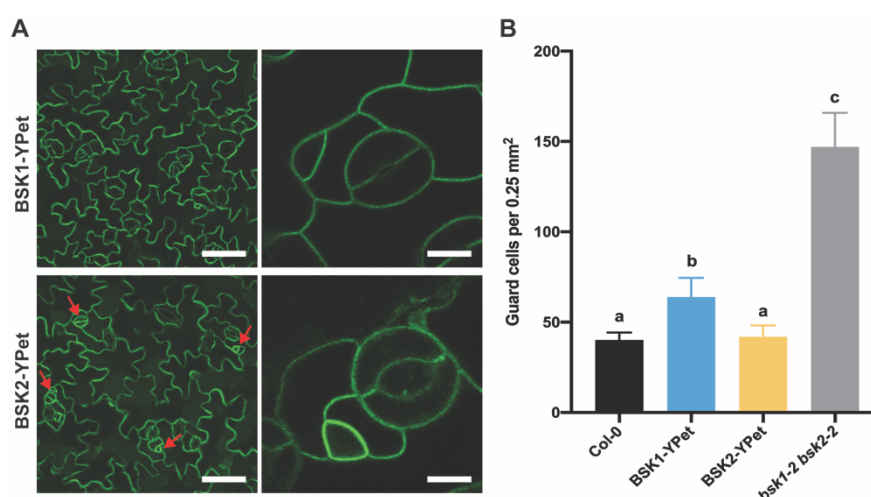


Fig.3 Expression specificity of BSK1 and BSK2 in stomatal lineage cells

(A) Subcellular localizations of BSK1 and BSK2 (green) in the abaxial cotyledons of 5-dpg seedlings. Scale bar, 50 µm for left pictures and 10 µm for right ones.

(B) Stomatal phenotypes in the abaxial cotyledon epidermis of 5-dpg seedlings (numbers of guard cells were measured in a 0.25 mm² region, $n \geq 12$ for each genotype).

Detection of phosphorylation sites and interacting partners

Phenotypic differences in the new knock-out alleles and differential expression patterns of tagged proteins suggested potential functional divergences between BSK1 and BSK2. Therefore, we applied CoIP-MS in the BSK1/2-YPet transgenic lines without external ligand treatments to find out the phosphorylation sites and potential interacting partners to gain a better understanding of the underlying mechanism (Fig.4A).

We succeeded in detecting the serine 230 (S230) in the BSK1 kinase activation loop as previously reported (Tang *et al.*, 2008) and a new phosphorylation site in threonine 353 (T353) located in the linker part between the kinase domain and the TPR domain (Fig.4B,

Supplementary table 2). The homologous site for T353 in BSK1 is only found in BSK10 (Fig.S1), while it is commonly seen in other BSK1 orthologues among angiosperm plants (Fig.S2). Three phosphorylation sites (serine 25, 208, 210) in BSK2 were captured, and the S210 is the homologous site of S230 in BSK1.

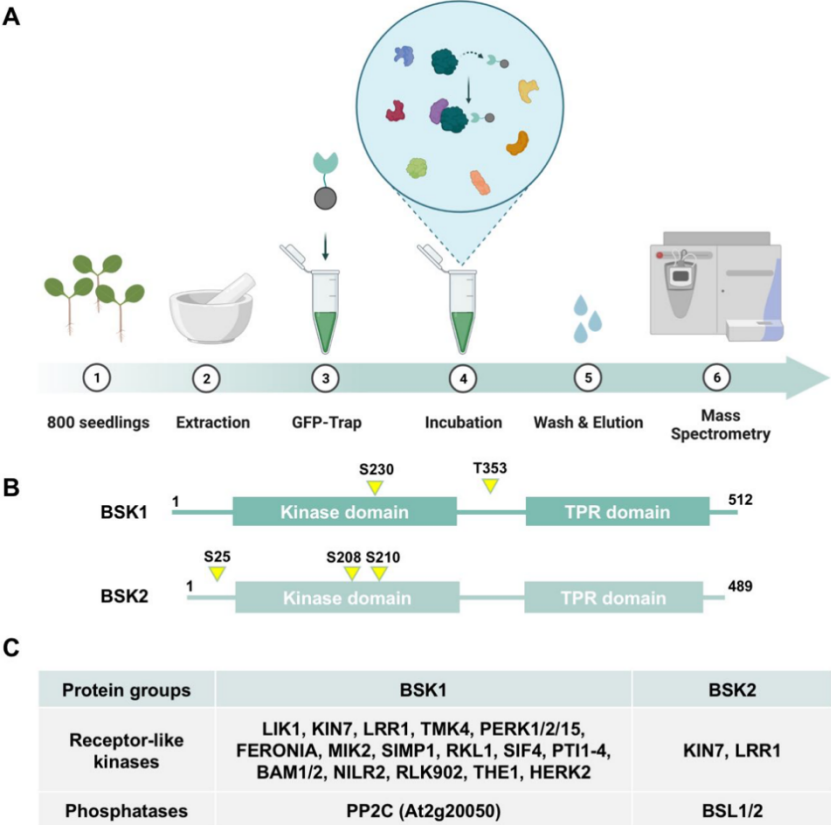


Fig.4. ColP-MS analysis for BSK1 and BSK2
 (A) ColP-MS workflow (produced by BioRender). Total proteins from transgenic seedlings (BSK1-YPet in *bsk1-2 bsk2-2* and BSK2-YPet in *bsk1-2 bsk2-2*) were extracted and incubated with GFP-Trap magnetic agarose beads. The eluted proteins were sent for MS analysis. YFP-Rab7 seedlings were used as control in parallel.
 (B) Phosphorylation sites detected by MS. The yellow triangle indicates the detected phosphorylation sites in BSK1/2.
 (C) Identification of BSK1/2-interacting proteins using ColP-MS. Two protein groups of our interest (receptor-like kinases and phosphatases) were listed in the table, and detailed information can be found in Supplementary Table 1 and Table 2.

In the protein group list, BSK1 associated with a plenty of receptor-like kinases on the membrane involved in various signaling pathways (Fig.4C, Supplementary Table 3). However, typical upstream interacting receptor kinases including BRI1 / FLS2 / ER (Tang *et al.*, 2008; Shi *et al.*, 2013; Wang *et al.*, 2021) and downstream components such as MAPKKK5 or protein phosphatase BSU1 were missing from the list (Kim *et al.*, 2009; Yan *et al.*, 2018). Only one potential protein phosphatase 2C member was found with BSK1. In the BSK2 dataset, we only captured two receptor-like kinases KIN7 and LRR1 with low abundance, but pulled down a large number of unique peptides from BSU1-LIKE 2 (BSL2) (Fig. 4C, Supplementary Table 4). Together, we identified the phosphorylation sites in BSK1 and BSK2 and disclosed the differential preference of the interacting partners without external ligand treatments.

Discussion

Overlapping but not identical functions of BSK1 and BSK2

The BSK family, from the RLCK XII subclade, has been proven to act as the signaling relay between upstream receptor complexes and downstream cytoplasmic protein targets (Cui *et al.*, 2018; DeFalco & Zipfel, 2021), which could be the key point for us to unravel the mystery of signaling specificity determination.

In previous publications, several BSK1 mutants were utilized for the phenotypic analysis. *bsk1-1*, the first reported mutant of BSK1, with a single amino acid substitution in the position 443 of the TPR domain (Arginine to Glutamine, R443Q) was found in the suppressor screen in the ENHANCED DISEASE RESISTANCE 2 mutant background and it exhibited the enhanced susceptibility to pathogens (Shi *et al.*, 2013). Sreeramulu *et al.* (2013) used the *bsk1-3* mutant (SAIL_140_C04) with a T-DNA insertion located in the 3' UTR region, and combined other BSK mutants to show that BSK members function redundantly in the BR signaling. Our group revisited the former BSK1 mutants and found that none of them were null alleles, and identified another *bsk1-2* allele (SALK_122120) with an insertion in the third intron near the BSK1 kinase domain of BSK1 as a loss-of-function mutant (Neu *et al.*, 2019). For BSK2, *bsk2-2* (SALK_001600) was confirmed to have an insertion in the kinase domain that interrupts the normal transcription of BSK2 (Neu *et al.*, 2019). Although *bsk1-2* and *bsk2-2* were identified as the loss-of-function mutants, we still could detect the transcripts before the insertion sites. And it is not clear whether the partial or chimera transcripts produced *in vivo* would have any effects. So, we generated the new null alleles *bsk1cr* and *bsk2cr* by CRISPR/Cas9 and confirmed the developmental defects in *bsk1-2 bsk2-2* double mutant. Interestingly, no obvious polarity defect has been reported in the single mutant *bsk1-2* in the stomata and embryo (Neu *et al.*, 2019), while *bsk1cr* showed the evident phenotypes with increased density in the stomata and decreased length of the zygotes (Fig.2 A, B, E, F, G). This phenotypic difference could be explained by two possibilities: the truncated proteins in the T-DNA mutant *bsk1-2* could be partially functional or it could still recruit other intact BSK members to compensate for its loss based on the finding of heterodimer between Arabidopsis BSK1 and BSK3 (Ren *et al.*, 2019). Only when introducing the mutation from another dominant player in this pathway like BSK2 in the ER pathway, we could observe strong defects as *bsk1-2 bsk2-2* showed, which further confirmed the leading role and overlapping functions of BSK1 and BSK2 in the context of ER-YDA signaling. Observations of the normal stomata and embryos in *bsk2cr* indicated that Arabidopsis seems to be more sensitive to the loss of BSK1 instead of BSK2 during the cell polarity formation.

Surprisingly, the new phenotyping data in CRISPR mutants contradicted the genetic complementary assay that showed BSK2-YPet fully rescued *bsk1-2 bsk2-2* in the cotyledon stomata, while BSK1-YPet only partially complemented (Fig.3B). The possible explanation is that C-terminal YPet fusion could not completely mimic the endogenous BSK1 in protein activity, considering the YDA recruitment function of the BSK1 TPR domain in the C-terminal (Neu *et al.*, 2019). Furthermore, the high-abundance expression of BSK2-YPet in the meristemoids or young guard cells, in contrast to equally expressed BSK1 in the epidermis (Fig.3A), allows a more stable and effective regulation in stomata even though the protein activity is partially compromised. This full rescued effect from the artificial BSK2-YPet construct suggested BSK2 could take over the stomatal functions of BSK1 to some extent, which recapitulates the redundancy between BSK1 and BSK2. It is also notable that *bsk1cr +/- bsk2cr* has small and compact rosette leaves similar to *bsk1cr bsk2cr* (Fig.2C), indicating that BSK2 could occupy a prominent place in the development of rosette although it still needs BSK1. The differential phenotypes and expression specificity of BSK2 further suggested the potential functional divergences with BSK1 in the signaling transduction, which would support the hypothesis that BSK1 and BSK2 have already diverged before basal angiosperms appeared during the land plant evolution (Neu *et al.*, 2019).

Robust trigger or stable sustainer? Evolutionary choices of BSKs!

Former genetic evidence basically confirmed the redundant but differential functions between BSK1 and BSK2, and the following proteomic analysis by CoIP-MS helped us to reveal the underlying mechanism of the functional divergences.

The serine sites in the activation loop of kinase domain seem to be evolutionally conserved in the plant BSK members (Fig.S1 and S2), and the phosphorylation on these sites in BSK1(S230) and BSK2(S210) is associated with BRI1 *in vivo* (Tang *et al.*, 2008). The phosphorylation from BRI1 receptor complex could release the auto-inhibitory from the intramolecular interaction between kinase domain and TPR domain in OsBSK3 (Zhang, B *et al.*, 2016), and this mechanism could be conserved in the plant BSKs except some newly evolved members like SSP/BSK12 in *Brassicaceae*.

However, it is not clear whether this intramolecular inhibition is solely controlled by the conserved S230, and we speculate the signaling specificity from diverse receptor complexes might follow other recruitment mechanism because of totally different downstream targets. Therefore, the new phosphorylation site on T353 would be a cutting point for us to dissect the signaling specificity of BSKs. Although T353 seems to be not common in Arabidopsis BSK members, it might act as a conservative site in BSK1

orthologues among angiosperms because it has already existed in the basal angiosperm *Amborella trichopoda* BSK1 gene (Fig.S2). In particular, the T353 site is localized at the linker part between the kinase domain and the TPR domain, and this small linker region could be the variable region of the BSKs. It would be possible T353 co-operates with S230 to regulate the intramolecular interaction between kinase domain and TPR domain to make sure the signal specificity. More research should be conducted to produce phosphor-inactive and phosphor-mimicking versions in S230 and T353 among BSK1 orthologues from the basal angiosperms to Arabidopsis, and to check the biological responses in BR signaling and ER pathway. Furthermore, *in vitro* assays, to detect the conformation changes in full-length BSK1 proteins when incubated with different receptors, would provide structural information in the BSK working model.

Why did BSK1 evolve to have another phosphorylation site whereas BSK2 did not? The interacting partners from CoIP-MS analysis would provide a potential answer for that. Epidermal equally expressed BSK1 showed a preference for multiple receptor-like kinases on the membrane (Fig. 3A, 4C and Supplementary Table 3) and some associated receptor-like proteins have been shown to be recruited into receptor kinase complexes in innate immunity such as LIK1(Miao *et al.*, 2020) and FERONIA (Stegmann *et al.*, 2017). Therefore, BSK1 may assembly with certain co-receptors or scaffold proteins in advance and waits for transient connections with FLS2 / BRI1 / ER to respond to various stimuli in a rapid way. The phosphorylation site T353 could provide an additional regulatory site for confining BSK1 conformation changes, which would function as a “safety catch” to prevent the accidental activation in other signaling pathways through the conserved “trigger” BSK1. On the contrary, BSK2, enriched expression in meristemoids, favored the downstream targets like BSL2 in the stomatal polarity complex (Guo *et al.*, 2021; Guo *et al.*, 2022) and γ -Tubulin component proteins in the cytoskeleton (Fig.4C, Supplementary table 4), suggesting that BSK2 exerts more influence on the cytoplasmic activities in the cell division instead of receiving signals from membrane receptor complexes. And the interaction between BSK2 and cytoplasmic targets should be tight and stable within meristemoid-like cells, which could therefore explain the strong complementary effect of BSK2-YPet in *bsk1-2 bsk2-2* (Fig.3B). So, BSK2 may not be necessary to evolve towards acquiring an extra phosphorylation site to further restrict its activity similar to BSK1 due to its expression specificity, and it is reasonable to regard BSK2 as a stable “sustainer” to maintain the robust signal in the particular cell types, especially in the ER-YDA pathway. Overall, the differential phosphorylation sites between BSK1 and BSK2 could be linked with the functional differences, which further indicates the evolutionary choices of BSK members in plant kingdom.

Taken together, I would like to propose a preliminary model for BSK1 and BSK2 (Fig.5): BSK1 functions as a common “trigger” for a rapid activation accompanying with many receptor-like kinases on the membrane and has evolved to acquiring an extra phosphorylation site to ensure the signaling specificity in multiple signaling pathways, while BSK2 associates specially with some cytoplasmic targets to act as a signal “sustainer” to guarantee the stable signal levels in the lineage cells during division process. BSK1 and BSK2 could partially take over the gene functions between each other in the ER pathway, and the communications (heterodimer formation or signal transduction) between them could happen after the ligand reception to co-regulate the specific signal transduction.

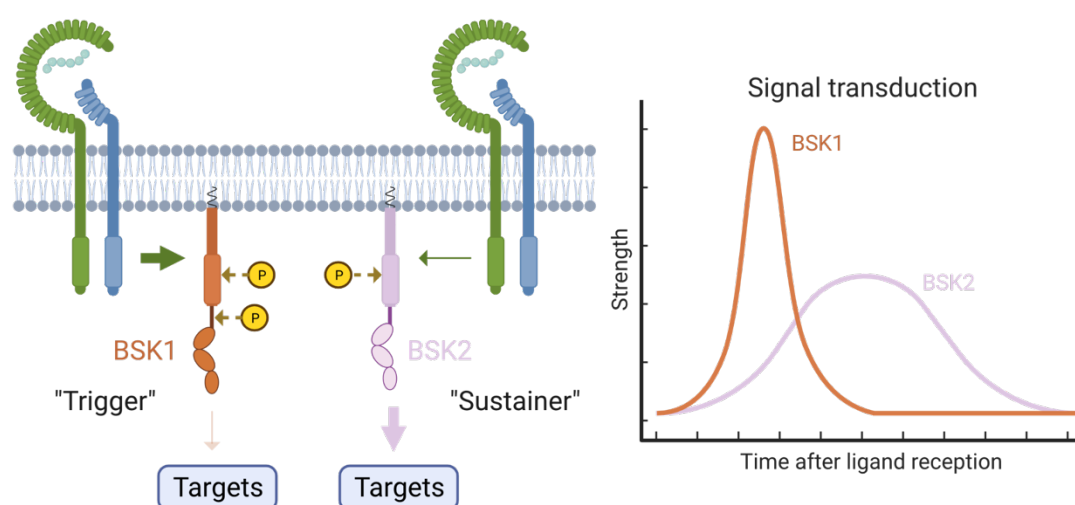


Fig. 5 Preliminary model for BSK1 and BSK2 in the ER pathway (produced by BioRender)

This study identified functional divergences between BSK1 and BSK2 based on mutant phenotypes, protein expression specificity and proteomic analysis, which further revealed evolutionary choices in phosphorylation sites and interacting partners during evolution among BSK members. The preliminary “Trigger and Sustainer” model would provide a totally new view of understanding the evolutionary mechanism between the BSK subfamilies and help us dissect the signaling determination mechanism in multiple signaling pathways. The next investigations should be conducted on the basis of the detailed characterization of the BSK working model from structural biology, and to check the biological responses in diverse signaling pathways to gain a comprehensive understanding of the BSK family.

Materials and methods

Plant materials

All Arabidopsis lines used in this chapter are from the Columbia-0 (Col-0) background and were grown in a plant chamber with a 16 h: 8 h, light: dark photoperiod at 23°C. *bsk1-2* (SALK_122120) and *bsk2-2* (SALK_001600) were described as previously (Neu *et al.*, 2019). *bsk1cr* and *bsk2cr* were newly generated by CRISPR/Cas9 and the methods were described in chapter 3. The genotyping primers were listed in the Supplementary Table S1.

Cloning and plant transformation

The binary plasmid backbone named *pBay-Basta* applied in this chapter was based on *pCambia3300* with some modifications (Wang *et al.*, 2021), and the In-Fusion cloning was used for the cloning steps by In-Fusion HD cloning Kit (TAKARA). Kai Wang produced the BSK1-YPet construct, which contains a 2780 bp BSK1 promoter, a BSK1 genomic sequence without the 6th intron, a C-terminal YPet and a terminator of Ubiquitin 10. The BSK1 promoter and genomic sequence was then replaced by the 2391 bp promoter and 2509 bp genomic sequence of BSK2 to generate BSK2-YPet. The constructed vectors were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation and the floral dip method was applied in the following plant transformation (Clough & Bent, 1998). The positive plants were screened on 1/2 Murashige and Skoog media containing 1%(w/v) sucrose, 0.8%(w/v) agar and 15 mg/L phosphinothricin.

Microscopy and imaging

The measurement of cotyledon stomata was performed with propidium iodide (PI) staining. The cotyledons of 5-day-old seedlings were incubated with 10 µg/ml PI solution (Sigma–Aldrich) for 10 min and rinsed with distilled water. The samples were then transferred to slides with the abaxial side on the top. The following imaging was carried out with a Zeiss LSM780 NLO confocal system (Carl Zeiss) with a 561 nm excitation laser and detectors (571–656 nm) for PI. The BSK1-YPet and BSK2-YPet signals were also detected by the Zeiss LSM 780 NLO microscope (laser with a wavelength of 514 nm for excitation; detectors record emission between 517 nm and 570 nm). Stomata numbers were measured with Fiji 2.0.0 and ZEN 2.0 blue edition software.

The embryonic phenotypes were captured by Differential Interference Contrast (DIC) imaging using a Zeiss Axio Imager. The ovules were dissected and cleared overnight in Hoyer's solution according to the previous article (Bayer *et al.*, 2009), and the lengths of the apical/basal cells at the 1-cell stage were measured by AxioVision 4 and ZEN 2.0 blue edition software.

Co-IP MS

The BSK1-YPet, BSK2-YPet and YFP-Rab7 transgenic lines were harvested after 7 days of growth in 1/2 Murashige and Skoog media and CoIP-MS analysis was performed as described (Singh *et al.*, 2018) with small modifications. The YFP-Rab7 line as control is kindly provided by Dr. Manoj Singh from the Center for Plant Molecular Biology (ZMBP), University of Tübingen. The plant materials were ground with liquid nitrogen and suspended in the lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1x cOmplete™ EDTA-free protease inhibitors cocktail (Roche) and 1x PhosSTOP™ phosphatase inhibitor (Roche). After 30 min of the protein solubilization step on ice, the cell debris was removed by twice centrifugation (10,000g, 15 min, 4°C) and the supernatant of each sample was incubated with 35 µl GFP-Trap magnetic agarose beads (ChromoTek) for 3 h in a cold room (4°C) for binding. The beads were then washed 4 times with wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) supplemented with 0.1% Triton X-100 and 1 time without Triton X-100. The final proteins were eluted with 100 µl 2x Laemmli buffer and sent to the University of Tübingen Proteome Center for MS analysis. Following a tryptic in-gel digestion, LC-MS/MS analysis was conducted on an Easy-nLC 1200 (Thermo Fisher Scientific) coupled to a QExactive HF mass spectrometer (Thermo Fisher Scientific) with the method (60 min, Top07 PHOSPHO, HCD). Data processing was performed with MaxQuant software (version 1.6.7.0) and the spectra were searched against the Arabidopsis thaliana database from Uniprot (<https://www.uniprot.org>). Raw data were processed with a setting of 1% for the FDR (False Discovery Rate), and phosphorus (STY) was set as the variable modification. The iBAQ values were enabled to measure the quantitative values by the ratio of the number of detected peptides in MS analysis to the number of theoretically predicted peptides from the matched proteins.

For each line, three biological replicates were performed in the workflow and the candidates from the list of protein groups would be omitted with the following standards: (1) proteins that interacted with YFP-Rab7 (control samples); (2) nuclear, mitochondrial and chloroplast proteins;

Phylogenetic tree analysis

Protein sequences of Arabidopsis BSK members were downloaded from The Arabidopsis Information Resource (TAIR, <https://www.arabidopsis.org/>) and the sequence information of BSK1-type orthologues in other plants were collected from Phytozome (<https://phytozome-next.jgi.doe.gov/>) as followed: *Physcomitrium patens* (Pp3c22_7510), *Sphagnum fallax* (Sphfalx16G053100), *Nymphaea colorata* (Nycol.I01688), *Amborella trichopoda* (evm_27.TU.AmTr_v1.0_scaffold00001.248), *Brachypodium distachyon* (Bradi3g32330), *Oryza sativa* (LOC_Os10g39670), *Zea mays* (Zm00001d029785) and

Brassica rapa (Brara.A00309). Collected protein sequences were submitted to Clustal Omega online tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) for the multiple sequence alignments and then colored by Jalview (<https://www.jalview.org/>).

Statistical analysis of quantitative Data

One-way ANOVA analysis with the tukey test used for statistical analysis. Diagrams and statistical analyses were made by Prism 9.3 (<https://www.graphpad.com/scientific-software/prism/>).

Supplementary Materials

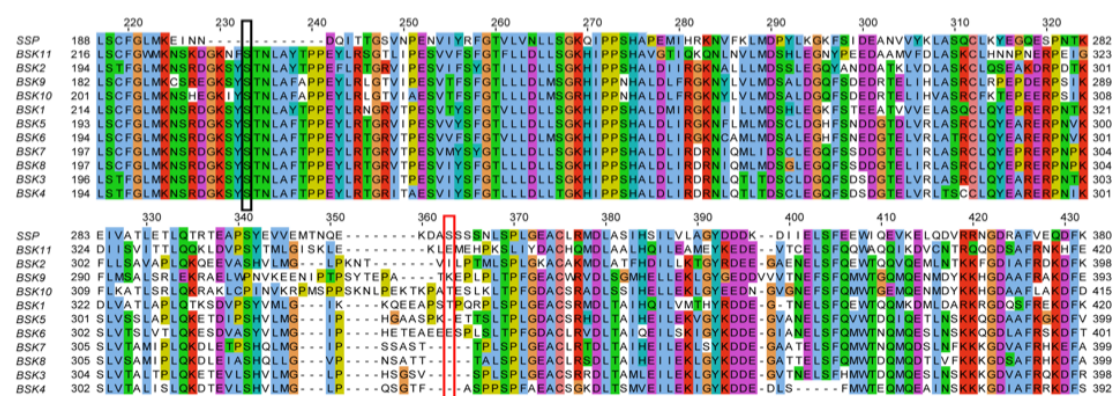


Fig.S1 Multiple sequence alignment of the BSK family in Arabidopsis

The black rectangle indicates the conserved serine site in the activation loop of BSKs, and the red rectangle shows the positions according to T353 in BSK1.

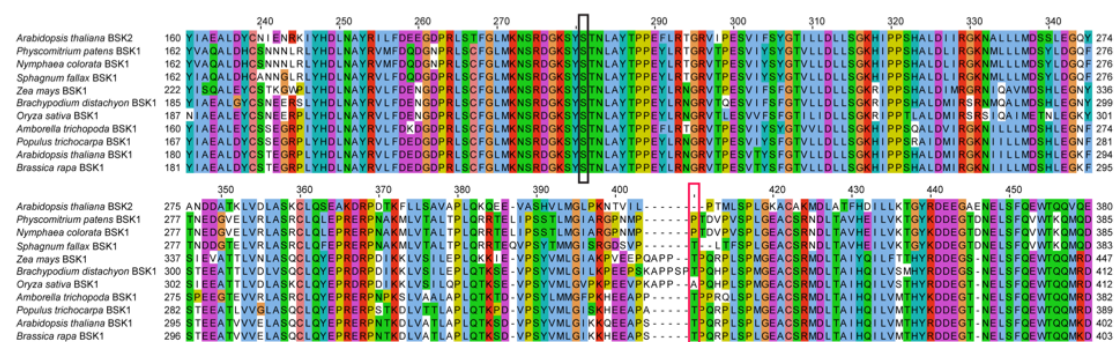


Fig.S2 Multiple sequence alignment of BSK1-type orthologues.

The black rectangle indicates the conserved serine site in the activation loop of BSKs and the red rectangle shows the positions according to T353 in BSK.

Table S1 Primers for mutant genotyping and plasmid construction

Primer names	Sequences	Purposes
bsk1-2 LP	ATGAGGTTGCGAGTAGGAT	bsk1-2 genotyping
bsk1-2 RP	CAAAAGCATGACCAATGAGAAG	bsk1-2 genotyping
bsk2-2 LP	GGAAGCGACTGGTGTGGGAAG	bsk2-2 genotyping
bsk2-2 RP	TGGTCGATCCTTTGCCTCTGA	bsk2-2 genotyping
LBb1.3	ATTTTGCCGATTTCCGAAC	SALK T-DNA primer
bsk1cr-F1	GAAGGAGACAATGTACAGAACTTACC	bsk1cr genotyping
bsk1cr-MD-R1	GCAAGAGTATCATTAGGCATGAACTC	bsk1cr genotyping
bsk1cr-R1	TCAGTACAATCTTGCTTACTAATGCCATG	bsk1cr genotyping
bsk2cr-F1	GTACCTTCAATTATGTACCAACGAGTG	bsk2cr genotyping
bsk2cr-MD-R1	TGATGGATCATCAGAAGAAGG	bsk2cr genotyping
bsk2cr-R1	CTGGGACAGGAACTCACAGATTCTC	bsk2cr genotyping

Table S2 Phosphorylation sites by mass spectrometry

Protein names	Positions within proteins	Localization probability
BSK1	230	0.979251
BSK1	353	0.977251
BSK2	208	0.999328
BSK2	210	0.986817
BSK2	25	0.999753

Table S3 BSK1 interacting protein group from CoIP-MS assay

Protein IDs	Protein names	No. Peptides	Seq coverage [%]	iBAQ
A0A654FW65; Q944A7; A0A178V289	Brassinosteroid-signaling kinase 1, BSK1	36	68.6	7438900000
A0A654F792; A0A5S9XC78; A0A178VFX9; C0LGN2	LYSM RLK1-Interacting kinase 1, LIK1	15	15.5	4244600
A0A384K9F3; C0LGM6; Q9M8T0	Probable inactive receptor kinase At3g02880, Kinase 7, KIN7	13	23.9	10226000
A0A384LF24; C0LGT4; Q9FMD7	Probable inactive receptor kinase At5g16590, Leucine Rich Repeat protein 1, LRR1	11	16.2	3137900
Q9M0A4; A0A384L7C9; A0A654EWQ3; A0A5S9XXR7; A0A1P8AYC4; F4JQC2; A0A1P8AYI8; O82226; A0A178VQ35; A0A178W6K7;	Putative cyclic nucleotide-gated ion channel 9/6/8, CNGC9/6/8	8	11.2	2517800
Q9LK43; A0A654FA27; A0A5S9XF12; A0A178VE58	Trans-membrane kinase 4, TMK4	7	9.3	2392800
F4IUD7; A0A654F559; A0A5S9WZS6; A0A178VM31; Q9SL76	Probable protein phosphatase 2C 19; Probable inactive cyclic nucleotide- dependent protein kinase At2g20050	7	6.6	1378100
A0A1P8AWI5; A0A654EJ81; A0A5S9WL85; A0A178WA51; Q9C821	Proline-rich receptor-like protein kinase PERK15	6	18.5	3355500

A0A178W908; Q9C4Z6; A0A178V9J1; Q9LV28	Receptor for activated C kinase 1B, RACK1B; Receptor for activated C kinase 1C, RACK1C	5	19	343890
A0A178WHX3; O24456-2; O24456	Receptor for activated C kinase 1A, RACK1	5	16.8	3394900
Q9SCZ4; A0A654FEW6; A0A178VHM4; A0A5S9XLJ9; A0A0B4UMR6	Receptor-like protein kinase FERONIA	5	5.8	51970000
Q0WV81; A0A654EPL0; A0A5S9WRG5; A0A384KH77; Q9S7Z8	Auxin efflux carrier component 3, PIN3	5	9.2	3182900
A0A654FMI7; A0A5S9XQJ1; A0A178UX41; Q8VZG8-2; Q8VZG8	MDIS1-Interacting receptor like kinase 2. MIK2	5	5.1	1219500
A0A1P8AXJ2; C0LGM1; A0A5S9X4U9; A0A178W0X1; Q9ZQC2; F4IPZ4; Q84WP2; F4IPZ3	Salt-induced Malectin-like domain-containing Protein1, SIMP1	4	5.3	1765600
Q9LP77; B5X571; A0A178WLI2	Probable inactive receptor kinase At1g48480, RKL1	4	5	662370
A0A178UG34; A0A654GAP0; O65717	Cyclic nucleotide-gated ion channel 1, CNGC1	4	5.4	657050
A0A178VVM7; O80837	Remorin 1.3	4	30.5	8483200

Q9ZSA2; A0A178USG2; A0A5S9XPV7; O49717; F4JKC7; A0A654FRP7; A0A1P8B8Z1; A0A178V2X2	Calcium-dependent protein kinase 21, CPK21; Calcium-dependent protein kinase 15, CPK15.	4	8.3	1623800
F4IB62; A0A178WF19; F4IB63; Q9FZB4; A0A178W7U5; A0A654EJ24; F4IB69; Q9C8I7; A0A5S9WLF9;	Probable LRR receptor-like serine/threonine- protein kinase At1g51820, Stress induced factor 4, SIF4	4	4.1	1706900
A0A1P8AZ55; A0A654F2N6; A0A178VQ50;	Probable receptor-like protein kinase At2g47060, PTO-Interacting 1-4, PTI1-4	4	18.6	2663600
A0A178V1B3; A0A5S9XVQ6; A0A1P8B8L9; A0A1P8B8K3; Q8L710; A0A654FS10; Q38991;	Cysteine-rich receptor-like protein kinase 17	4	6.5	852110
A0A5S9XJG4; A0A178VJB0; Q9M2Z1; A0A5S9YJF0; A0A178UM62; O49545	Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM1/2	4	4.1	232170
A0A5S9WPM4; A0A178WLP5; C0LGG9-2; C0LGG9; F4HRH4; A0A5S9WLG4; A0A178WAA7; C0LGG8-2; C0LGG8	Probable LRR receptor-like serine/threonine- protein kinase At1g53430, Nematode-induced LRR-RLK 2, NILR2	3	5.5	257290
Q9LV48-2; Q9LV48; A0A654FBM9; A0A5S9XF95; A0A178VAK0; A0A178VFC1;	Proline-rich receptor-like protein kinase PERK1/2	3	5.2	2142800
A8MQX0; A0A178WI81; A0A654ECC1; A0A5S9VNI1; A0A178WIH0; Q940Y5-2;	Auxin efflux carrier component 7, PIN7	3	5.4	445000
A0A654F3D6; A0A5S9WXY3; A0A178W0J4; Q9ASQ5	Calmodulin-binding receptor-like cytoplasmic kinase 3, CRCK3	3	5.3	980580

C0LGN3; A0A5S9XDA8; A0A384LKD1; Q9LVI6	Probable inactive receptor kinase RLK902	3	4	196630
Q9LK35; A0A178U7Z8; A0A654GAW2	Receptor-like protein kinase THESEUS 1,	3	3.5	748540
A0A1I9LQD4; A0A5S9XLR7; A0A178VB71; Q6NLQ6; Q6NLQ6-2	Calcium-dependent protein kinase 32, CPK32	3	6.3	950690
A0A178V4I3; Q42479	Calcium-dependent protein kinase 3, CPK3	3	7.6	862760
Q9LUT0; A0A384L906	Putative receptor-like cytoplasm kinase 1, CARK1	3	10.2	457340
A0A178WLW6; A0A5S9WF06; Q9SA72	Probable receptor-like protein kinase Hercules receptor kinase 2, HERK2	2	2.9	81457

Table S4 BSK2 interacting protein group from CoIP-MS assay

Protein IDs	Protein names	No. Peptides	Seq coverage [%]	iBAQ
A0A654G8G5; Q9LS26; A0A178UM21	Brassinosteroid-signaling kinase 2, BSK2	49	77.1	28987000
F4HW26; B9DFZ9; A0A178WHH8; Q9SJF0	Serine/threonine-protein phosphatase, BSL2	21	27.8	14580000
A0A178UJQ8; P38558	Tubulin gamma-2 chain, TUBG2	7	12	1871700
A0A178VJV9; P38557	Tubulin gamma-1 chain, TUBG1	7	12	423820
A0A654FYV7; A0A5S9Y1Z7; A0A178UFG2;	Gamma-tubulin complex component 3, GCP3	4	6.2	290410
A0A178V3F1; A0A654FLE0; Q8L7U5	Serine/threonine-protein phosphatase BSL1	4	5.1	521400
A0A384K9F3; C0LGM6; Q9M8T0	Kinase 7, KIN7	3	5.6	1288800
A0A384LF24; C0LGT4; Q9FMD7	Leucine Rich Repeat protein 1, LRR1	3	5.4	452050
Q0WV81; A0A654EPL0; A0A5S9WRG5;	Auxin efflux carrier component 3, PIN3	2	4.2	17654000
A0A178UTQ0; A0A654G204; A0A5S9Y5S1;	Gamma-tubulin complex component 2, GCP2	2	3.7	226880

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Chapter 2: SCREAM in Arabidopsis embryogenesis

Draft manuscript:

MAP Kinase 6-Dependent Activation of the bHLH Transcription Factor SCRM Promotes Polarization of the Arabidopsis Zygote

Contributions:

Houming Chen and Martin Bayer designed experiments and wrote the manuscript together. Houming Chen constructed binary plasmids and generated transgenic plants. Houming Chen and Daniel Slane set up the CRISPR/Cas9 system and produced knock-out lines. Houming Chen acquired DIC and confocal images and conducted the measurement of embryo and stomata with the help of Kai Wang and Yingjing Miao. Houming Chen conducted the luciferase assays. Feng Xiong performed yeast-one-hybrid assays. Yimin Hu tested prokaryotic expression and conducted alphafold predictions. Rebecca Schwab took charge of *in situ* hybridizations. Feng Xiong and Thomas Laux provided experimental materials related to WRKY2 and WOX8. All authors commented on the manuscript and final revisions were performed by Houming Chen and Martin Bayer.

MAP Kinase 6-Dependent Activation of the bHLH Transcription Factor SCRM Promotes Polarization of the Arabidopsis Zygote

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Keywords: bHLH transcription factor, zygotic polarity, phosphorylation, MAP Kinase pathway.

Abstract

Cell polarity formation and accompanying asymmetric cell division initiate the cell differentiation processes and contribute to diverse cell types or tissues in eukaryotes. In Arabidopsis embryogenesis, a Mitogen-activated protein kinase (MAPK) pathway including the MAPKK Kinase YODA (YDA) or MKKK4 regulates zygotic polarity and asymmetric division by activating the downstream transcription factor (TF) WRKY2 to control cell fate. Besides, YDA pathway mediates stomatal polarity and division by degrading basic helix-loop-helix (bHLH) TFs such as SPCH and SCRM. Little is known about why the YDA pathway applies distinct downstream TF targets in embryos and stomata, and whether there is a conserved target existing across the aforementioned developmental contexts. Here, we recapitulate SCRM as a phosphorylation target of the YDA pathway during embryogenesis, whereas embryonic SCRM are activated by phosphorylation in contrast to the phosphorylation-dependent destabilization in stomata. SCRM and WRKY2 work synergistically to positively regulate WOX8 expression without direct interaction, suggesting independent TF complexes in between. Further phenotypic screen detected a close paralogue bHLH35 which might participate in the TF complex of SCRM. These findings expand our understanding of regulatory networks under the control of the MAPK pathway in cell differentiation.

Introduction

Multi-cellularity enables organisms to develop specialized cell and tissue types with diverse functions throughout their lifespan, thus contributing to their health and fitness. Asymmetric cell division is a key instrument to break symmetry and to initiate different developmental programs in the daughter cells (Sunchu & Cabernard, 2020; Sugioka, 2021; Bogaert *et al.*, 2022; Guo & Dong, 2022).

In many angiosperms, the first division of the zygote is asymmetric, accompanied by the establishment of a primary apical-basal axis (Lindsey & Topping, 1993). In *Arabidopsis*, the zygote divides asymmetrically to yield a small apical cell with embryo fate and a large basal cell primarily contributing to the extraembryonic suspensor (Mansfield & Briarty, 1991). Polarity establishment in the *Arabidopsis* zygote is controlled by the evolutionarily conserved ERECTA-YODA mitogen-activated protein (MAP) kinase pathway (Lukowitz *et al.*, 2004). Currently known members of this pathway comprise receptor kinases of the ERECTA (ER) family (ERf), that are presumably activated by a yet unknown extra-cellular ligand (Wang *et al.*, 2021). The signal is then transduced by two members of the membrane-associated BRASSINOSTEROID SIGNALING KINASE (BSK) family BSK1 and BSK2 (Neu *et al.*, 2019; Wang *et al.*, 2021). Independent of receptor activation, YDA can be activated by the sperm-derived BSK protein SHORT SUSPENSOR (SSP/BSK12), which seems to represent a naturally occurring constitutively active variant of BSK protein (Wang *et al.*, 2007; Bayer *et al.*, 2009; Zhang, M *et al.*, 2017; Neu *et al.*, 2019). The YDA-dependent MAP kinase cascade includes the MAP3K YODA (YDA) (Lukowitz *et al.*, 2004), MAP KINASE KINASE 4 (MKK4) and MKK5, as well as the MAP KINASES 3 (MPK3) and MPK6 (Wang *et al.*, 2007; Zhang, M *et al.*, 2017). MPK6 activates the downstream transcription factor (TF) WRKY2 in a phosphorylation-dependent manner to regulate the early suspensor-specific gene *WUS-RELATED HOMEODOMAIN 8* (*WOX8*) (Ueda *et al.*, 2017). Defects in zygote elongation and polar division plane positioning are morphological hallmarks of reduced YDA activation and are easily detectable in *er/ER erl1 erl2* triple, *ssp* single, *bsk1 bsk2* double and *wrky2* single mutants (Bayer *et al.*, 2009; Ueda *et al.*, 2017; Neu *et al.*, 2019; Wang *et al.*, 2021).

Although our understanding of YDA signaling in the early zygote has increased significantly in the last years, most of the conceptual knowledge about this pathway has been gained by studying its function in stomata development (Guo *et al.*, 2021; Torii, 2021). Interestingly, while cell lineage initiation in stomata development and zygote polarization seem to rely by and large on the same upstream signal transduction components, there seem to be distinct downstream TF targets. ERf activation is modulated by extracellular peptides that compete for ER binding (Lee *et al.*, 2015; Lin *et al.*, 2017). The epidermis-specific EPIDERMAL PATTERNING FACTOR 2 (EPF2) activates the ER receptor complex while mesophyll-derived STOMAGEN, also called EPF9, binds to ER without kinase activation

(Hunt & Gray, 2009). During the first steps of stomata development, ERF- and BSK1/2-dependent activation of the YDA-MKK4/5-MPK3/6 cascade leads to phosphorylation of the basic helix-loop-helix (bHLH) transcription factor SPEECHLESS (SPCH), marking it for ubiquitination and degradation (Gudesblat *et al.*, 2012). SPCH controls the asymmetric entry division that forms a smaller meristemoid mother cell and a larger stomata lineage ground cell (Wang *et al.*, 2007; Lampard *et al.*, 2008). This first step is reminiscent of YDA function in the early embryo. MUTE and FAMA, two close homologs of SPCH, fulfil consecutive functions in the development of the meristemoid to guard cells. MUTE regulates the differentiation of the meristemoid to a guard mother cell, and is, as in the case of SPCH, negatively controlled by ER-YDA signaling (MacAlister *et al.*, 2007; Pillitteri *et al.*, 2008; Qi *et al.*, 2017). A single round of cell division in the guard mother cell and final differentiation of the pair of guard cells is controlled by FAMA. Here, a MAP kinase cascade including YDA, MKK7/9, and MPK3/6 positively regulates activity (Lampard *et al.*, 2009). However, MUTE and FAMA are not direct targets of MPK6 phosphorylation as they lack a MAP kinase target domain (MPKTD).

SCREAM (SCRM), also named INDUCER OF CBF EXPRESSION 1 (ICE1), a ubiquitously expressed bHLH protein, has been reported as a central regulator in both freezing tolerance and stomata development (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008). Transcriptional activity of SCRM is essential for induction of *C-REPEAT BINDING FACTOR* (CBF) and further cold-responsive genes in cold stress (Chinnusamy *et al.*, 2003; Lee *et al.*, 2005; Kim *et al.*, 2015). Further studies have identified SCRM as a phosphorylation substrate of the MKK4/5-MPK3/6 cascade and MPK3/6-directed phosphorylation on SCRM protein negatively regulates tolerance of freezing *in planta* (Li *et al.*, 2017; Zhao *et al.*, 2017). In stomata development, SCRM and its close paralogue SCRM2 form heterodimers with SPCH, MUTE and FAMA to orchestrate stomata initiation, commitment, and terminal differentiation, respectively (Kanaoka *et al.*, 2008). Loss of SCRM leads to stomata defects and *scrm scrm2* double mutant phenocopies the *spch* mutant without mature guard cells on the cotyledon surface (Kanaoka *et al.*, 2008). Recent research has shown that SCRM functions as a scaffold to bridge MPK3/6 and SPCH via a bipartite motif, the MAPK docking site and the KRAAM sequence (Putarjuna *et al.*, 2019). In a previously isolated gain-of-function mutant *scrm-D*, a single amino acid substitution in the KRAAM motif (R236H) disrupts normal interaction between MPK6 and SCRM and blocks degradation of SCRM (Putarjuna *et al.*, 2019). Consequently, the *scrm-D* mutation leads to increased activity of the SPCH/SCRM-D heterodimer and a strong gain-of-function phenotype with an epidermis almost entirely consisting of stomata.

Here, we show that SCRM also acts as a target of the embryonic YDA pathway and plays a crucial role in zygote polarization and establishment of different cell identities in the daughter cells. Our results suggest that, in contrast to its function in stomata, SCRM activity

is positively controlled by MPK6-directed phosphorylation. Moreover, genetic evidence reveals that SCRM functions in parallel with WRKY2 to ensure normal expression of the patterning gene *WOX8*. In a reverse genetic screen for potential interaction partners of the SCRM embryonic TF complex, we have identified bHLH35 as a potential co-factor for the early embryonic function of SCRM. Our work refines our knowledge of the embryonic YDA pathway and the activation of early patterning genes in plants. It also highlights the diversity of regulation mechanisms on downstream targets in MAPK signaling.

Results

SCRM is a critical factor in zygote polarization

In stomata development, asymmetric division of the stomata lineage ground cell and differentiation of the meristemoid into a pair of guard cells depends on bHLH transcription factors forming heterodimeric complexes with the bHLH transcription factor SCRM (Kanaoka *et al.*, 2008). These bHLH complexes have been described as direct phosphorylation targets of MPK6 in the ERECTA-YODA pathway (Herrmann & Torii, 2021). As the asymmetric division of the zygote is controlled by the same pathway and SCRM transcripts can be detected in the zygote and early embryo (Fig. S1A) (Zhao *et al.*, 2019), a similar function of SCRM in embryo development might be possible.

To determine whether SCRM is a downstream target of the YDA pathway in embryogenesis, we first performed a phenotypic analysis of a loss-of-function allele by measuring the lengths of apical and basal cells at the one-cell stage. At this stage, the combined length of the apical and basal cell represents the final length of the zygote as cell division occurs after the elongation phase (Mansfield & Briarty, 1991). The ratio of apical to basal cell size is hence used as a quantitative read-out of cell polarization as it directly reflects the asymmetric, apical positioning of the division plane in the zygote (Ueda *et al.*, 2017; Wang *et al.*, 2021).

We used a previously published *scrm* T-DNA allele (Kanaoka *et al.*, 2008) as well as a novel CRISPR *scrm-cr* mutant allele in these measurements (Fig. S1B). In comparison to wildtype, *scrm* mutants exhibited shorter zygote length (Fig.1A, B, Fig.S1C) as well as impaired polarity (Fig.1C). Because *yda* homozygous plants are sterile and produce no embryos, we used a CRISPR/Cas9-mediated deletion mutation in the *SSP* gene, termed *ssp-6* (Fig.S1B), as positive control for phenotypic embryo analysis. Whereas either single mutant showed a similar degree of phenotypic defects in zygotes and one-cell stage embryos, enhanced phenotypes were observed in the *scrm ssp-6* double mutant (Fig.1A-E). As loss of SSP does not lead to a full loss of YDA-dependent signaling (Wang *et al.*, 2021), additional loss of SCRM would further impair the pathway and might explain this additive effect. At the transition stage, a similar tendency was evident in suspensor lengths (Fig.1D, E). The embryonic defects mentioned in the above mutants, are hallmarks of YDA-dependent pathway mutants and suggest SCRM as an embryonic downstream target of the YDA cascade.

In a previous study, the *WOX8* gene has been reported as transcriptional target of the embryonic YODA pathway (Ueda *et al.*, 2017). When we analyzed expression of a *gWOX8* Δ -YFP marker line (Ueda *et al.*, 2011) in the *scrm* mutant, we found that the YFP signal in the zygote was significantly reduced compared to wildtype background (Fig.1F, G). Taken together, these data suggest that SCRM is required for cell elongation and the

first asymmetric division in zygotes, as well as strong activation of WOX8 expression.

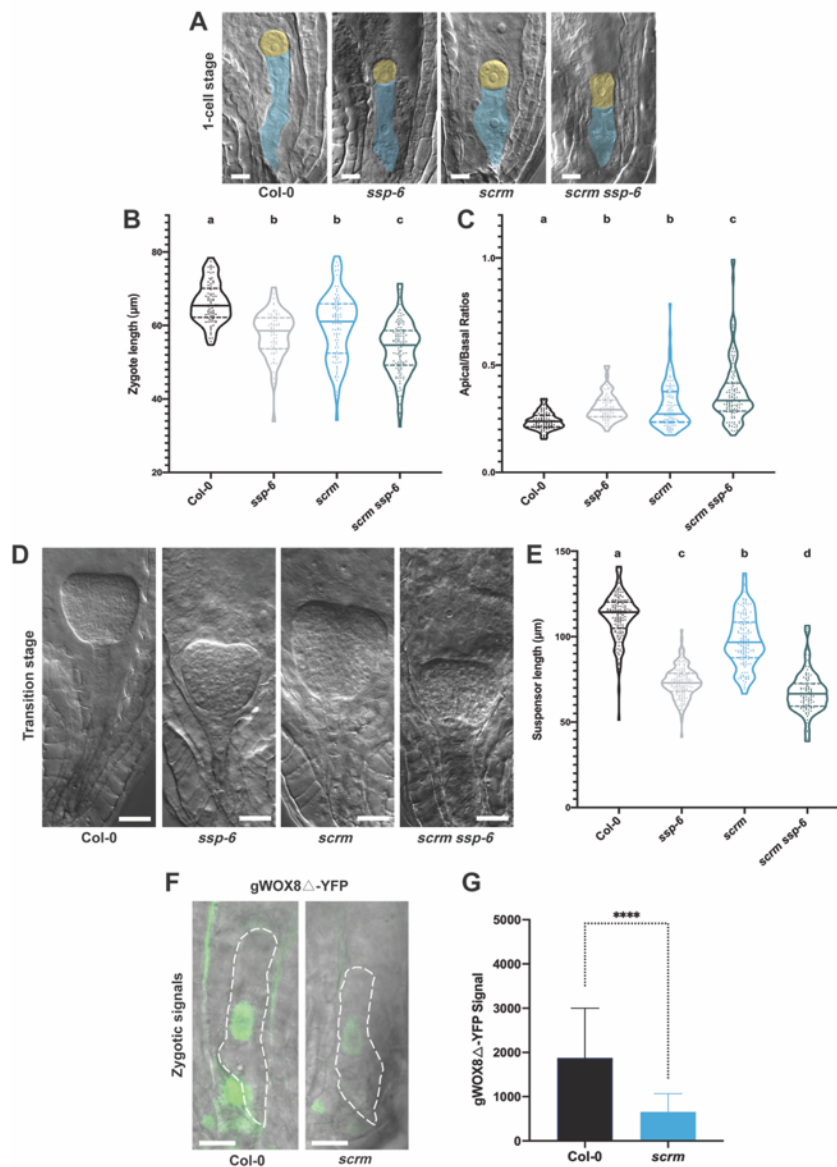


Fig.1 SCRM is required for zygotic polarity and elongation.

(A) One-cell stage embryos of genotypes as indicated (apical cells colored in yellow and basal cells colored in blue). Scale bars, 10 μ m. (B) Zygote length (sum of apical and basal cell lengths at one-cell stage, $n \geq 65$ for each genotype). (C) Zygotic polarity (ratios of apical and basal cell lengths, $n \geq 65$ for each genotype). (D) Transition stage embryos of genotypes as indicated. Scale bar, 20 μ m. (E) Suspensor length in transition stage embryos ($n \geq 95$ for each genotype). (F) Zygotic gWOX8 Δ -YFP signal in zygotes in wildtype and *scrm*. Scale bar, 10 μ m. (G) gWOX8 Δ -YFP signal strength indicated by arithmetic mean intensity in wildtype ($n = 23$) and *scrm* ($n = 22$). Data are means $\pm$ SD and (****) $P < 0.0001$ by Student's *t* test. Letters on plots represent significantly associated categories in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$) in B, C and E.

Embryonic functions of SCRM rely on phosphorylation

Judging by the phenotypic similarity of the *scrm* mutant to mutants of the embryonic YDA pathway and the known regulation of SCRM by MPK6 during stomata development and cold stress, we speculated that SCRM could also be a direct target of MPK6 in the embryonic YDA pathway. To address this, we analyzed the embryonic phenotype of the

stomatal gain-of-function *scrm-D* mutant (Fig.2A-E) (Kanaoka *et al.*, 2008). *scrm-D* is a missense mutation that interferes with MPK6 interaction and MPK6-dependent phosphorylation of the SCRM-D protein. The single amino acid substitution (R236H) in the KRAAM motif leads to ectopic SCRM protein accumulation and strong gain-of-function phenotypes during stomata development (Fig. S2A) (Putarjunan *et al.*, 2019). Interestingly, at the one-cell stage and transition stage, reduced zygote lengths and shorter suspensors were detected in *scrm-D* embryos (Fig.2A-E), reminiscent of *scrm* loss-of-function alleles. Loss of zygote polarization was even more pronounced in *scrm-D* than in *scrm* (Fig.2C), suggesting a dominant negative effect. This would indicate that loss of SCRM can be partially compensated by other factors. A newly produced *YPet-SCRM-D* transgenic line also essentially mimicked the phenotypes of *scrm-D*, showing more stomata in cotyledons (Fig.S2A) and reduced elongation and polarization of zygotes (Fig.S2C, D).

The phenotype of the *scrm-D* allele would suggest that MPK6-dependent phosphorylation is required for SCRM activity in the early embryo, emphasizing on a previously unknown mode of SCRM regulation. In contrast, in the context of stomata development, phosphorylation of the SPCH/SCRM heterodimeric complex leads to ubiquitination and proteolysis. The activity of SCRM is therefore negatively controlled by the ER-YDA pathway-mediated phosphorylation in the epidermis.

To gain insight into the protein abundance of SCRM during embryogenesis, we genetically complemented the *scrm* mutant with transgenic constructs containing the SCRM genomic locus with either a N-terminal or C-terminal translational fusion to the *YPet* fluorescence protein-coding sequence, respectively. Both constructs complemented the *scrm* mutant phenotypes in embryo and epidermis (Fig.2 B, C, E; Fig. S2A, B). Microscopic analysis of the SCRM-YPet reporter lines revealed no or very low expression in pollen and egg cell (Fig. 2F). After fertilization, there was an immediate increase in YPet signal in the zygote nucleus that was persistent in all cells of the later embryo (Fig. 2F). These findings are consistent with published transcriptomic data from isolated embryos (Fig.S1A) (Zhao *et al.*, 2019).

Observing YPet fluorescence at the one-cell stage revealed no obvious difference in the amount of SCRM-YPet protein in the nuclei of the two daughter cells. This further supports our observation that the regulation of SCRM-YPet activity in the early embryo does not rely on cell type-specific degradation and therefore differs from the mode of regulation during stomata development.

The SCRM protein is predicted to harbor six potential MAPK phosphorylation sites (Li *et al.*, 2017; Zhao *et al.*, 2017). To determine whether SCRM functions depend on phosphorylation by the YDA pathway, we mutated all six phosphorylation sites as previously described by Li *et al.*, 2017 to produce phospho-mimetic (Serine or Threonine to Aspartic acid) and non-phosphorylatable (Serine or Threonine to Alanine) SCRM

versions and named them YPet-SCRM 6D and YPet-SCRM 6A, respectively. Phospho-mimetic YPet-SCRM 6D fully restored SCRM function in *scrm* embryos (Fig. 2B, C, E), while non-phosphorylated YPet-SCRM 6A was incapable of suppressing the embryonic defects (Fig. 2B, C, E), supporting the notion that phosphorylation is necessary for SCRM function. This finding was corroborated in a previously reported transgenic line carrying a non-phosphorylatable version of SCRM used in a study by Li et al., 2017, ICE1 6A-GFP. In agreement with our findings for the YPet-tagged version of SCRM 6A, this previously published line did not functionally complement SCRM function in *scrm* mutant embryos (Fig. S2C, D), whereas it gave rise to an increase of stomata index (Fig. S2A, B). These findings demonstrated that the embryonic function of SCRM is dependent on interaction with MPK6 and MPK6-dependent phosphorylation. Notably, the observed gain-of-function stomatal phenotypes and loss-of-function embryonic phenotypes from non-phosphorylated SCRM were consistent with the results obtained with *scrm-D*, highlighting the differences in regulation by the ERECTA/YDA pathway in different developmental contexts.

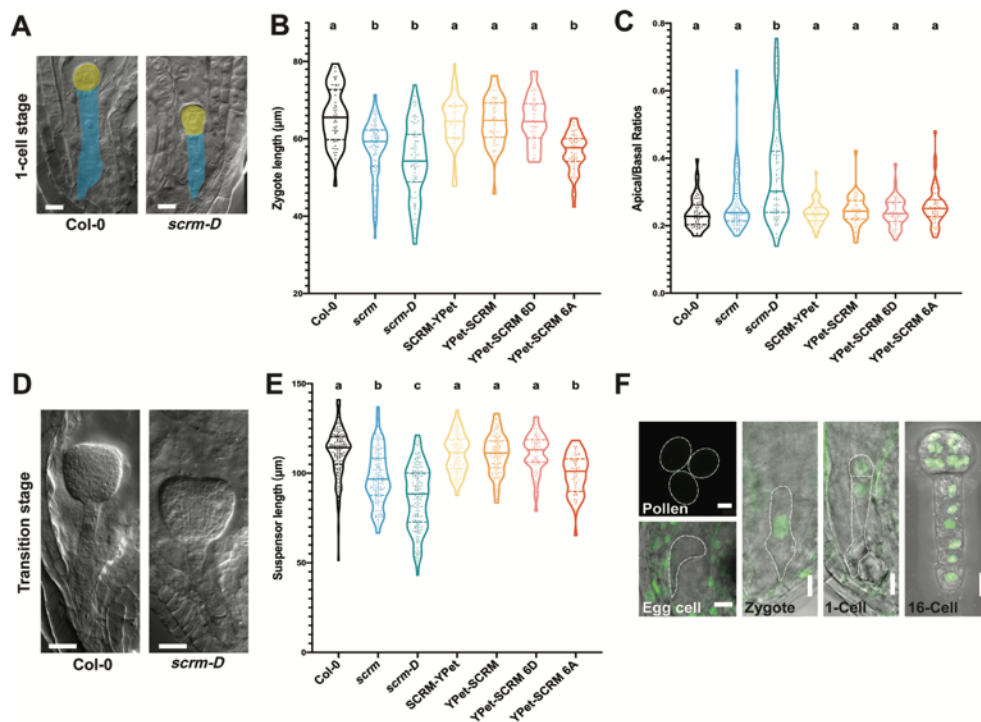


Fig.2 SCRM regulates zygotic asymmetry and patterning via phosphorylation. (A) One-cell stage embryos of genotypes as indicated (apical cells colored in yellow and basal cells colored in blue). Scale bars, 10 μm. (B) Zygote length ($n \geq 45$ for each genotype). (C) Zygotic polarity ($n \geq 45$ for each genotype). (D) Transition stage embryos of genotypes as indicated. Scale bar, 20 μm. (E) Suspensor length in transition stage embryos ($n \geq 50$ for each genotype). (F) Confocal microscopy images of SCRM-YPet in gametes and early embryos. Cell shapes are outlined with dotted line. Scale bars, 10 μm. Letters on graphs indicate significantly associated categories in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$) in B, C and E.

SCRM functions in parallel with WRKY2

Our results confirmed SCRM as a novel target of the embryonic ER-YDA pathway. The reduced *WOX8* expression in *scrm* mutants is reminiscent of WRKY2 function, a

transcription factor that was previously described as a target of the embryonic ER-YDA pathway (Ueda *et al.*, 2017).

To understand the genetic relationship between *SCRM* and *WRKY2*, we analyzed *scrm* and *wrky2-1* single and *scrm wrky2-1* double mutants. Simultaneous loss of *SCRM* and *WRKY2* strongly enhanced the embryonic phenotypes of *scrm* or *wrky2* single mutants with extremely short zygotes and high apical/basal ratios at the one-cell stage (Fig. 3A, B, C), reminiscent of homozygous *yda* embryos. Disordered embryo development with barely a recognizable suspensor, as described for homozygous *yda* embryos (Lukowitz *et al.*, 2004), were detectable in double mutant embryos at later stages (Fig. S3A, B).

The effect on gWOX8 Δ -YFP reporter gene expression in the *scrm wrky2* double mutant was following this trend and showed a further reduction in signal intensity in comparison to the single mutants and wildtype (Fig. 3D).

The additive effects of *scrm* and *wrky2* mutants in the early embryo would suggest that these two transcription factors act independently and are not part of a common transcription factor complex. To address this question, we used a split-luciferase assay using full-length SCRM protein in pairwise combinations with WRKY2 and SPCH. We were unable to detect any interaction between SCRM and WRKY2 (Fig.S3C, D), while the previously published interaction of the SCRM-SPCH heterodimer was readily detectable. This could be an indication that SCRM and WRKY2 act independently in *WOX8* activation (Fig. S3C). We tested a 2.5 kb promoter fragment of *WOX8* in a dual luciferase assay in Arabidopsis suspension cell protoplasts and could detect increased expression of the *pWOX8::LUC* construct in the presence of SCRM, whereas there was no increase in expression of *pWOX2::LUC*. As positive control, we used the known transcriptional target of SCRM in cold stress response *pCBF3::LUC*, which was activated in this assay in the same order of magnitude as *pWOX8::LUC* (Fig. S3E).

In a previous study, cis-regulatory elements for *WOX8* activation by WRKY2 have been mapped to a region termed *cisB* present in the second intron of *WOX8*. In the same study, an additional region upstream of the start codon (*cisA*) was identified as a site sufficient for *WOX8* activation (Ueda *et al.*, 2011). As we could detect activation of *LUC* expression by SCRM with a construct harboring this region, we tested this fragment in a dual luciferase assay. *LUC* activity was strongly increased by SCRM when this element was fused to a minimal 35S minimal promoter (Fig. 3E). Taken together, these findings suggest that *WOX8* activation by SCRM is direct but independent of and additive to WRKY2 function.

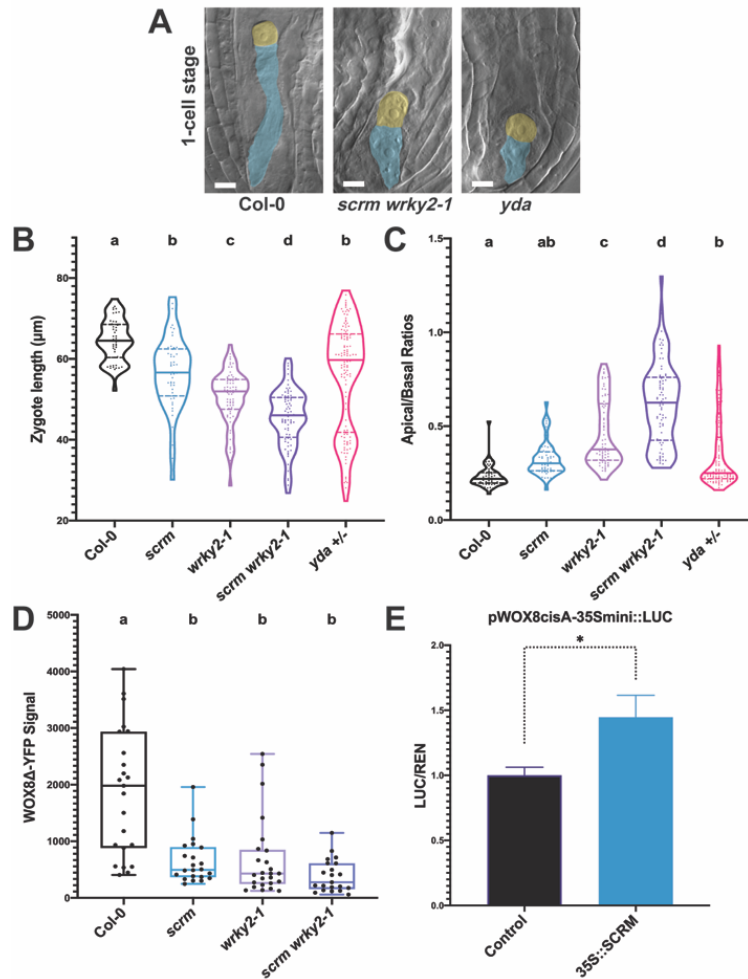


Fig.3 SCRM and WRKY2 work synergistically in zygote asymmetry

(A) One-cell stage embryos of genotypes as indicated (apical cells colored in yellow and basal cells colored in blue). Scale bars, 10 μm . (B) Zygote length ($n \geq 50$ for each genotype). (C) Zygotic polarity ($n \geq 50$ for each genotype). (D) gWOX8 Δ -YFP signal measurements in zygotes ($n \geq 21$ for each genotype). (E) SCRM transcriptionally activates WOX8 cisA element in the dual-luciferase assay. Three biological replicates were applied. Data are means $\pm$ SD and (-) $P < 0.05$ by Student's t test. Letters on plots indicate significantly associated categories in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$) in B and C and D.

Phenotypic screening identifies bHLH35 as potential co-factor of SCRM

Research in stomata development has indicated that SCRM functions as a ubiquitous cofactor in various cell type-specific heterodimers with bHLH proteins of the bHLH 1a subfamily, which contains SPCH, MUTE and FAMA (Herrmann & Torii, 2021). With the assumption that SCRM acts similarly as heterodimer during embryogenesis, we conducted a phenotypic screening of loss-of-function mutants in various bHLH genes based on their detectable expression in early embryos according to publicly available RNA-seq data as well as their close phylogenetic relationship to known interaction partners of SCRM (Fig. S4A) (Toledo-Ortiz *et al.*, 2003; Carretero-Paulet *et al.*, 2010; Zhao *et al.*, 2019). In the aforementioned bHLH 1a subclade, genes of the known interaction partners SPCH, MUTE, and FAMA are not expressed in the zygote and the early embryo. MYC70 showed an increase in RNA level during zygote elongation (Fig. S4A), however a T-DNA

insertion line in *MYC70* did not show zygotic defects and *scrm myc70* double mutant embryos were indistinguishable from *scrm* single mutants (Fig. S4B, C).

To expand our search, we also examined the bHLH IIIb subclade, which contains homologs of *SCRM* (Fig. S4D). The closely related gene *SCRM2*, which has largely overlapping functions with *SCRM* in stomata development, is basically not expressed in embryos (Fig. S4D) and no zygotic or suspensor defects were evident in *scrm2* and *scrm/+scrm2* mutants (Fig. S4E-G). Another homologous gene, *bHLH90* shows low expression during embryo development and its mutant showed no detectable deviation from wildtype zygote elongation and apical/basal ratios (Fig. S4D, H, I). The combination of *scrm* and *bhlh90* also exhibited no significant enhancement of the *scrm* phenotype (Fig. S4H, I). Intriguingly, a more distant homolog in the bHLH IIIb subclade, *bHLH35*, showed increased transcription accompanying zygote elongation and would therefore be expressed at the right time to be a possible interaction partner for *SCRM* (Fig. S4D). Although an obvious polarity defect for the *bhlh35* T-DNA mutant was not observed at the one-cell stage (Fig. 4B), lengths of zygotes and suspensors were shorter than wildtype and similar to *scrm* (Fig. 4A, C). Double homozygous mutant *scrm bhlh35* showed no significant differences in zygote length to *scrm* or *bhlh35* single mutants (Fig. 4A) but a further decline in suspensor length (Fig. 4C). Average value in the ratios of zygotic polarity of *scrm bhlh35* were close to those of *bhlh35*, though it was clear that half of the data points were distributed to higher numbers (Fig. 4B), suggesting a weak loss-of-polarity effect when combining mutation in *scrm* and *bhlh35* mutants.

We then tested the effect of *SCRM* and *bHLH35* on *WOX8* expression in a dual-luciferase assay in *Arabidopsis* suspension cell protoplasts, we observed a strong synergistic activity (Fig. 4D). While expression of *SCRM* lead to an increase in *pWOX8*-driven *LUC* activity, *bHLH35* by itself did not have any measurable effect on *LUC* activity. However, the co-expression of *bHLH35* and *SCRM* lead to synergistic activity and a strong increase of *LUC* activity in comparison to *SCRM* expression alone. This would argue that *bHLH35* is capable of activating *WOX8* expression in the presence of *SCRM*.

In the plant embryo, loss of *bHLH35* leads to a similar decrease in *gWOX8* Δ -YFP expression as in *scrm* mutants when compared to wildtype (Fig. S4J), further supporting their common function.

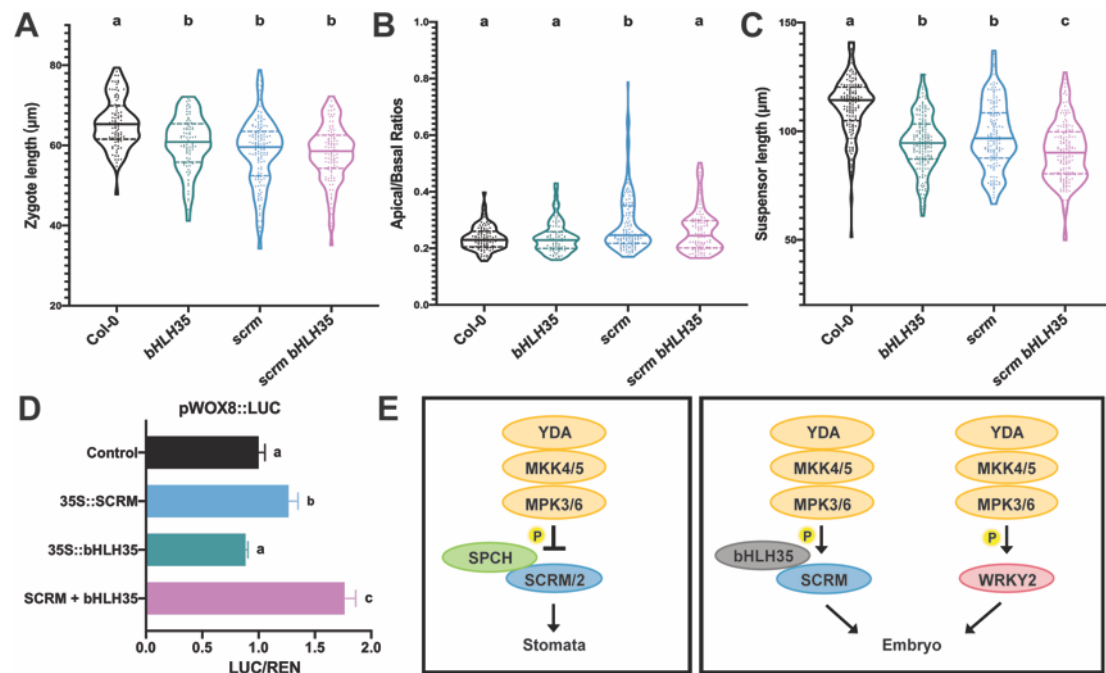


Fig.4 bHLH35, a potential interaction partner of SCRM

(A) Zygote length ($n \geq 80$ for each genotype). (B) Zygotic polarity ($n \geq 80$ for each genotype). (C) Suspensor length in transition stage embryos ($n \geq 144$ for each genotype). (D) Regulation model of zygote polarity. The characters on plots indicate the significance between genotypes ($n \geq 35$ for each group). $P < 0.05$ by one-way ANOVA with a post hoc Tukey test.

Discussion

SCRM, also known as ICE1, has been demonstrated to participate in multiple biological processes *in planta*, including freezing tolerance, seed dormancy, endosperm breakdown and stomata development (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008; Denay *et al.*, 2014; Hu *et al.*, 2019; MacGregor *et al.*, 2019). Former publications have confirmed that it functions as the downstream target of a MAPK pathway (Li *et al.*, 2017; Zhao *et al.*, 2017), and detailed analysis in stomata development places SCRM downstream of the YDA-MKK4/5-MPK3/6 MAP kinase cascade, where it cooperates with the transcription factor SPEECHLESS (SPCH) to control the asymmetric entry division and amplifying divisions to initiate meristemoid cells (Kanaoka *et al.*, 2008; Putarjunan *et al.*, 2019). In zygotes, the ER-YDA pathway phosphorylates WRKY2 to mediate the expression of the basal identity gene *WOX8* (Ueda *et al.*, 2011; Ueda *et al.*, 2017). We found that the expression pattern of *SCRM* displays early zygotic activation in RNA and protein levels, consistent with the model that ER-YDA signaling occurs immediately after fertilization (Lukowitz *et al.*, 2004; Bayer *et al.*, 2009; Neu *et al.*, 2019). The observed phenotypic similarity of zygotic elongation, zygotic polarity and suspensor length between *scrm* and *ssp-6* strongly indicates that they both function in the same pathway, a possibility further supported by stronger phenotypes observed in *scrm ssp-6* mutants, approaching those of the *yda* mutant. Additional evidence for this mutual involvement is provided by our finding that SCRM works as a transcriptional activator of *WOX8*, a known target gene of the embryonic YDA pathway.

Our identification of SCRM as a novel target of the ERECTA-YODA pathway during embryogenesis highlights the multiple roles this protein fulfils in various developmental and stress response pathways (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008; Denay *et al.*, 2014; Hu *et al.*, 2019; MacGregor *et al.*, 2019). In these different contexts, SCRM appears to be universally controlled by MPK6-dependent phosphorylation. Therefore, SCRM appears to be a central hub for linking MAP kinase pathways to bHLH transcription factor complexes as reported by Putarjunana *et al.*, 2019 for the SPCH-SCRM complex.

Phosphorylation-dependent activation of SCRM in zygotes

In the stomatal lineage, MPK3/6 interact with and phosphorylate SPCH and SCRM to reduce their protein stability and transcriptional activity (Lampard *et al.*, 2014; Putarjunan *et al.*, 2019). SCRM functions as a scaffold to recruit MPK3/6 via the MAPK docking site and KRAAM (KiDoK) motif to bridge the MAPKs and SPCH. Underlining this, a *scrm-D* mutation in the KRAAM motif has been shown to abolish associations between SCRM/SPCH and MAPKs (Putarjunan *et al.*, 2019).

Previous studies have shown that the loss-of-interaction *scrm-D* mutant and loss-of-phosphorylation transgenic line give rise to gain-of-function stomata phenotypes due to a lack of phosphorylation-dependent degradation (Kanaoka *et al.*, 2008; Li *et al.*, 2017; Putarjunan *et al.*, 2019). In cold response, the MPK3/6 cascade also acts as a negative regulatory loop to maintain a dynamic SCRM protein level (Li *et al.*, 2017; Zhao *et al.*, 2017). However, our finding that zygote asymmetry defects arising from the *scrm-D* mutant and phosphorylation-deficient transgenic lines were similar to loss-of-function mutants (Fig.2A-E), point to a phosphorylation-dependent activation of SCRM by the embryonic YDA pathway. This is reminiscent of a positive influence on the activity of the FAMA-SCRM heterodimer by MPK6-dependent phosphorylation during guard cell differentiation (Lampard *et al.*, 2009). These findings would suggest that the effect of MAPK phosphorylation on SCRM activity is specific to the cell type, probably depending on interacting partners, such as F-box proteins. Notably, the SCRM ortholog OsICE1 in rice was demonstrated to be phosphorylated by OsMPK3 under freezing stress to prevent its ubiquitination by the E3 ligase OsHOS1, leading to accumulation of phosphorylated OsICE1 and upregulation of *OsTPP1* gene expression (Zhang, Z *et al.*, 2017). This is a further example that the effect of MAPK phosphorylation on SCRM activity is context-dependent. Furthermore, our results raise the question whether activation of SCRM-dependent bHLH complexes by MAP kinase-dependent phosphorylation is a more widespread phenomenon, which might be overlooked in contexts where a possible activation is followed by proteasomal degradation of the SCRM-bHLH complex.

Independent TF complexes synergistically polarize Arabidopsis zygotes

Our phenotypic comparisons of mutant combinations implied that establishment of zygotic polarity and asymmetry requires a complex regulatory network comprising synergistic and parallel-acting factors. We found an enhancement of the loss-of-function phenotype in the *scrm wrky2-1* double mutant in comparison to the single mutants but at the same time, we could not show physical interaction between SCRM and WRKY2 protein. This suggests that these proteins might belong to independent TF complexes which synergistically regulate *WOX8* expression. Notably, in contrast to the specific expression of *WRKY2* in suspensor cells (Ueda *et al.*, 2017), *SCRM* seems to be ubiquitously expressed in all cells of the early embryo. This might further support an independent function of these transcription factors. In addition, the HD-ZIP transcription factors HDG11/12 have been identified as activators of *WOX8* expression, however the connections between SCRM and HDG11/12 still need to be further investigated. It is worth noting that HDG2, a paralogue in the HD-ZIP IV family close to HDG11/12, has a stomata defect in loss-of-function mutants similar to that of *scrm* (Peterson *et al.*, 2013). It would be interesting to elucidate the connections between SCRM and the HD-ZIP IV family in setting up stomata and embryo patterning. Furthermore, HDG11 has been shown to influence *ERECTA* expression, the receptor kinase gene acting upstream of the YDA-dependent MAP kinase cascade (Guo *et al.*, 2019). As *hdg11* mutants show a similar maternal effect as *er* mutants during embryogenesis, HDG11 might also have a pronounced effect on the expression of the upstream receptor complex (Ueda *et al.*, 2017; Wang *et al.*, 2021). This could indicate a complex regulatory network that would warrant further investigation. The independent activation of the same target gene by different transcription factors, that are both controlled by the same MAP kinase pathway, might give the possibility to integrate multiple signaling inputs and also to provide robustness to the signaling output.

To gain more insight into the embryonic SCRM TF complex, we conducted a phenotypic screen in the bHLH Ia and IIIb subclades and identified bHLH35 as a potential partner. Although we were unable to detect direct interaction in a Split-LUC assay (data not shown), synergistic induction of the *WOX8* promoter by a combination of SCRM and bHLH35 indicates the possibility that they could be involved in a higher order complex. MYB15, a R2R3 MYB transcription factor, has been shown to interact with SCRM to repress CBFs expression in freezing tolerance (Agarwal *et al.*, 2006) and to bind both SCRM and bHLH35 to control phosphate accumulation under zinc deficiency (Pal *et al.*, 2017). A recent study unveiled MYB15 as a phosphorylation target of MPK6 in response to cold stress (Kim *et al.*, 2017), which suggests SCRM, bHLH35 and MYB15 could form a phosphorylation-dependent higher-order complex in plants. Functional characterization of MYB15 and detailed dissection of the complex of SCRM, bHLH35 and MYB15 in embryogenesis would be a critical first step in investigating this possibility.

Collectively, this study reveals that the embryonic functions of SCRM are activated by

phosphorylation of the YDA pathway, which differs significantly from the phosphorylation-dependent degradation model in stomata development. It provides a new example of how the MAPK pathway alters regulation mechanisms on a single target to establish functions in different development contexts. Our findings uncover the parallel regulation by SCRM and WRKY2, which not only provides new perspectives on the common bHLH functional model, but also extends the understanding of regulatory networks in embryogenesis.

Methods and materials

Plant Materials and Growth Conditions

Arabidopsis strains used in this research are all from the Columbia-0 (Col-0) background and were grown in a plant chamber with a 16 h: 8 h, light: dark photoperiod at 23 °C. The mutants in this study were described previously: *scrm* (*ice1-2*, SALK_003155), *scrm-D* (R236>H), *wrky2-1* (SALK_020399), *bHLH35* (SALK_016841), *scrm2-1* (SAIL_808_B10), *myc70* (SALK_069605) and *yda-11* (SALKseq_078777) (Kanaoka *et al.*, 2008; Pal *et al.*, 2017; Ueda *et al.*, 2017; Ohta *et al.*, 2018; Wang *et al.*, 2021). *bHLH90* (SALK_052405C) carrying a T-DNA insertion in the 5th intron was ordered from the Nottingham Arabidopsis Stock Centre (NASC). The gWOX8 Δ -YFP marker line was provided by Prof. Thomas Laux's lab (Ueda *et al.*, 2011) and ICE1-GFP 6A was from Prof. Shuhua Yang's lab (Li *et al.*, 2017). Genotyping primers and details are listed in the Supplementary Table1.

Molecular Cloning and Generation of Transgenic Plants

In-fusion cloning by the In-Fusion HD cloning Kit (TAKARA) was used for plasmid construction. The YPet coding sequence (CDS) following a Nos terminator was cloned into a *pBay-Hyg* vector with Sall and KpnI (pCambia backbone with some modifications, details in Wang *et al.*, (2021) to generate *YPet-pBay-Hyg*. An *SCRM* genomic fragment containing 2589 bp upstream sequence before the start codon and 1835 bp genomic region without a stop codon was in-frame introduced between BamHI and Sall into *YPet-pBay-Hyg* to generate *SCRM-YPet*. For *YPet-SCRM*, a 5134 bp upstream region of *SCRM* was firstly cloned into BclI/BamHI sites of the *YPet-pBay-Hyg* backbone, then Nos terminator was digested by ApaI/KpnI and replaced by a 1838 bp *SCRM* genomic sequence with stop codon and 611 bp downstream sequence. *SCRM* genomic fragments were amplified from genomic DNA with Q5[®] High-fidelity polymerase (NEB). *YPet-SCRM-D*, *YPet-SCRM 6D* and *YPet-SCRM 6A* were produced by site-directed mutagenesis PCR using *YPet-SCRM* as a template. In-Fusion cloning and mutagenesis primers were included in Supplementary Table 2.

The binary vectors were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation for the floral dip method in Arabidopsis transformation (Clough & Bent, 1998). The positive transformants were screened on 1/2 Murashige and Skoog media containing 1%(w/v) sucrose, 0.8%(w/v) agar and 20 mg/L hygromycin.

CRISPR/Cas9-Generated Plant Mutants

The CRISPR system in this research was established according to former methods. Plant codon-optimized pcoCas9 sequence (Li *et al.*, 2013) combined with *rbcS* terminator driven by egg-cell specific promoter *pEC1.2-EC1.1* (Wang *et al.*, 2015) was cloned into *pBay-Bar* backbone and the seedcoat specific promoter *pAT2S3* connected with mCherry sequence

(Gao et al., 2016) was subsequently introduced to generate CRISPR destination vector *pBay-Bar-CRmCherry*. To produce the sgRNA cassettes, a pair of 20 bp target sequences was picked according to CRISPR-P 2.0 (Liu et al., 2017) and introduced into shuttle-in vectors pEF004/005 via mutagenesis PCR as described (Wu et al., 2018). The two sgRNA cassettes were subcloned into *pBay-Bar-CRmCherry* between XmaJI and MluI by In-Fusion assembly. The destination constructs were then transformed into Col-0 wildtype by the floral dip method (Clough & Bent, 1998). Positive T1 plants were selected by phosphinothricin resistance on 1/2 MS media containing 50 mg/L phosphinothricin and Cas9-free T2 or T3 mutants were identified by seedcoat mCherry marker. To generate *scrm-cr* and *ssp-6*, sgRNA cassettes were designed to remove genomic sequence (Fig.S1B) and the genotyping primers are detailed in the Supplementary Table 1.

Microscopy Imaging

Differential interference contrast (DIC) imaging with a Zeiss Axio Imager was applied to measure the lengths of apical/basal cells at the 1-cell stage and suspensors at transition stage from ovule samples, which were dissected and cleared overnight in Hoyer's solution as described in Bayer *et al.*, (2009). Length measurements were conducted by AxioVision 4 and ZEN 2.0 blue edition software.

SCRM-YPet embryonic expression and gWOX8 Δ -YFP signals were detected by a Zeiss LSM 780 NLO microscope (Laser with 514nm wavelength for excitation, detectors record emission for wavelength between 517nm and 570nm). Immature seeds were immersed in desalt water and nuclear signal intensity in zygotes was measured by ZEN 2.0 blue edition software.

For propidium iodide (PI) staining, cotyledons from 5-day-old seedlings were dissected and submerged in 10 μ g/ml PI (Sigma–Aldrich) for 10 min. Cotyledon samples were rinsed 3 times with water and then transferred to slides with abaxial side on the top. PI signal detection (571 - 656 nm) was under excitation of 561 nm wavelength laser and measured by Fiji 2.0.0 and ZEN 2.0 blue edition software.

Split-Luciferase Complementation Assay

A split-luciferase complementation assay was conducted as previously described (Zhou *et al.*, 2018). Full-length CDS of *SCRM* and *WRKY2* were cloned into *pCAMBIA1300-Nluc* and *pCAMBIA1300-Cluc* respectively by In-Fusion assembly. Meanwhile *SPCH-pCAMBIA1300-Cluc* was generated as control. Constructs were transferred into *Agrobacterium tumefaciens* GV3101 and positive clones were cultured for 16 h and suspended in induction buffer (10 mM MES pH5.7, 10 mM MgCl₂ and 150 mM acetosyringone) with OD600 at 0.5. Agrobacterial suspension was infiltrated into leaves of 4-week-old *Nicotiana benthamiana* from the abaxial side and tobacco plants were kept in

the plant chamber for 48 h. Then leaf discs (0.6 cm diameter) from plants were poked out and immersed into luciferin substrate (1 mM luciferin aqueous solution with 0.02% Silwet L-77) to detect relative luminescence by Infinite F200 Fluorescence Microplate Reader (Tecan). For western blotting, *SCRM-Nluc* was detected by Anti-luciferase antibody (Sigma, L0159) and Cluc constructs were immunoblotted by Anti-CLuc antibody (Sigma, L2164).

Dual-Luciferase assay

The dual-luciferase assay was carried out according to the published protocol (Hellens *et al.*, 2005) with some modifications. 2511 bp of the *WOX8* promoter was cloned into reporter plasmid *pGREENii-0800LUC* with BclI and NcoI by In-Fusion reaction. 416 bp length of *WOX8 cisA* element (Ueda *et al.*, 2017) was fused with a 35S minimal promoter and together inserted into *pGREENii-0800LUC* between BamHI and NcoI. The effector constructs were generated by inserting CDS of SCRM and bHLH35 after 2 x 35S promoters in *pJIT60* vectors. Reporter and effector plasmids were co-transformed into 125ul protoplast cell suspension with the standard protoplast transfection protocol (Mehlhorn *et al.*, 2018) in the Transformation Facility of the Center for Plant Molecular Biology (ZMBP), University of Tübingen. The luciferase activity was detected using Dual-Luciferase[®] Reporter Assay System (Promega, E1910) by Infinite F200 Fluorescence Microplate Reader (Tecan).

Phylogenetic Tree Analysis

bHLH Protein sequences were downloaded from The Arabidopsis Information Resource (TAIR, www.arabidopsis.org) and submitted to Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) for multiple sequence alignment and phylogenetic tree construction using neighbor-joining method with 1000 bootstraps.

Statistical Analysis of Quantitative Data

One-way ANOVA analysis with tukey test and student's t test and were used for statistical analysis. Diagrams and statistical analyses were made by Prism 9.3 (<https://www.graphpad.com/scientific-software/prism/>).

Supplementary Figures

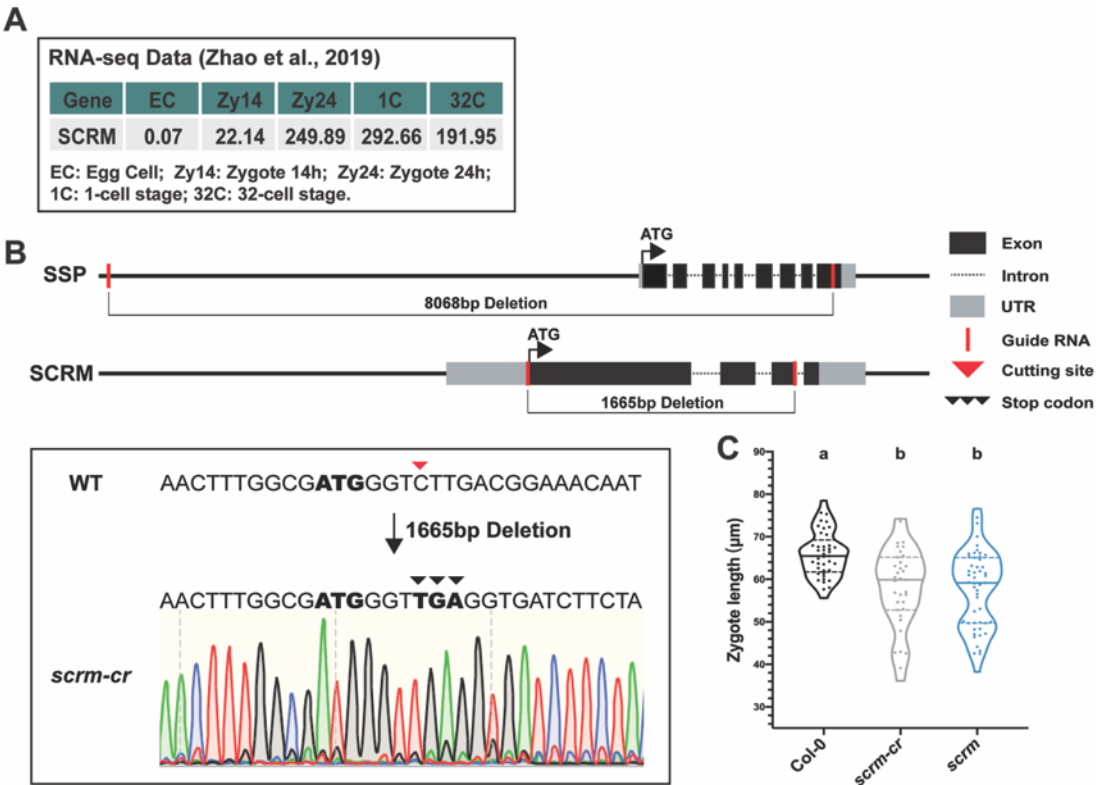


Fig.S1 CRISPR/Cas9-generated *scrm-cr* mutant exhibits zygotic defects.
(A) SCRM RNA-seq data in early embryos (reported in Zhao *et al.*, 2019).
(B) Diagrams for *ssp-6* and *scrm-cr* CRISPR deletions in genome.
(C) Zygote length ($n \geq 35$ for each genotype).

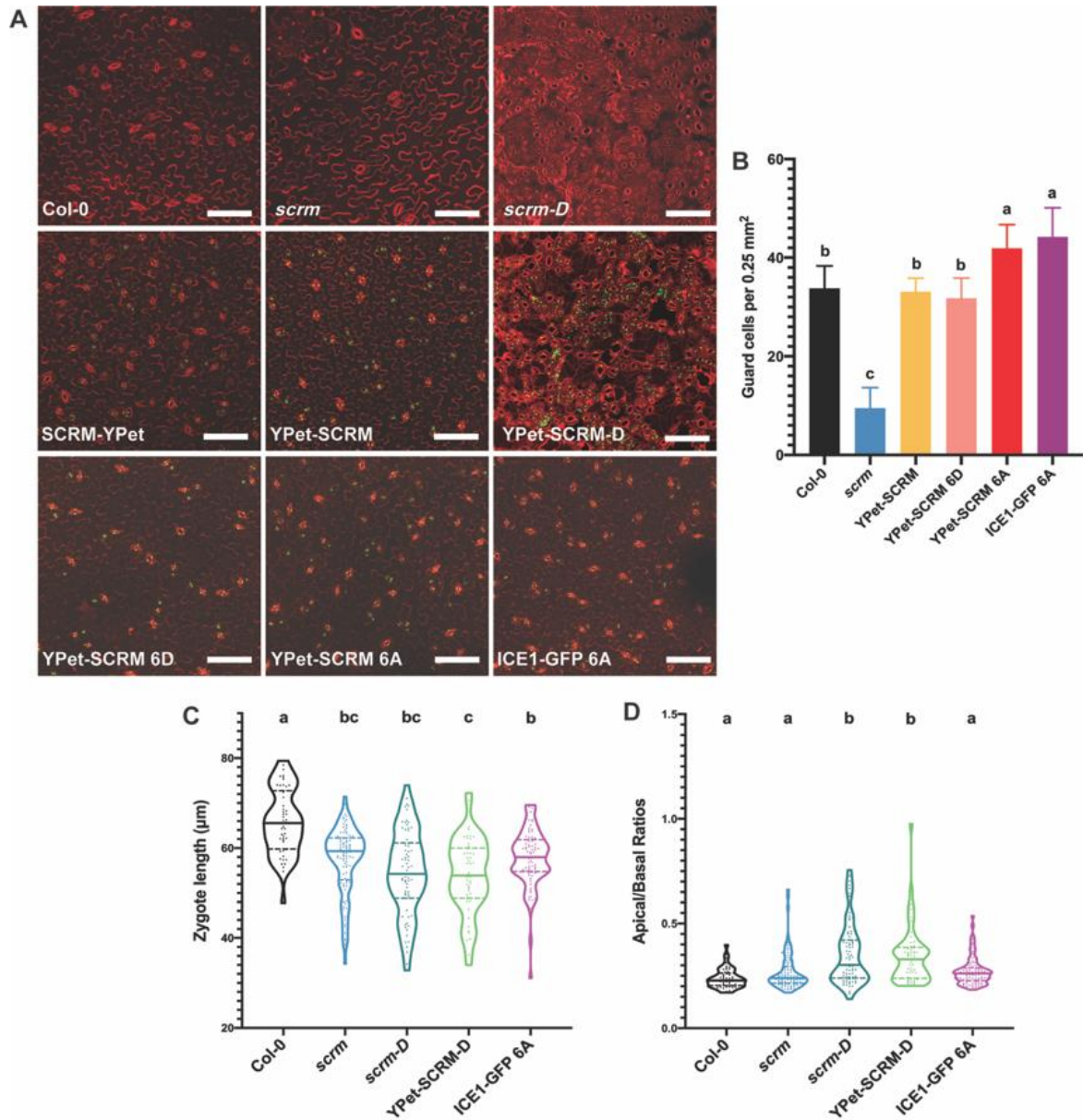


Fig.S2 Stomatal and embryonic phenotypes in transgenic SCRM variants
 (A) Confocal images of 5 dpf (short for 5day post germination) abaxial cotyledon of genotypes as indicated. Scale bar, 100 μ m.
 (B) Guard cell density in SCRM variants driven by native promoter (numbers of guard cells are measured in a 0.25mm² region).
 (C) Zygote length ($n \geq 50$ for each genotype). (D) Zygotic polarity ($n \geq 50$ for each genotype).
 Letters on graphs indicate significantly associated categories in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$) in C and D.

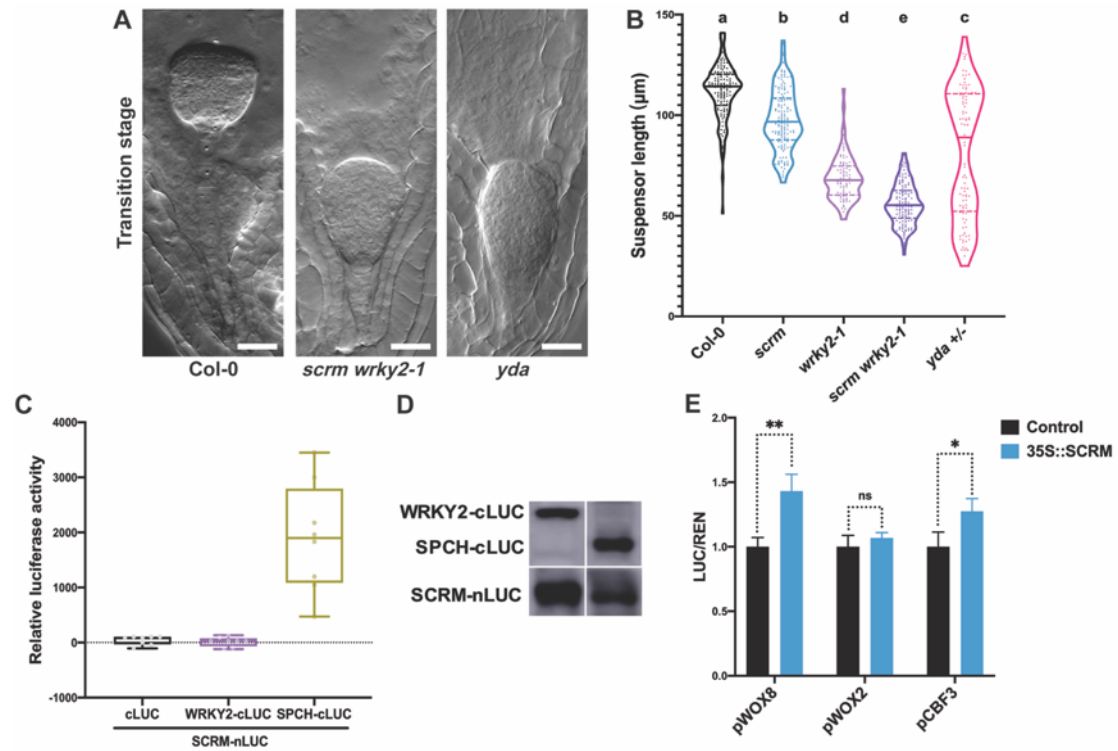


Fig.S3 SCRM shows no interaction with WRKY2
 (A) Transition stage embryos of genotypes as indicated. Scale bar, 20 μ m.
 (B) Suspensor length in transition stage embryos ($n \geq 80$ for each genotype). Letters on graphs indicate significantly associated categories in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$).
 (C) Split-Luciferase assay. 8 replicates for each combination and protein expression identified by Western Blot in (D).
 (E) Dual-Luciferase assays. Three biological replicates were applied. Data are means $\pm$ SD and (·) $P < 0.05$ / (··) $P < 0.01$ by Student's t test.

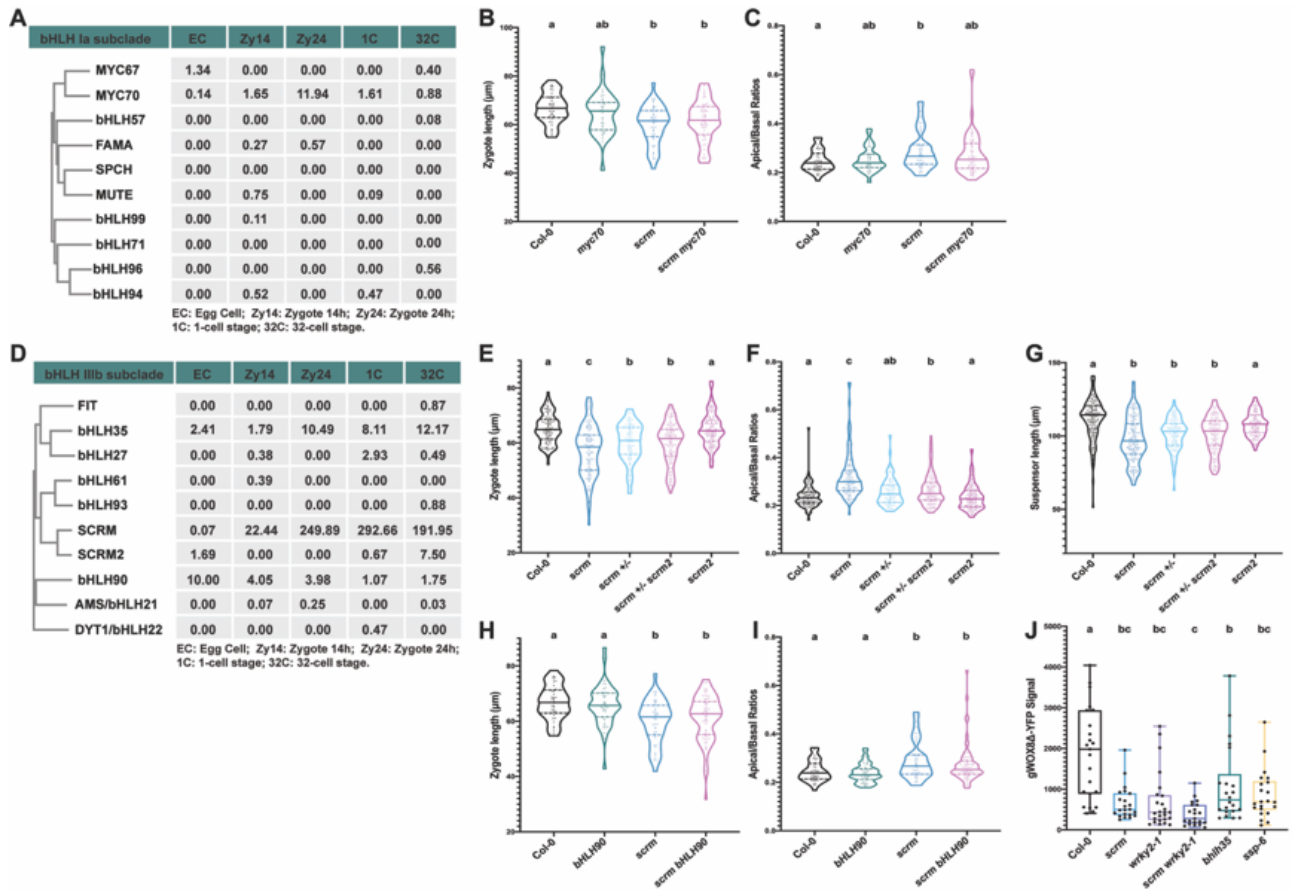


Fig.S4 Phenotypic screening for SCRM interaction partners
 (A) Phylogenetic tree combing RNA-seq data of bHLH Ia subclade.
 (B-C) MYC70 embryonic phenotyping data.
 (D) Phylogenetic tree combing RNA-seq data of bHLH IIIb subclade.
 (E-G) SCRM2 embryonic phenotyping data.
 (H-I) bHLH90 embryonic phenotyping data.
 (J) gWOX8 Δ -YFP signal measurements in zygotes ($n \geq 21$ for each genotype). Data points from Fig.1G and Fig.3D were included in this plot.
 The characters on plots indicate the significance between genotypes ($n \geq 35$ for each group). $P < 0.05$ by one-way ANOVA with a post hoc Tukey test.

Supplementary Table1

Genotyping primer list

Primer Name	Sequences
scrm(SALK_003155)-LP	ATTCTTTGCTCTGCCTCTTCC
scrm(SALK_003155)-RP	TTTGTAGGGCCTTTGTTGTTG
wrky2-1(SALK_020399)-LP	CCAAGAATTTGGCTGAATCTC
wrky2-1(SALK_020399)-RP	TGTTAGAACACGAATCACCCC
bhlh035(SALK_016841)-LP	TGGACCAAATTACAATAATTGTGG
bhlh035(SALK_016841)-RP	CTCACCTCTTTCTCTGGCATG
scrm2-1(SAIL_808_B10)-LP	TGCATCCGACATCTTTTATCC
scrm2-1(SAIL_808_B10)-RP	TTGTTGTTGTTGCTGTGAAGC
myc70(SALK_069605C)-LP	AAACTGCCACAGCCAATAATG
myc70(SALK_069605C)-RP	TGGGTTGAATGGAAGTTCTTG
yda-11(SALKseq_078777)-LP	GGGTTGTTGATTAGTAAACCATAT
yda-11(SALKseq_078777)-RP	TAGTAGGAGACCCAGTAGTT
bHLH90(SALK_052405C)-LP	GTTTTGGCAGGAAATGTTGAG
bHLH90(SALK_052405C)-RP	TCGTGGACTTCAATTTTCACC
LBb1.3	ATTTTGCCGATTTTCGGAAC
SAIL-LB3	TAGCATCTGAATTTTCATAACCAATCTCGATACAC
scrm-cr-LP	TATATAAAGACAGAGACAGTGACAACAGAGAC
scrm-cr-MRP	AGTAGGCTGTGCACCAGACGATGCT
scrm-cr-RP	GATCATACCAGCATACCCTGCTGTATC
ssp-6-LP	ATGGTTCTGGTTGGTCCAGGGGA
ssp-6-MLP	TGTTCAAGAAACGGTGATCGAGC
ssp-6-RP	ACTGGTCTTGGCAAAAGGGAGATTCA

Supplementary Table2

Cloning and mutagenesis primer list

Primer Name	Sequences
SCRM-YPet-InF	TAGAACTAGTGGATCCAGACAACCGGACCACCGTCAATAACAT
SCRM-YPet-InR	CACCATGGATCCGCCTCCACCGATCATACCAGCAT
pSCRM(5134)-InF	ATGATTACGAGCTCTCTAGAACTAGTCCTAGGATCTAAAGCCACCTCATTG
pSCRM(5134)-InR	CAACTCCTCACCTTTAGACACCATCGCCAAAGTTGACACCTTTACC
YPet-SCRM-InF	ATGAACGAGTTGTATAAAGGGCCCATGGGTCTTGACGGAAACAATGGTGG
YPet-SCRM-InR	GCCAAGCTGACGTCAGGTACCTAACCGCCATTAACATATGTCTCCTC
SCRM-D-muF	[Phos]-TTCCAGAAACATGCAGCTATGCGTCAGAGCTCT
SCRM-D-muR	[Phos]-GCATAGCTGCATGTTTCTGGAACAGAGTAGGCT
SCRM-S94A-muF	[Phos]-TCTTCTTCTTGCTCCTTCTCAAGCTT
SCRM-S94A-muR	AAGCTTGAGAAGGAGCACAGAAGAAGA
SCRM-S94D-muF	[Phos]-TCTTCTTCTTGATCCTTCTCAAGCTT
SCRM-S94D-muR	AAGCTTGAGAAGGATCACAGAAGAAGA
SCRM-S203A-muF	[Phos]-GGAAGGTTTTGGTGCTCCTGCTAATGGT
SCRM-S203A-muR	ACCATTAGCAGGAGCACCAAAACCTTCC
SCRM-S203D-muF	[Phos]-GGAAGGTTTTGGTGACCCTGCTAATGGT
SCRM-S203D-muR	ACCATTAGCAGGGTCACCAAAACCTTCC
SCRM-T366A-muF	[Phos]-TGAACCTGAGTCAGCTCCTCCTGGATCT
SCRM-T366A-muR	AGATCCAGGAGGAGCTGACTCAAGTTCA
SCRM-T366D-muF	[Phos]-TGAACCTGAGTCAGCCCCTCCTGGATCT
SCRM-T366D-muR	AGATCCAGGAGGGGCTGACTCAAGTTCA
SCRM-T382A T384A-muF	[Phos]-AAGCTTCCATCCGTTGGCACCTGCACCGCAAAC
SCRM-T382A T384A-muR	GTTTGCGGTGCAGGTGCCAACGGATGGAAGCTT
SCRM-T382D T384D-muF	[Phos]-AAGCTTCCATCCGTTGGACCCTGACCCGCAAAC
SCRM-T382D T384D-muR	GTTTGCGGGTCAGGGTCCAACGGATGGAAGCTT
SCRM-S403A-muF	[Phos]-GTTGTGTCCCTCTTCTTTACCAGCTCCTAAAGGCCAGCAA
SCRM-S403A-muR	TTGCTGGCCTTTAGGAGCTGGTAAAGAAGAGGGACACAAC
SCRM-S403D-muF	[Phos]-CTCTTCTTTACCAGACCCTAAAGGCCAG
SCRM-S403D-muR	CTGGCCTTTAGGGTCTGGTAAAGAAGAG

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Conclusions and Prospects

This thesis applies a comparative view to conduct a delicate dissection of the ER-YDA pathway with a specific focus on the upstream receptor-like cytoplasmic kinases BSK1/2 and the downstream transcription factor target SCRM.

The overlapping but differential functions of BSK1/2 have been confirmed according to phenotypic differences of loss-of-function mutants, specificity of expression patterns and preference for particular interacting partners, which enables us to propose a preliminary model for BSK1/2 in the ER-YDA pathway. The model regards the ER receptor complex as the single signal input, whereas recent evidence in stomata development suggests the receptor kinase HAESA-LIKE 1 could function as the second signal input. Meanwhile, when several pathways activate simultaneously, BSK1/2-associated proteins could be altered by the competition or crosstalk in signaling components between pathways. Therefore, BSK1/2 binding affinity with upstream receptor components and downstream MAPKs or protein phosphatases is worthy of study. The nanoscale dynamic by live-cell imaging *in vivo* and structural analysis *in vitro* would provide more information for further modeling and verification. The putative model also indicates an evolutionary divergence in the BSK family, therefore, the characterization of the ancient orthologues in the lower plants would gain a better understanding of the evolution of the BSK family. It would be more intriguing to investigate whether co-evolution occurs between BSKs and receptor kinases, which could further explain the signaling specificity in the BSK family.

In addition, SCRM is recapitulated as a phosphorylation target of the ER-YDA pathway during embryogenesis, whereas embryonic SCRM are activated by phosphorylation in contrast to the phosphorylation-dependent degradation in stomata. Synergistic effect but no direct association between SCRM and WRKY2 suggests the complicated regulatory networks or a higher order transcription factor complex which could include bHLH35 as a potential partner. My results showed that SCRM is a conserved target of the ER-YDA pathway. However, the phosphorylation regulation mechanism switches from degradation in stomata to activation in the embryo. Further investigations should be conducted to discover the composition of the embryonic SCRM transcription complex and its structural conformation changes when phosphorylation occurs, and to examine whether a potential feedback loop exists in the ER-YDA pathway.

Publications

Accepted publications:

Independent parental contributions initiate zygote polarization in *Arabidopsis thaliana*.

Wang K, **Chen H**, Ortega-Perez M, Miao Y, Ma Y, Henschen A, Lohmann JU, Laubinger S, Bayer M. 2021. *Current Biology* 31(21): 4810-4816.

Square one: zygote polarity and early embryogenesis in flowering plants.

Wang K, **Chen H**, Miao Y, Bayer M. 2020. *Current opinion in plant biology* 53: 128-133.

Manuscripts ready for submission:

MAP kinase 6-dependent phosphorylation of SCRM promotes polarization of the Arabidopsis zygote.

Chen H, Xiong F, Hu Y, Wang K, Miao Y, Schwab R, Laux T, Bayer M.

A tailored SNP-Ratio Mapping method for paternal-defective fertilization mutations.

Miao Y, Vorbrugg S, **Chen H**, Henschen A, Bayer M.

A filament-like embryo system to study the suspensor-embryo transition.

Wang K, Miao Y, Ortega-Perez M, **Chen H**, Bayer M.

Book chapter:

Chen H, Miao Y, Wang K, Bayer M. 2021. Zygotic Embryogenesis in Flowering Plants.

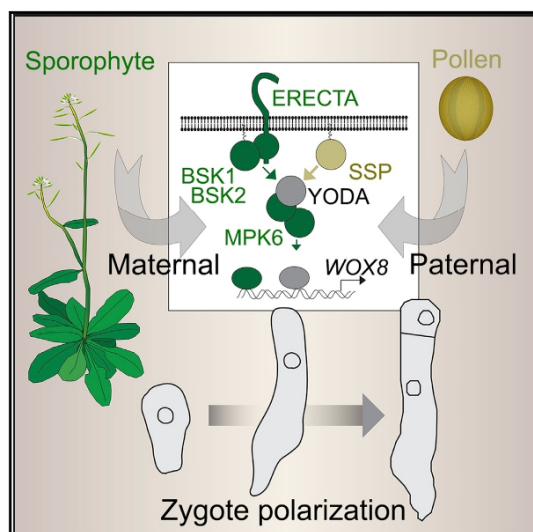
Chapter 4 in Seguí-Simarro JM. 2021. Doubled Haploid Technology: Volume 2: Hot Topics, *Apiaceae*, *Brassicaceae*, *Solanaceae*, *Methods in Molecular Biology*, vol. 2288: Springer US.

Current Biology

Report

Independent parental contributions initiate zygote polarization in *Arabidopsis thaliana*

Graphical abstract



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In brief

Cell polarization and asymmetric division of the zygote initiate axis formation of the embryo in the model plant *Arabidopsis*. Wang et al. show in this context that a maternal receptor complex and a paternally provided constitutively active signaling protein independently contribute to MAP kinase signaling controlling zygote polarization.

Highlights

- Independent maternal and paternal factors influence *Arabidopsis* zygote polarity
- Maternal ERECTA family receptor kinases activate the MAP3K YODA in the zygote
- Paternal SHORT SUSPENSOR protein activates YODA independently of maternal ERECTA

Wang et al., 2021, Current Biology 31, 1–7
November 8, 2021 © 2021 Elsevier Inc.
<https://doi.org/10.1016/j.cub.2021.08.033>

CellPress

Report

Independent parental contributions initiate zygote polarization in *Arabidopsis thaliana*

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<https://doi.org/10.1016/j.cub.2021.08.033>

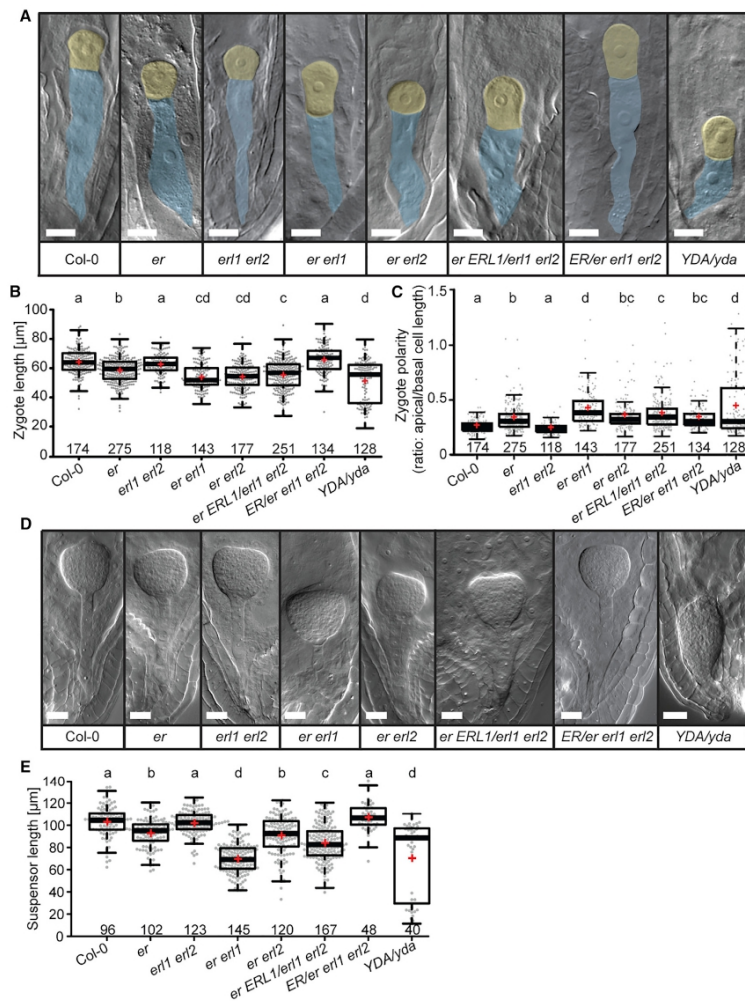
SUMMARY

Embryogenesis of flowering plants is initiated by polarization of the zygote, a prerequisite for correct axis formation in the embryo. The daughter cells of the asymmetric zygote division form the pro-embryo and the mostly extra-embryonic suspensor.¹ The suspensor plays a pivotal role in nutrient and hormone transport and rapid growth of the embryo.^{2,3} Zygote polarization is controlled by a MITOGEN-ACTIVATING PROTEIN (MAP) kinase signaling pathway including the MAPKK kinase (MAP3K) YODA (YDA)⁴ and the upstream membrane-associated proteins BRASINOSTEROID SIGNALING KINASE 1 (BSK1) and BSK2.^{5,6} Furthermore, suspensor development is controlled by cysteine-rich peptides of the EMBRYO SURROUNDING FACTOR 1 (ESF1) family.⁷ While they act genetically upstream of YDA, the corresponding receptor to perceive these potential ligands is unknown. In other developmental processes, such as stomata development, YDA activity is controlled by receptor kinases of the ERECTA family (ERf).^{8–12} While the receptor kinases upstream of BSK1/2 in the embryo have so far not been identified,¹ YDA is in part activated by the sperm cell-derived BSK family member SHORT SUSPENSOR (SSP) that represents a naturally occurring, constitutively active variant of BSK1.^{5,13} It has been speculated that SSP might be a paternal component of a parental tug-of-war controlling resource allocation toward the embryo.^{2,13} Here, we show that in addition to SSP, the receptor kinase ERECTA plays a crucial role in zygote polarization as a maternally contributed part of the embryonic YDA pathway. We conclude that two independent parental contributions initiate zygote polarization and control embryo development.

RESULTS AND DISCUSSION

Despite its central role in zygote polarization and controlling embryonic versus non-embryonic development, it is still unclear if the embryonic YDA pathway is activated in response to extracellular signals. In many aspects of *Arabidopsis* development, YDA activity is controlled by receptor kinases of the ERECTA family, including ERECTA (ER), ER-LIKE 1 (ERL1), and ERL2.^{9,10,12} As transcripts of *ER* can be detected in the zygote (Table S1),¹⁴ a critical function of *ER* in controlling zygote polarity seems plausible. Homozygous *er erl1 erl2* triple mutants are sterile and produce no embryos.¹¹ We therefore addressed a possible function of *ER* in early embryogenesis by examining homozygous single, double, and segregating triple mutants of *ERf* genes. As mutants in *Ler* background used in previous studies contain a non-functional *ER* gene, we performed all genetic experiments with alleles in Col-0 background.^{4,13,15} When comparing *er* mutants with wild type, weak defects in zygote elongation, zygote polarization, and suspensor length became apparent (Figure 1) as well as aberrant division plane orientations in the suspensor (Figure S1). These defects are hallmarks of reduced YDA activity⁴ and mimic the

phenotypes of *ssp* or *bsk1 bsk2* double mutants.^{5,13} Loss of *ERL1* and *ERL2* function only showed detectable defects in the absence of functional *ER* by enhancing the *er* phenotype, indicating that *ERL1* and *ERL2* play a minor role in early embryogenesis but can partially take over *ER* function in its absence (Figure 1), as previously described for *ERf* function during stomata development.^{10,16–18} To test if *ER* acts upstream of YDA in the context of zygote polarization as it does in other developmental contexts,^{10,18,19} we performed genetic rescue experiments with a constitutively active version of YDA (*yda-CA*;⁴ Figure S1). Constitutive YDA activity rescued the *er erl2* double mutant phenotype and caused increased zygote elongation, suggesting that YDA acts downstream of *ER* in a common signaling cascade. As *yda-CA* was hemizygous in the parental plant and therefore segregated in the analyzed embryos, a quarter of the embryos did not carry the *yda-CA* transgene, resulting in slightly lower median values in the *er erl2* background compared to wild-type background. These results suggest that the *ER*/YDA pathways involved in embryogenesis and stomata development share a common overall architecture.²⁰ Triple *ERf* mutants with segregating *er (ER/erl1 erl2)*, however, did not show defects in zygote



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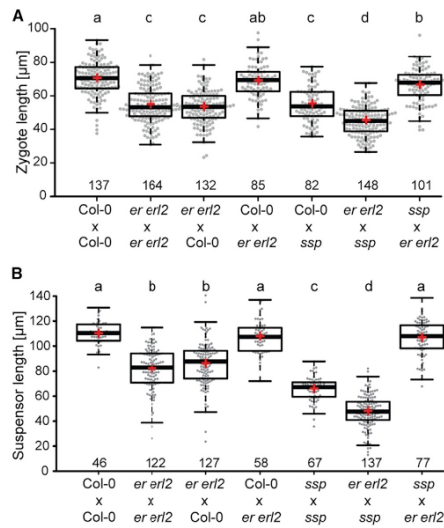


Figure 2. Parental effects in reciprocal crosses

Boxplot diagrams of zygote elongation (A) and suspensor length (B) in F1 embryos of reciprocal crosses. Genotypes are given as female × male. Boxplot data presentation as described in Figure 1. Different letters above boxes refer to individual groups in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$). See also Figures S2 and S4 and Table S3.

elongation or suspensor formation despite normal transmission of the *er* mutant allele (Figure 1; Table S2). The fact that phenotypic defects in homozygous *er* embryos only became apparent if the parental plants were homozygous for *er*, but not in homozygous offspring of heterozygous *ER/er* parents, led us to test for possible parent-of-origin effects. In reciprocal crosses of *er erl1* double mutants and wild type (Figures 2 and S2), we could only observe defects in F1 embryos if maternal plants carried the *er erl1* mutations, but not if these alleles were transmitted by the pollen donors (Figures 2 and S2). Furthermore, zygote elongation defects could not be observed if maternal plants were heterozygous for the *er* mutation (Figure 1), although slightly altered zygote polarity can be observed in segregating triple *ER/er erl1 erl2* mutants (Figure 1). The phenotype of the embryo therefore seems to be predominantly determined by the genotype of the maternal sporophyte while loss of *ER* function in the haploid phase or the embryo in segregating plants did not have a major effect on the embryonic phenotype. This unusual parental effect may explain why a role of *ERECTA* in zygote polarization has so far been overlooked.

Two possible scenarios could explain the sporophytic maternal effect: zygote polarization could be affected by a non-cell-autonomous function of *ER* in sporophytic tissue. Such a scenario has, for example, been described for early auxin signaling in the embryo.²¹ Alternatively, *ER* could function in the zygote but rely on *ER* transcripts and/or *ER* proteins inherited to the zygote via the pre-meiotic megaspore mother cell (MMC).

A transcriptional (*ER_{pro}::3xVenus-N7*) and a functional translational fusion (*ER_{pro}::ER-YPet*) reporter gene as well as RNA *in situ* hybridization revealed *ER* expression in the sporophytic seed coat as well as the female germline (Figure S3). As *ER* transcripts can be detected at low level in isolated zygotes¹⁴ (Table S1) but are below detection limit in RNA *in situ* hybridization and very weak *ER-YPet* expression was sufficient to rescue the mutant phenotype in seedlings, we were not able to draw a definite conclusion about the location of *ER* function based on the observed expression pattern. However, *yda* has been reported as a zygotic recessive mutant,^{4,15} and we confirmed this for the *yda-11* allele (Figures 1 and S1). On the other hand, *SSP* acts as sperm-derived activator.^{5,13} Both genes would therefore argue for a function of the embryonic *ERECTA/YDA* pathway in the zygote. This is further supported by the function of *WRKY2*, a direct downstream target of *MPK6* phosphorylation.²² Consistently, loss-of-function *wrky2* null mutations are zygotic recessive without detectable parent-of-origin effects.²² When looking at mRNA stability, *ER* transcripts showed a moderate half-life in seedlings at roughly the same level as typical house-keeping genes, such as *ACTIN2*, for which rapid mRNA turnover does not appear necessary (Table S3).²³ *ER* transcripts can be detected in the egg cell, but transcript levels decline after fertilization, indicating that there is little or no *de novo* transcription of *ER* in the zygote (Table S1).¹⁴ In order to address the question of where *ER* function is necessary for zygote polarization, we specifically reduced *ER* protein function in the zygote using the *ER_{pro}::ER-YPet* line in *erl1 erl2* background. Genetically encoded anti-GFP nanobodies fused to ubiquitin ligases have been used as an elegant tool to specifically target GFP/YFP-tagged proteins for degradation.^{24,25} To test its functionality, we expressed the NSImb-vhhGFP4 nanobody under the *ER* promoter. The resulting seedlings mimicked the *erl1 erl2* triple-mutant phenotype,¹¹ albeit with a slightly weaker phenotype (Figure 3), indicating that the nanobody is functional but does not completely abolish *ER-YPet* activity. In the next step, we expressed the nanobody under the strong egg cell-specific *EC1* promoter²⁶ to specifically reduce *ER-YPet* levels in the egg cell/zygote. This resulted in a significant reduction of zygote length in the *ER_{pro}::ER-YPet* rescue line (Figure 3), indicating that zygote polarization critically depends on functional *ER* protein in the zygote. Taken together, the available data suggest that the sporophytic maternal control of *ER* function can be explained by the inheritance of pre-meiotically produced *ER* transcripts/proteins to the zygote and that very low amounts of *ER* are sufficient to provide function in the zygote.

In (B)–(E), the sample size is given above the x axis. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; red crosses represent sample means; data points are plotted as gray dots. Letters above boxes refer to individual groups in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$). In embryos of heterozygous *yda* plants, two distinct populations of data points can be observed, with approximately a quarter showing strong zygote elongation (B) and polarity defects (C), and reduced suspensor length (E) possibly representing the homozygous offspring. See also Figure S1 and Tables S1–S3.

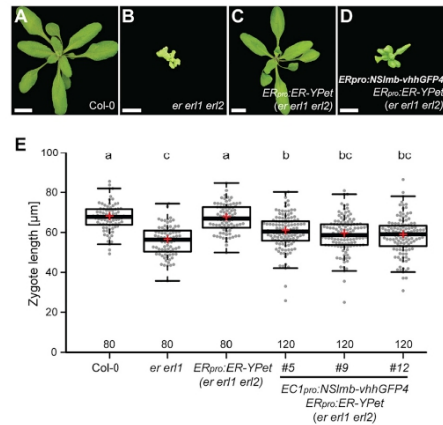


Figure 3. Cell-autonomous function of ER protein in the zygote
(A–D) Vegetative growth phenotype of wild-type Col-0 (A), *erl1 erl2* triple homozygous (B), genetically rescued plants (*ERpro:ER-YFPet* in *erl1 erl2*; C), and genetically rescued plants expressing anti-GFP nanobody (*ERpro:ER-YFPet* in *ERpro:ER-YFPet* *erl1 erl2*; D). Scale bar, 1 cm. See also Figure S3. (E) Egg cell-specific expression of the anti-GFP nanobody (*EC1pro:NSlimb-vhhGFP4*) in the *ERpro:ER-YFPet* *erl1 erl2* plant leads to reduced zygote length. Boxplot diagram of zygote elongation. Boxplot data presentation as described in Figure 1. Different letters above boxes refer to individual groups in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$).

Different mechanisms that cause maternal effects on plant embryogenesis including inheritance of cytoplasmic factors have been proposed more than two decades ago.²⁷ Furthermore, sporophytic maternal effects have previously been identified in the mutant *sin1-2* allele of the SHORT INTEGUMENTS 1 (*SIN1*)/DICER-LIKE 1 (*DCL1*) gene.^{28,29} A comprehensive survey of essential genes in *Arabidopsis* showed that many mutations in essential genes can be transmitted through the female germline and only become deleterious in the early embryo.³⁰ This suggests that genetic compensation by premeiotic gene products prevents lethality in the female gametophyte for many essential genes and might therefore not be a rare event.³⁰

BSK1 and BSK2 have been shown to act upstream of YDA, presumably linking ERECTA signaling with YDA activation.⁵ Reciprocal crosses with *bsk1 bsk2* double mutants and wild type showed similar sporophytic maternal effects as observed for *er* (Figure S4). As maternal effects have been described for mutations in genes acting downstream of YDA,^{31,32} not just *ER* but several components of this signaling pathway appear to be under maternal sporophytic control. This is quite intriguing, as the non-canonical BSK family member SSP activates YDA as paternal factor contributed by the sperm cell.¹³ SSP has been shown to resemble a naturally occurring, constitutively active form of BSK that directly interacts with YDA via its TPR motif.⁵ This raises the question of whether the two parental contributions to YDA activation can function independently. We tested this hypothesis in seedlings, where ectopic expression of *SSP* leads to constitutive

activation of the YDA pathway^{5,13} resulting in cotyledons lacking mature stomata. This was also observed for *SSP* expression in *erl1 erl2* triple-mutant background, indicating that *SSP* can activate YDA in the absence of a functional receptor complex (Figure 4A). Overexpression of *BSK1* in *bsk1 bsk2* double-mutant background rescued the *bsk1 bsk2* phenotype, indicating that the construct is functional. In contrast to *SSP*, however, it failed to rescue the *erl1 erl2* triple-mutant phenotype, despite having similar or higher transcript levels when compared to *35Spro:SSP* (Figures 4B and 4C).

If *SSP* serves as an independent signal for YDA activation in the zygote, loss of *SSP* should have an additive effect to loss of *ER* function. The further loss of *SSP* in *erl1* and *erl2* mutants indeed led to further reduction in zygote length and an almost complete loss of zygote polarity (Figures 2 and S2), indicating that *SSP* provides additional YDA activation independently of *ER* function. If the embryonic YDA pathway indeed relies on two independent signaling inputs, additional *SSP* activity should compensate for the loss of *ER* function. To test this, we introduced an additional copy of *SSP* (*SSPpro:SSP-YFP*) in the *erl2* double mutant and found that additional *SSP* activity indeed partially rescued the loss-of-function phenotype of *erl2* (Figure 4D).

Taken together, our data outline a signaling pathway (Figure 4E) with a maternally controlled receptor complex and a paternally provided activating protein that converge at the level of MAP3K activation. *SSP* is a *Brassicaceae*-specific gene while *ER*, *BSK1*, and *BSK2* are evolutionarily conserved in flowering plants.^{33,34} *SSP* has been shown to be critical for correct suspensor development,¹³ an organ that serves as a conduit for nutrients to the embryo,³ and for rapid development of the embryo.² Therefore, suspensor development could be a possible target of a parental conflict over nutrient allocation to the embryo.³⁵ It has recently been observed that there are preferentially maternal expressed genes in the suspensor while there is mainly biparental expression in descendants of the apical cell.³⁶ This maternal bias in the suspensor could be interpreted in the context of the parental conflict theory enforcing equal nutrient distribution to all embryos by the maternal genome.³⁵ In this context, it might be significant that *ER* function is under sporophytic control as the sporophytic maternal effect would ensure uniform suspensor development of all embryos irrespective of segregating maternal alleles. In this scenario, *SSP* could be seen as a *Brassicaceae*-specific paternal contribution that circumvents the maternal control of suspensor development, bypassing ERECTA signaling as a strong independent activator of the YDA pathway.

This raises the question if suspensor development in other plant species is also under maternal control and whether the two independent inputs for YDA activation indeed are part of a parental tug-of-war over nutrient allocation to the embryo. Or are *Brassicaceae* shifting from ligand/receptor-based zygote polarization to a sperm-based activation mechanism via *SSP* to fine-tune the timing of YDA activation as *SSP* naturally links fertilization with YDA activation?

The receptor kinase *ER* implies extra-cellular ligands in the control of zygote polarization, and the known ESF1 peptides are prime candidates. However, at present it is unclear if they have a function in zygote polarization or influence suspensor development after zygote polarization.⁷ Future research will be needed to address the question of where the ligand for the *ER*

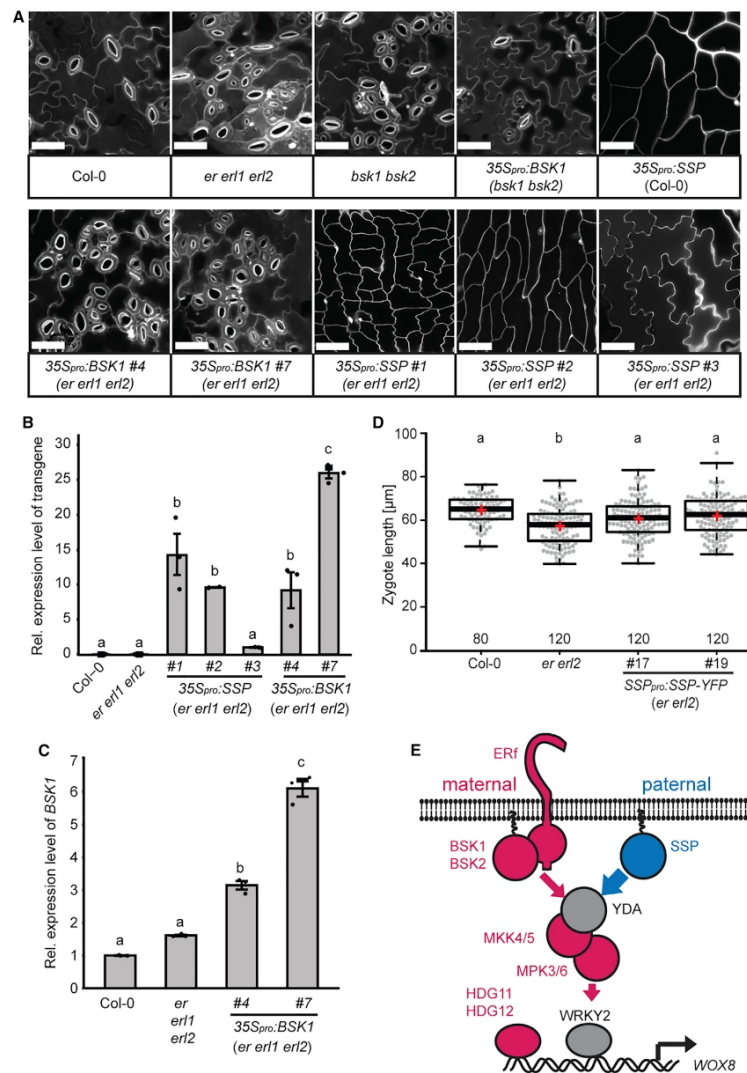


Figure 4. Independent parental contributions to YDA activation in the zygote

(A) Maximum projections of epidermal confocal images. Genotypes are given under each figure panel. Cell walls are stained with propidium iodide. Scale bar, 20 μm.

(B) Expression level of 35Spro:SSP and 35Spro:BSK1 in *erl1 erl2* seedlings shown in (A) as determined by qRT-PCR. Bar graph shows mean values of three technical replicates with standard error.

(legend continued on next page)

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receptor kinase is originating and how conserved this mode of zygote polarization is in land plants.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2021.08.033>.

ACKNOWLEDGMENTS

We would like to thank Gerd Jürgens for helpful discussions and comments on the manuscript. We also thank the Salk Institute Genomic Analysis Laboratory as well as the Nottingham *Arabidopsis* Stock Centre (NASC) for providing the sequence-indexed *Arabidopsis* tDNA insertion mutants and the Light Microscopy Facility at the Max Planck Institute for Developmental Biology for support. Research in our groups is supported by the German Research Foundation (Deutsche Forschungsgemeinschaft - DFG SFB1101/B01 to M.B. and SFB1101/B07 to J.U.L.), the Chinese Scholarship Council (fellowship no. 201806320131 to Y. Miao), and the Max Planck Society.

AUTHOR CONTRIBUTIONS

K.W. and M.B. designed the study with critical comments and modifications by H.C., M.O.-P., Y. Miao, Y. Ma, J.U.L., and S.L. K.W., H.C., M.O.-P., Y. Miao, and A.H. performed the experiments. K.W., H.C., M.O.-P., Y. Miao, Y. Ma, A.H., J.U.L., S.L., and M.B. evaluated the data and interpreted the experimental results. K.W. and M.B. wrote the initial manuscript draft, and all authors commented on and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 26, 2021

Revised: July 9, 2021

Accepted: August 11, 2021

Published: September 7, 2021

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(C) Expression level of *BSK1* in Col-0, *erl1 erl2*, and *35S_{pro}::BSK1 erl1 erl2* seedlings. Bargraph shows mean values of three technical replicates with standard error.

(D) An additional copy of *SSP* (*SSP_{pro}::SSP-YFP*) partially rescues the *erl2* loss-of-function phenotype in the zygote. The boxplot diagram shows zygote length. Boxplot data presentation as described in Figure 1.

Different letters above bars and boxes in (B)–(D) refer to individual groups in a one-way ANOVA with a post hoc Tukey test ($p < 0.05$).

(E) Simplified schematic depiction of the embryonic YDA pathway. YDA is activated by the maternally supplied receptor complex consisting of ER and the membrane-associated proteins BSK1 and BSK2. YDA is also activated by the paternally contributed BSK family member SSP. Signaling components are colored according to genetic contribution: maternal effect (magenta), paternal effect (blue), and zygotic recessive (gray).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Stellar competent cells	Takara	Cat#636763
<i>Agrobacterium tumefaciens</i> GV3101	³⁷	N/A
Chemicals, peptides, and recombinant proteins		
Murashige and Skoog Plant Salts	SERVA	Cat#47516.03
Propidium iodide	SIGMA-ALDRICH	Cat#P4170
SCR1 Renaissance 2200	Renaissance Chemicals Ltd	N/A
Triton X-100	Carl Roth GmbH	Cat#3051.1
Glycerol	SIGMA-ALDRICH	Cat#G5516
Para-formaldehyde	SIGMA-ALDRICH	Cat#P6148
GUM arabic	SIGMA-ALDRICH	Cat#G9752
Chloral hydrate	Carl Roth GmbH	Cat#K318.1
Digoxigenin-11-UTP	Roche	Cat#11209256910
Anti-Digoxigenin-AP	Roche	Cat#11093274910; RRID: AB_514497
DIG-labeled Control	Roche	Cat#11585746910
Protease (Pronase)	SIGMA-ALDRICH	Cat#73702
T7 Polymerase	Thermo Scientific	Cat#EP0111
Q5 High-Fidelity DNA polymerase Kit	NEW ENGLAND BioLabs	Cat#M0491
In-Fusion HD Cloning Kit	TAKARA	Cat#639650
Dream Taq Green PCR Master Mix	Thermo Scientific	Cat#K1081
RNeasy Plant Mini Kit	QIAGEN	Cat#74904
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific	Cat#K1622
Luna Universal qPCR Master Mix	NEW ENGLAND BioLabs	Cat#M3003
Phosphinothricin	SIGMA-ALDRICH	Cat#45520
Hygromycin B	Carl Roth GmbH	Cat#CP13.1
Experimental models: Organisms/strains		
<i>Arabidopsis thaliana</i> : Col-0	N/A	N/A
<i>Arabidopsis thaliana</i> : er	⁵	SALK_066455
<i>Arabidopsis thaliana</i> : er1	⁵	GK_109G04
<i>Arabidopsis thaliana</i> : er2	⁵	GK_486E03
<i>Arabidopsis thaliana</i> : YDA/yda-11	NASC ³⁸	SALK_078777
<i>Arabidopsis thaliana</i> : bsk1-2	⁵	SALK_122120
<i>Arabidopsis thaliana</i> : bsk2-2	⁵	SALK_001600
<i>Arabidopsis thaliana</i> : BSK1/bsk1 bsk2	⁵	N/A
<i>Arabidopsis thaliana</i> : ssp-2	¹³	SALK_051462
<i>Arabidopsis thaliana</i> : er er1	This paper	N/A
<i>Arabidopsis thaliana</i> : er er2	This paper	N/A
<i>Arabidopsis thaliana</i> : er1 er2	This paper	N/A
<i>Arabidopsis thaliana</i> : er er1 er2	⁵	N/A
<i>Arabidopsis thaliana</i> : ER/er er1	This paper	N/A
<i>Arabidopsis thaliana</i> : ER/er er1 er2	This paper	N/A
<i>Arabidopsis thaliana</i> : er ERL1/er1 er2	⁵	N/A
<i>Arabidopsis thaliana</i> : er er1 ssp	This paper	N/A
<i>Arabidopsis thaliana</i> : ER _{pro} :3xVenus-N7	This paper	N/A
<i>Arabidopsis thaliana</i> : ER _{pro} :ER-YPet; er er1 er2	This paper	N/A
<i>Arabidopsis thaliana</i> : YDA _{pro} :yda-CA	This paper	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Arabidopsis thaliana</i> : YDA _{pro} :yda-CA; <i>er</i> 1 <i>er</i> 2	This paper	N/A
<i>Arabidopsis thaliana</i> : ER _{pro} :NSlmb-vhhGFP4; ER _{pro} :ER-YFP; <i>er</i> 1 <i>er</i> 2	This paper	N/A
<i>Arabidopsis thaliana</i> : EC1 _{pro} :NSlmb-vhhGFP4; ER _{pro} :ER-YFP; <i>er</i> 1 <i>er</i> 2	This paper	N/A
<i>Arabidopsis thaliana</i> : 35S _{pro} :BSK1; <i>bsk1 bsk2</i>	This paper	N/A
<i>Arabidopsis thaliana</i> : 35S _{pro} :BSK1; <i>er</i> 1 <i>er</i> 2	This paper	N/A
<i>Arabidopsis thaliana</i> : 35S _{pro} :SSP	This paper	N/A
<i>Arabidopsis thaliana</i> : 35S _{pro} :SSP; <i>er</i> 1 <i>er</i> 2	This paper	N/A
<i>Arabidopsis thaliana</i> : SSP _{pro} :SSP-YFP; <i>er</i> 1 <i>er</i> 2	This paper	N/A
Oligonucleotides		
Listed in Table S4	This paper	N/A
Recombinant DNA		
pCambia 3300	38,40	N/A
pCambia 1300	38,40	N/A
pBay-bar	5	N/A
pBay-hyg	5	N/A
ER _{pro} :3xVenus-N7	This paper	N/A
ER _{pro} :ER-YFP	This paper	N/A
YDA _{pro} :yda-CA	4	N/A
ER _{pro} :NSlmb-vhhGFP4	This paper	N/A
EC1 _{pro} :NSlmb-vhhGFP4	This paper	N/A
35S _{pro} :BSK1	This paper	N/A
35S _{pro} :SSP	This paper	N/A
SSP _{pro} :SSP-YFP	5,13	N/A
Software and algorithms		
R version 3.6.1	N/A	https://www.r-project.org/
R: multcompView package	N/A	https://cran.r-project.org/web/packages/multcompView/index.html
Adobe Illustrator CS6	Adobe	https://www.adobe.com/products/illustrator.html
Adobe Photoshop CS6	Adobe	https://www.adobe.com/products/photoshop.html
BoxPlotR	41	http://shiny.chemgrid.org/boxplotr/
ImageJ 1.52p	42	https://imagej.nih.gov/ij/
ZEN 2.0 blue edition	ZEISS	https://www.zeiss.de/messtechnik/produkte/software.html
AxioVision 4	ZEISS	https://www.micro-shop.zeiss.com/en/de/softwarefinder/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Martin Bayer (martin.bayer@tuebingen.mpg.de).

Materials availability

All plasmids and *Arabidopsis* lines generated in this study are available from the Lead Contact without restrictions.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.



EXPERIMENTAL MODEL AND SUBJECT DETAILS

All *Arabidopsis thaliana* mutant and transgenic lines used in this study are in Col-0 background. Seeds were washed with 70% (v/v) ethanol for 5 min, followed by washing with 100% ethanol for 1 min. Then seeds were dried under clean bench. Surface-sterilized seeds were stratified at 4°C in dark for 2 days on half-strength Murashige and Skoog (½ MS) medium containing 1% (w/v) sucrose and 1% (w/v) agar (pH = 5.7)⁴³ then transferred to long-day conditions (16 h at 3000 photons per square meter per second, 8 h at dark) at 23°C for 1–2 weeks in custom-built walk-in growth chambers (Induplan, 8118140). Afterward, seedlings were transferred to soil and were grown under long-day conditions (16 h at 80 micromoles per square meter per second, 8 h at dark) at 23°C and 65% relative humidity as described before.² Mature seeds were harvested in seed bags and stored at room temperature.

METHOD DETAILS

Mutant lines

The *ssp-2* (SALK_051462), *bsk1-2* (SALK_122120), *bsk2-2* (SALK_001600), *er* (SALK_066455), *erl1* (GK_109G04), *erl2* (GK_486E03)^{5,13} have been described previously. The *yda-11* (SALKseq_078777) allele in Col-0 background carries a T-DNA insertion in the second exon. As the truncated gene product lacks essential parts of the YDA coding region⁴ and the mutant phenotype is similar to *yda-1*,⁴ the *yda-11* presumably represents a null allele. Insertion lines were provided by the Nottingham *Arabidopsis* Stock Center (NASC).³⁸ *bsk1 bsk2* double mutant, *er ERL1/erl1 erl2* and *er erl1 erl2* triple mutants have been described before.³ All other homozygous or heterozygous multiple mutant combinations were obtained by crossing. Crosses were performed by manual dissection of anthers before anthesis, followed by manual pollination approximately 24 h later.

Genotyping

For genotyping of mutant plants, PCR was performed with Dream Taq Green PCR Master Mix (Thermo Scientific). Gene-specific primers (LP and RP; Table S4) were used for the wild-type allele. Left border primers in combination with a gene-specific primer (RP) were used to detect the insertion allele. Segregation analysis of *ER/er erl1 erl2*, *er ERL1/erl1 erl2* and *BSK1/bsk1 bsk2* was based on counting the number of strong dwarfed mutant seedlings (phenotype of the *er erl1 erl2* triple homozygous and *bsk1 bsk2* double mutant seedlings) in the offspring.

Plasmid construction

Q5 High-Fidelity DNA polymerase Kit (NEW ENGLAND BioLabs) was used to clone DNA fragments from *Arabidopsis* Col-0. Plasmid construction was performed by in-fusion cloning using In-Fusion HD Cloning Kit (TAKARA) and transformed into Stellar competent cell (TAKARA). Oligonucleotide sequences for molecular cloning are summarized in Table S4. For plant transformation, transgenes were constructed in pCambia 3300³⁹ as well as pBay-bar and pBay-hyg binary vectors.² pBay-bar and pBay-hyg are modified versions of the pCambia binary vectors pCambia 3300 and pCambia 1300,³⁹ respectively. pCambia 3300 and pCambia 1300 (<https://cambia.org>) were modified from pPZP vectors.⁴⁰ In pBay-bar and pBay-hyg, the T-DNA has been replaced by a synthetic DNA fragment harboring unique restriction enzyme sites as well as plant codon-optimized selectable marker genes (conferring phosphinothricin and hygromycin resistance, respectively) under control of the *Arabidopsis* *RPL10A* promoter (−960 bp to +287 bp of At1g14320 including the first intron).⁵ To generate *ER_{pro}:3xVenus-N7*, a 1934 bp genomic region immediately upstream of the start codon of *ER* was transcriptionally fused with the coding region of 3 copies of Venus YFP with a C-terminal N7 nuclear localization sequence and *UBQ10* terminator in pCambia 3300. *ER_{pro}:ER-YPet* contains a fragment of the *ER* locus including a 1934 bp region upstream of the start codon and the genomic sequence. The *ER* genomic sequence was fused in-frame at the 3' end with the sequence of YPet YFP and *UBQ10* terminator. To generate *ER_{pro}:NSImb-vhhGFP4*, the *ER-YPet* sequence in *ER_{pro}:ER-YPet* was replaced by the *NSImb-vhhGFP4* coding sequence,²⁵ and the whole cassette was introduced into pBay-hyg. For *EC1_{pro}:NSImb-vhhGFP4*, a 465 bp region of *EC1.1* was fused with *NSImb-vhhGFP4* in pBay-hyg. To generate *35S_{pro}:BSK1* and *35S_{pro}:SSP*, *BSK1* CDS and *SSP* CDS, respectively, were fused with 2x 35S promoter and 35S terminator in pBay-bar backbone. *SSP_{pro}:SSP-YFP* and *YDA_{pro}:yda-CA* have been described previously.^{4,13} In *SSP_{pro}:SSP-YFP*, 6630 bp *SSP* 5' UTR, whole *SSP* genomic sequence and 153 bp *SSP* 3' UTR of *SSP* were cloned to pCambia 3300. Citrine YFP sequence was fused in between the kinase domain sequence and TPR domain sequence of *SSP*. In *YDA_{pro}:yda-CA*, 3762 bp *YDA* 5' UTR and 1217 *YDA* 3' UTR were fused with truncated *YDA* genomic sequence in pCambia 3300. The truncated *YDA* genomic sequence contains a 447 bp deletion (+650 bp to +1096 bp) in the second exon.

Transgenic plants

All initial transgenic plants were transformed by floral dip using *Agrobacterium tumefaciens* GV3101.³⁷ *ER_{pro}:3xVenus-N7* was transformed into Col-0. *ER_{pro}:ER-YPet*, *35S_{pro}:BSK1* and *35S_{pro}:SSP* were transformed into *er erl1/ERL1 erl2* plants. *35S_{pro}:BSK1* and *35S_{pro}:SSP* were also transformed into *BSK1/bsk1 bsk2* and Col-0, respectively. *YDA_{pro}:yda-CA* and *SSP_{pro}:SSP-YFP* were transformed into *er erl2*. T₁ transgenic seedlings of these constructs were screened on 3/4 3/4 MS plates containing 1% (w/v) sucrose and 50 mg/L phosphinothricin. *ER_{pro}:NSImb-vhhGFP4* and *EC1_{pro}:NSImb-vhhGFP4* in pBay-hyg were transformed into *ER_{pro}:ER-YPet #4* in *er erl1 erl2*, and T₁ seedlings were screened on 3/4 3/4 MS plates containing 1% (w/v) sucrose, 50 mg/L phosphinothricin

and 20 mg/L hygromycin. T₃ plants were used for phenotypic analyses, except for 35S_{pro}:SSP, 35S_{pro}:SSP *er1 er2* and 35S_{pro}:BSK1 *er1 er2*, where T₁ seedlings were directly used because of sterility of the transgenic plants. Two independent transgenic lines of YDA_{pro}:yda-CA in *er1 er2* (#16 and #19) were crossed with Col-0 and offspring homozygous for the *ER ERL2* wild-type alleles were selected in the F₂ generation. Transgene expression was determined by RT-PCR using M13rev and transgene-specific reverse primers (listed in Table S4).

DIC and Confocal microscopy

For differential interference contrast (DIC) imaging, ovules were dissected by hand and incubated overnight in Hoyer's solution (7.5g gum arabic, 5 mL glycerol, 100 g chloral hydrate and 30 mL water, diluted 2:1 with 10% (w/v) gum arabic solution). DIC images were taken with a Zeiss Axio Imager. Z1 microscope equipped with AxioCam HRC camera and AxioVision 4 software as described before.⁵ Confocal microscopy was conducted with a Zeiss LSM 780 NLO microscope with ZEN 2.0 blue edition. SCR1 Renaissance 2200 (SR2200) staining were performed according to published protocols^{44,45} and confocal images were obtained with excitation at 405 nm and detection wavelength from 415 nm to 475 nm. For SR2200 staining of early ovules containing developing megaspore mother cell, early ovules were incubated in SR2200 solution (0.1% (v/v) SR2200, 1% (v/v) DMSO, 0.05% (w/v) Triton X-100, 5% (w/v) glycerol, 4% (w/v) para-formaldehyde in PBS buffer, pH 8.0) for 5 min. Then ovules were washed with water once and incubated in 5% (w/v) glycerol for imaging. For SR2200 staining of ovules containing megaspores or mature female gametes, both incubation and washing steps were performed with gentle vacuum to have a better staining. For YFP imaging, a 514 nm laser wavelength was used for excitation, a wavelength between 526 nm and 553 nm was recorded. For propidium iodide (PI) staining, cotyledons of seedlings (5 days after germination) were dissected and incubated in 10mg/L PI solution in water for 30 min, followed by brief washing with water. Afterward, cotyledons were transferred to microscopy slides and the abaxial side was imaged. PI fluorescence was detected from 571 nm to 656 nm with an excitation wavelength of 561 nm.

RNA in situ hybridization

RNA *in situ* hybridization were conducted according to Mayer et al.⁴⁶ with modifications (see below). The probes were amplified directly from cDNA from Col-0 seedlings using the primers ER-insitu-s: GTAAAGATCTCGGTGTGG and T7+ER-insitu-as: TAATAC-GACTCACTATAGGGCTGAAGACATATTCACA (T7 promoter sequence is the first 20bp) modified from Pillitteri et al.⁴⁷ They were transcribed and labeled with up to a 1:3 ratio of UTP: Digoxigenin-11-UTP (Roche) using the T7 Polymerase (Thermo Scientific). High specific labeling was by dot blot analysis using DIG-labeled Control RNA (Roche) as reference. For the plant material, unfertilized siliques were collected from Col-0, *ER/er*, and *er* plants. They were fixed and embedded as described previously,⁴⁸ shortening the ethanol series to 45 min and using 10 μ m sections. Paraffin was removed by immersing twice in HistoClear (National Diagnostics) for 10 min. The samples were first rehydrated by ethanol series, then digested with 0.125 mg/mL Pronase (Sigma) for 10 min, treated shortly with Glycine 0.2% (w/v) in 1x PBS and 1x PBS before fixation in 4% (w/v) PFA for 10 min. Sections were dehydrated by ethanol series and 200 μ l hybridization solution was applied per slide. After incubation in a humid box at 50°C overnight, the samples were shortly immersed in 2x SCC and washed three times in 0.2x SCC for 50 min at 50°C. The slides were incubated in 0.5% (w/v) blocking reagent (Boehringer) in TBS for 45 min. The Anti-Digoxigenin-alkaline-phosphatase-coupled antibody (Roche) was diluted 1:1250 in BXT (1% (w/v) BSA, 0.3% (w/v) Triton X-100 in TBS), 160 μ l/slide applied and incubated for 1.5 h. Sections were washed 4 times in BXT for 20 min and incubated for 5 min in detection buffer (100 mM Tris (pH 9.5), 50 mM MgCl₂, 100 mM NaCl). Hundred twenty microliters of staining solution (450 μ g/mL NBT, 175 μ g/mL BCIP, 2% (w/v) Polyvinyl alcohol in detection buffer) were applied per slide and incubated overnight. They were immersed shortly in detection buffer, added 15% (w/v) glycerol to each slide and applied a coverslip for brightfield microscopy.

Quantitative RT-PCR analysis

RNA was extracted with the RNeasy Plant Mini Kit (QIAGEN) and quantified with a Nanodrop spectrophotometer. Col-0, *er1 er2* and transgenic T₁ seedlings were grown on ½ MS media containing sucrose and agar for 14 days under long-day conditions. After 4 h in light, seedlings were collected and directly frozen in liquid nitrogen. 12 seedlings were collected for Col-0, *er1 er2* and 35S_{pro}:BSK1 *bsk1 bsk2* each. Single T₁ seedlings were collected for 35S_{pro}:SSP, 35S_{pro}:SSP *er1 er2* and 35S_{pro}:BSK1 *er1 er2*. cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) from 1 μ g total RNA of each sample, and was diluted 2-fold before quantitative PCR (qPCR) reaction. The qPCR was performed in a CFX Connect (Bio-Rad) with Luna Universal qPCR Master Mix (NEW ENGLAND Biolabs) in a total reaction volume of 20 μ L (10 μ L Luna Universal qPCR Master Mix, 0.5 μ L 10 μ M/L forward primers and reverse primers, 2 μ L cDNA and 7 μ L H₂O). The qPCR program was run as suggested by the qPCR Master Mix supplier (95°C for 60 s, 40 cycles: 95°C for 15 s and 60°C for 30 s). Melting curves were generated by increasing temperature from 65°C to 95°C with measurement every 0.5°C increment. Gene-specific primers were used for amplification (Table S4). *Actin2* (*At3g18780*) was used to normalize gene expression.⁴⁹ Relative gene expression level was shown as 2^{- $\Delta\Delta$ Ct}.⁵⁰ Three replicates per reaction were carried out. Biological replicates were not possible because T₁ lines of 35S_{pro}:SSP, 35S_{pro}:SSP *er1 er2* and 35S_{pro}:BSK1 *er1 er2* were tiny and sterile.

Phenotypic analysis of rosette leaves

For Images of rosette leaves, three-week old plants were photographed with Canon EOS 1000D camera. To increase the visual contrast, images of rosette leaves were separated from background soil using Adobe Photoshop.



RNA stability

Arabidopsis RNA half-life (stability) was derived from published data by Szabo et al.²³ using an exponential decay model. All data, a detailed description of the analysis and a hands-on protocol have been previously published.²³

QUANTIFICATION AND STATISTICAL ANALYSIS

Measurement of zygote/suspensor length

After DIC imaging, size measurements were performed using measurement tools of ImageJ software (version 1.52p).⁴² The length of the fully elongated zygote was inferred from the sum of apical and basal daughter cell length of the 1-cell embryo. Zygote polarity was determined as ratio of apical and basal cell length. Suspensor length during transition stage was measured from the micropylar end of the basal suspensor cell to the center of uppermost suspensor cell. In crossing experiments, anthers were removed before anthesis, followed by manual pollination approximately 24 h later. Ovules were collected 24–30 h after pollination to image the 1-cell embryos and 4 days after pollination to image transition stage embryos.

Statistical analysis

Boxplot diagrams were made online with BoxPlotR.⁴¹ For statistical analysis of phenotypic data and expression data, the one-way ANOVA analysis with post hoc Tukey test was performed in R (version 3.6.1) with multcompView package (<https://cran.r-project.org/web/packages/multcompView/index.html>). The number of data points in each group was listed at the bottom of each diagram. The segregation analysis was statistically analyzed with Chi-Square test.



Square one: zygote polarity and early embryogenesis in flowering plants

Kai Wang, Houming Chen, Yingjing Miao and Martin Bayer



In the last two decades, work on auxin signaling has helped to understand many aspects of the fundamental process underlying the specification of tissue types in the plant embryo. However, the immediate steps after fertilization including the polarization of the zygote and the initial body axis formation remained poorly understood. Valuable insight into these enigmatic processes has been gained by studying fertilization in grasses. Recent technical advances in transcriptomics of developing embryos with high spatial and temporal resolution give an emerging picture of the rapid changes of the zygotic developmental program. Together with the use of live imaging of novel fluorescent marker lines, these data are now the basis of unraveling the very first steps of the embryonic patterning process.

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Current Opinion in Plant Biology 2020, 53:128–133

This review comes from a themed issue on **Growth and development**

Edited by **Marcus Heisler** and **Alexis Maizel**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 11th November 2019

<https://doi.org/10.1016/j.pbi.2019.10.002>

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Introduction

In embryogenesis, a single cell generally develops into a miniature version of the adult organism. In plants, however, the final body architecture is determined post-embryonically, likely as adaption to their sessile life. Plant embryos represent primitive versions of seedlings and only a few basic cell types are established during embryogenesis [1] (Figure 1).

Fertilization of the egg cell marks the beginning of a new sporophytic generation, starting with the zygote. In many angiosperm species, the zygote divides asymmetrically, often after an initial phase of cell elongation [2]. The apical daughter cell will always form at least part of the embryo. The basal daughter cell contains a large central vacuole and its descendants will mainly contribute extra-embryonic

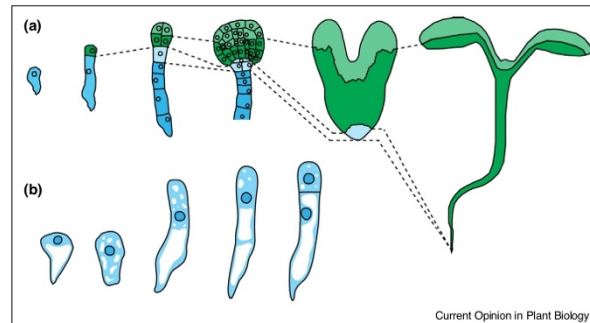
tissue [3]. In *Brassicaceae*, embryogenesis follows an invariable series of cell divisions, in which the apical cell forms a spherical proembryo (Figure 1a). The basal daughter cell undergoes a series of transverse mitotic divisions to form the filamentous suspensor. Except the uppermost cell, the suspensor is extra-embryonic, connects the embryo with maternal seed tissue, and does not contribute to the later plant seedling. Therefore, in *Brassicaceae*, the first zygotic division already marks the boundary between embryonic and extra-embryonic development [4]. In other flowering plants, however, the patterning process can be less stereotypic and in some cases there is no clear boundary between embryonic and suspensor tissue (Figure 2). Cells derived from the first apical cell can also differentiate into suspensor cells, as seen in some members of the *Caryophyllaceae*. In a similar manner, descendants of the basal cell might also contribute to large portions of the proembryo as in some *Asteraceae* family members [3]. Therefore, patterning seems to be mainly influenced by position-dependent cues and the concept of cell lineages apparently does not apply in the early plant embryo. Nonetheless, a universal feature of embryogenesis in seed plants is the polarity of the first zygotic division with the apical daughter cell always contributing to the embryo. This division seems to be an essential step in axis formation of the angiosperm embryo and it is therefore a fundamental question how the zygote is polarized.

Early morphological changes

In flowering plants, already the egg cell is a polar structure with the nucleus at the apex and a large central vacuole at the basal pole [5] (Figure 1b). Microtubules are preferentially oriented in a longitudinal direction [6*]. Directly after fertilization, however, this polarity seems to be lost as the large vacuole is broken up into smaller, evenly distributed vacuoles and the nucleus moves to a central position [5,7]. After this transient apolar phase, polarity is reestablished and the zygote starts to elongate in a similar fashion as tip-growing cells such as pollen tubes or root hair cells [6*,7]. In *Arabidopsis*, the zygote grows three-fold in size whereas in many grasses there is barely any noticeable growth at this stage [3]. The first hallmark of polarity reestablishment happens at this stage as the nucleus moves out of the center to an apical position, followed by an asymmetric division. What kind of positional information determines the direction of cell elongation and the position of the cell division plane?

In many animals, the sperm entry site can function as positional cue [8,9]. In a similar fashion, the brown alga *Fucus* relies on the position of sperm entry in the absence

Figure 1



Embryogenesis in Arabidopsis.

(a) After the first zygotic division, the apical cell forms the spherical embryo (green) while the basal cell forms the mostly extra-embryonic suspensor (blue). The final seedling is mainly formed by the upper tier (light green) and lower tier (dark green) of the embryo proper. Only the upper-most suspensor cell (light blue) in direct contact with the embryo proper contributes to the embryonic root. The rest of the suspensor (dark blue) remains extra-embryonic. (b) In accordance with morphological criteria, egg cell polarity is lost in the early zygote. The large central vacuole (white) at the basal pole of the egg cell is partitioned into smaller evenly distributed vacuoles. During cell elongation, the zygote repolarizes and the nucleus moves to the apex before the first asymmetric division. During zygotic cell elongation, a large central vacuole is reformed at the basal pole.

of other polarizing cues [10]. In flowering plants, however, *in vitro* experiments with rice gametes would argue against positional information provided by the sperm entry site as there was no correlation between its position and polarity of the zygote [11]. Fusion with the sperm, on the other hand, seems to be a necessary trigger for egg activation as it causes Calcium influx and initiates karyogamy and the activation of a zygotic transcriptional program [12] which cannot be achieved by artificial fusion of two egg cells.

In rice, egg activation possibly involves the paternally provided AP2 transcription factor OsASGR-BBML1 [13^{**}]. Overexpression of BBML1 in the egg cell leads to proliferation without fertilization. Dominant negative versions of BBML1 lead to developmental arrest if expressed in the zygote. As ectopic overexpression of BBM induces embryogenesis in somatic cells in Arabidopsis [14], this family of transcription factors could be part of a two-component system of maternal and paternal factors that initiate a zygotic transcriptional program similar to the BELL/KNOX transcriptional regulators in the unicellular alga *Chlamydomonas reinhardtii* [15]

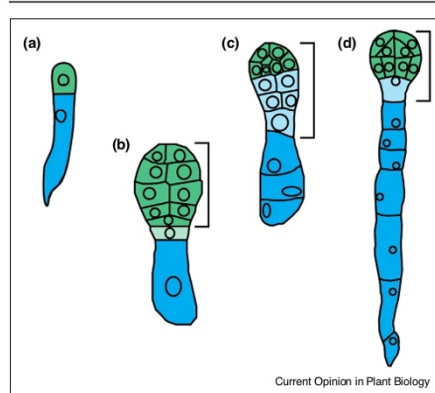
Zygotic gene expression

There have been conflicting reports about the timing of the maternal-to-zygotic transition and the degree of maternal and paternal contribution to early embryogenesis of flowering plants [16–21,22^{*}]. Recent advances in manual isolation of single cells made it possible to

generate transcriptional profiles of gametic cells and early embryos with much higher temporal and spatial resolution and lower contamination by surrounding maternal tissue [13^{**},23^{**},24^{**}]. With these latest transcriptomic data from grasses and Arabidopsis, a clear picture is emerging.

In animals, the maternal-to-zygotic transition follows two phases. At the beginning, development is regulated purely post-transcriptionally by maternal mRNAs and proteins stored in the oocyte. Later, as the zygotic genome is activated, maternal RNAs are depleted and zygotic mRNAs take over functionally [25]. In flowering plants, there seems to be in principle a similar progressive activation of zygotic bi-parental transcripts [13^{**},23^{**},24^{**}]. However, this transition seems to happen rapidly in the zygote. In Arabidopsis, sperm-specific transcripts can be detected in zygotes at 14 hours after pollination (hap) but are dramatically reduced or absent in zygotes at 24 hap. At the same time, egg cell-specific transcripts are rapidly eliminated and several thousand genes are upregulated in the zygote compared to the egg cell [24^{**}]. In Arabidopsis, it has been shown that already the first zygotic cell division relies on *de novo* transcription [24^{**}]. The onset of zygotic transcription is accompanied by rapid turn-over of gametic histones and replacement by zygotic histones [26]. These new data therefore further support the view that zygotic genome activation happens very rapidly in flowering plants as previously proposed by Nodine and Bartel [20]. However,

Figure 2



Clonal origin of embryonic structures (after [3], modified).

(a) The first cell division gives rise to an apical (green) and basal (blue) daughter cell. (b) Suspensor cells derived from the apical cell in *Sagina procumbens* (Caryophyllaceae); shown in light green. (c) Cells originating from the basal cell contribute substantially to the embryo proper in *Geum urbanum* (Asteraceae); depicted in light blue. (d) In *Brassicaceae*, only the upper-most descendent of the basal cell (light blue) contributes to the embryo. Cells contributing to the later seedling are marked by brackets.

these rapid transcriptome changes and the observed intracellular reorganization of the zygote raise the question if polarization of this cell relies on pre-existing parental factors or if polarity establishment is an intrinsic zygotic process.

MAP kinase signaling

Polarity of the zygote and differentiation of the first daughter cells seem to be at least in part controlled by a MAP kinase-dependent signaling pathway. This pathway includes the MAP kinases MPK3 and MPK6, the MAPK kinases MKK4 and MKK5 as well as the MAPKK kinase YODA (YDA) [27–29] (Figure 3). Loss of YDA impairs zygote elongation and results in symmetric cell division. Furthermore, both daughter cells adopt embryo-like division patterns, resulting in embryos lacking a recognizable suspensor. Constitutive activation of the YDA pathway leads to structures entirely consisting of suspensor-like cells [27]. This pathway therefore seems to promote zygote elongation and polarity and suppresses embryonic development in the basal daughter cell [30].

One of the direct phosphorylation targets of this signaling cascade is the transcription factor WRKY2. Together with the HOMEODOMAIN GLABROUS (HDG) transcription factors HDG11/12, WRKY2 activates expression of *WUSCHEL-RELATED HOMEBOX 8* (*WOX8*) [31,32].

WOX8 and *WOX9* are necessary for correct suspensor development and expression of several developmentally important genes in the embryo [33]. *WOX* genes generally play important roles as transcriptional repressor in stem cell niches. However, these members of the evolutionarily more ancient *WOX9* clade rather seem to act as transcriptional activators [34].

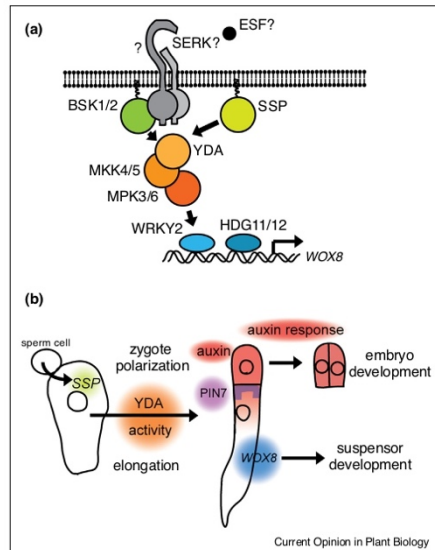
As the *wrky2* mutant recapitulates certain aspects of the *yda* phenotype but lacks others, there are likely additional targets of the YDA-dependent signaling cascade in the zygote. Potential targets could be transcription factor complexes including the RWP-RK protein GRD/RKD4, although GRD itself does not seem to be a direct target [35,36]. Mutant *grd* embryos in principle resemble *yda* embryos albeit the phenotype is less pronounced. Some members of the *RKD* family play a pivotal role in the egg-to-zygote transition [37,38]. It is therefore tempting to speculate that YDA might be involved in setting up a zygotic genome program via GRD regulation. As the overexpression of *RKD4* can induce somatic embryogenesis [36], a role of *RKD4* in activating a zygotic transcriptome seems at least plausible.

YDA is activated in the zygote by the BRASSINOSTEROID SIGNALING KINASE family members BSK1 and BSK2 [39**]. These membrane-associated proteins directly interact with the YDA kinase domain, possibly in a phosphorylation-dependent manner. BSK family proteins have been described as integral part of SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)-dependent receptor complexes in various signaling pathways [40–42]. These leucine-rich repeat containing co-receptors are involved in YDA-dependent developmental processes [43]. However, the receptor teaming up with the SERK co-receptors upstream of YDA in the embryo as well as possible ligands are still unknown.

Possible candidates include peptides of the EMBRYO SURROUNDING FACTOR (ESF) family. Downregulation by RNAi causes mild suspensor defects that can be rescued by expression of a constitutively active variant of YDA [44]. The spatial and temporal information provided by these possible ligands is, however, presently unclear.

In *Brassicaceae*, an additional way of YDA activation exists in the zygote. The unusual BSK family member SHORT SUSPENSOR (SSP/BSK12) accumulates specifically in the zygote, apparently translated from paternally inherited transcripts [45]. The SSP protein lacks a regulatory intra-molecular interaction typically seen in BSK proteins [39**]. Resembling a constitutively active version of BSK1, SSP can activate YDA directly after fertilization possibly without involving canonical receptor activation (Figure 3). As *ssp* mutants show slower embryonic development and a less defined boundary between embryo and suspensor, much like many species outside the

Figure 3



Signaling events in the early embryo.

(a) Schematic depiction of the embryonic YDA signaling pathways. The MAPKKK YDA can be activated by a possible receptor kinase complex including the membrane-associated proteins BSK1/2. In addition, paternally provided SSP directly activates YDA in *Brassicaceae*. **(b)** Consecutive signaling events in the early embryo. YDA activity contributes to zygote elongation, asymmetric cell division, and differential WOX8 expression. A local, PIN7-mediated auxin maximum in the apical daughter cell initiates 3-dimensional growth of the embryo proper.

Brassicaceae family, this paternal input might provide a beneficial boost of YDA activity contributing to the rapid life cycle of *Brassicaceae* family members [45,46].

Auxin signaling

Many developmental processes in plants start with an asymmetric division and almost universally seem to involve the plant hormone auxin [47–49]. In the zygote, however, direct evidence for a role of auxin in regulating the first asymmetric division is lacking [30]. As the zygote seems to elongate in a tip-growing manner similar to pollen tubes or root hair cells, there is the possibility that this division actually resembles a rather specialized case independent of auxin signaling. The resulting daughter cells, however, show differential expression of genes involved in auxin response and transport. PIN7 in the basal cell is thought to initiate basal-to-apical auxin

transport [50]. According to this model, three-dimensional growth of the embryo is then initiated by an auxin response maximum in the apical cell, re-orienting the cell division plane [48]. Auxin responses in the apical cell, therefore, seem to be an essential part of initiating embryonic development. Since the differential auxin response in the two zygotic daughter cells relies on already differentially expressed genes of auxin transport and response [50,51], this raises the question if auxin signaling in this context mainly amplifies and firmly establishes already pre-existing, transient differences.

Auxin transport is intimately linked to auxin synthesis and recent seemingly conflicting reports indicate possibly several sources [52–54,55**]. Consistent with PIN7-mediated transport from the basal cell, auxin might initially be synthesized in maternal cells in the micropylar region that are in direct contact with the basal daughter cell [55**]. However, once suspensor differentiation is initiated in the basal cell, the suspensor itself seems to become an auxin source [54].

Conclusions

Recent advances in live imaging and novel fluorescent markers [6*,7,56] as well as transcriptomic approaches [13**,23**,24**] have yielded new insights into the enigmatic processes that lead to symmetry breaking in the zygote. Polarity information seems to be ubiquitous in plant cells and interpreted at least in part by the recently discovered SOSEKI proteins [57**]. Their role in zygote polarization, however, has to be investigated in detail. It is still an open question if cell polarity in the zygote is inherited from the parental generation or established *de novo*. Does this process rely on external cues or is it directed by an intrinsic mechanism possibly including mechanical forces? How does the MAP kinase-WRKY-WOX pathway integrate with auxin signaling in establishing different cell identities? Recently described MPK-dependent phosphorylation of PIN proteins might suggest that this interaction could be rather direct [58*,59]. With the recently developed and refined methods, future research will help to answer these fundamental questions at the very first step of plant development.

Conflict of interest statement

Nothing declared.

Acknowledgements

We apologize to our colleagues whose work we have not included in this manuscript due to space restrictions. We would like to thank Daniel Slane and Gerd Jürgens for critical reading of the manuscript and helpful discussions. Research in our group is supported by the German Science Foundation (Deutsche Forschungsgemeinschaft – DFG: SFB1101/B01 to M.B.), the Chinese Scholarship Council (Fellowship No. 201806320131 to Y.M.), and the Max Planck Society. The authors declare no conflict of interest.

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Chapter 4

Zygotic Embryogenesis in Flowering Plants

Houming Chen, Yingjing Miao, Kai Wang, and Martin Bayer

Abstract

In the context of plant regeneration, in vitro systems to produce embryos are frequently used. In many of these protocols, nonzygotic embryos are initiated that will produce shoot-like structures but may lack a primary root. By increasing the auxin-to-cytokinin ratio in the growth medium, roots are then regenerated in a second step. Therefore, in vitro systems might not or only partially execute a similar developmental program as employed during zygotic embryogenesis. There are, however, in vitro systems that can remarkably mimic zygotic embryogenesis such as *Brassica* microspore-derived embryos. In this case, the patterning process of these haploid embryos closely follows zygotic embryogenesis and all fundamental tissue types are generated in a rather similar manner. In this review, we discuss the most fundamental molecular events during early zygotic embryogenesis and hope that this brief summary can serve as a reference for studying and developing in vitro embryogenesis systems in the context of doubled haploid production.

Key words Axis formation, Pattern formation, Radial patterning, Root formation, Shoot apical meristem, Zygote polarization, Zygotic embryogenesis

1 Introduction

Land plants form most of their body shape postembryonically in response to environmental cues [1, 2]. During embryogenesis, therefore, only a simple yet stereotypic manifestation of a small plant is set up. This seedling invariably consists of a few readily recognizable structures along its apical–basal axis, namely, the embryonic leave(s), the hypocotyl, and the embryonic root. Less obviously, the stem cell niches (meristems) for postembryonic development of the shoot and the root are also already established at the opposite ends of the apical–basal axis. The plant embryo is further partitioned along the radial axis into vasculature, ground tissue, and epidermis.

These different tissue types are generated by consecutive steps of patterning along the apical–basal as well as the radial axis [3]. Many of the molecular players orchestrating this development

have been identified in *Arabidopsis thaliana*, but other model species such as maize and rice have also significantly contributed to our understanding of embryonic patterning in plants. In this review, we mainly focus on *Brassicaceae* embryogenesis as the stereotypic patterning process in this plant family facilitates the identification of different cell types by position. The invariant patterning also makes it possible to trace the origin of cells back to decisive cell divisions without live imaging [4]. In order to keep this review concise, we limit our overview to key factors that seem to have a conserved function in flowering plants and will refer to more comprehensive or specialized reviews where necessary.

2 Early Steps of Axis Formation

2.1 Zygote Polarization

In *Arabidopsis*, the fertilized egg cell (zygote) seems to undergo a brief phase in which cell polarity is lost, before it repolarizes, elongates about threefold, and finally divides asymmetrically to form an apical and a basal cell [5, 6] (Fig. 1).

The apical cell will invariably develop into the embryo. Descendants of the basal cell will foremost contribute to an extraembryonic support structure, called suspensor. In *Brassicaceae*, the suspensor is a filamentous structure formed by a series of stereotypic horizontal divisions of the basal cell and its descendants. The early embryo and suspensor are clonally derived from the apical and basal cell, respectively. However, in other angiosperms, this boundary is less clear and cells originating from the apical cell can also contribute to the suspensor (i.e. in members of the *Caryophyllaceae*) or cells derived from the basal cell can form part of the embryo, as in members of the *Asteraceae* family [7, 8].

In *Arabidopsis*, the elongation of the zygote is accomplished by tip growth [9]. This rather specialized mechanism of cell elongation can also be seen during pollen tube growth and root hair growth. While zygote elongation is a common feature of many angiosperm species [7], it does not seem to be a necessity for zygote polarization. In some members of the grasses, such as maize and rice, the egg cell undergoes an asymmetric division right after fertilization without any apparent cell elongation [10, 11].

The correct positioning of the zygote nucleus during this repolarization phase depends on actin filaments and vacuolar dynamics [9, 12]. The intrinsic polarity of the zygote does not necessarily result in size differences of the daughter cells and there are quite a number of plant species in which the apical cell is equal in size or larger than the basal cell [10, 13]. Independently of their size, however, the apical cell pointing toward the central cell and the chalaza will always form (at least part of) the embryo proper. The basal cell that is positioned toward the micropyle and is in physical

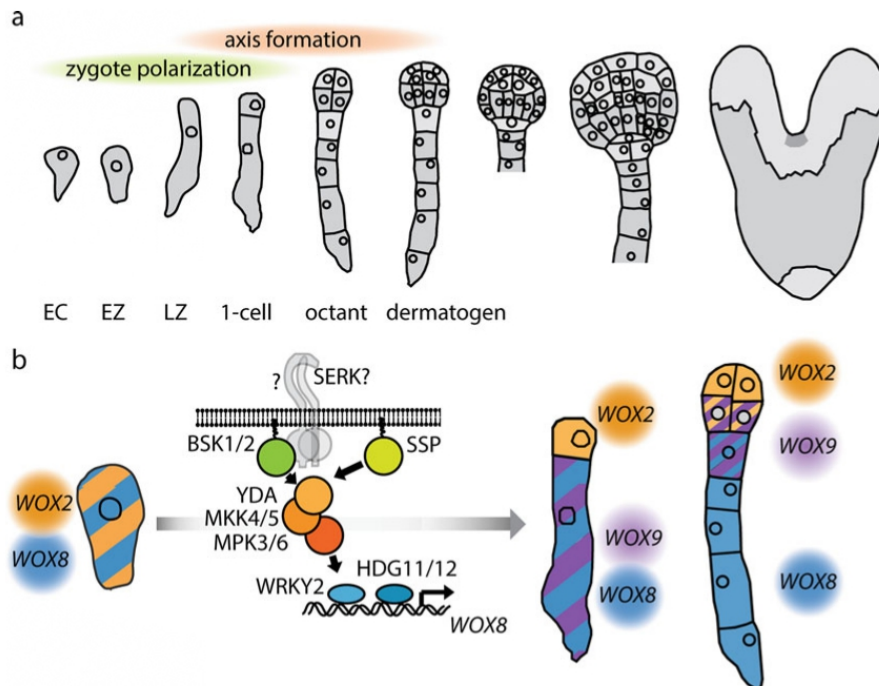


Fig. 1 Apical–basal axis formation. **(a)** Overview of early embryo morphology in Arabidopsis. Developmental stages are indicated below the graphic. *EC* egg cell, *EZ* early zygote, *LZ* late zygote. **(b)** Zygote polarization and patterning along the apical–basal axis. A simplified model of the embryonic YDA pathway is shown on the left. Differential expression of *WOX* gene family members along the apical–basal axis is depicted on the right

contact with sporophytic tissue of the seed coat will always contribute to the extraembryonic suspensor [14].

2.2 MAP Kinase Signaling

Zygote elongation and zygote polarization are at least in part regulated by a MITOGEN- ACTIVATED PROTEIN (MAP) kinase-dependent pathway (Fig. 1). This MAP kinase cascade includes the MAP2K kinase (MAP3K) YODA (YDA) [15], the MAPK kinases (MAP2K/MKK) MKK4 and MKK5, and the MAP kinases (MAPK/MPK) MPK3 and MPK6 [16, 17]. Upstream of YDA in this signaling cascade, two members of the BRASSINOS TEROID SIGNALING KINASE (BSK) family—BSK1 and BSK2—work in parallel [18]. These membrane-associated proteins typically function as signaling relay in SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK)-dependent receptor kinase pathways [19]. It is therefore a likely scenario that the YDA-dependent MAP kinase cascade is controlled by an extracellular signal received by a SERK-dependent receptor complex

[18]. Possible candidates for the extra-cellular signal include a family of small proteins termed EMBRYO SURROUNDING FACTOR [20] and the possible peptide ligand CLAVATA3/ESR-RELATED (CLE) 8 [21]. These extra-cellular molecules influence suspensor formation, but their direct involvement in activating the embryonic YDA pathway has not been shown and is therefore still hypothetical.

In *Brassicaceae*, an additional mechanism of YDA activation exists [22]. The atypical *BSK* family member *SHORTSUSPENSOR* (*SSP*) evolved as sister gene of *BSK1* [23]. In contrast to *BSK1*, which functions in a phosphorylation-dependent manner [24], the *SSP* protein seems to constitutively adopt an activated conformation [18]. *SSP* is tightly controlled at both transcriptional and translational levels. *SSP* transcripts accumulate at high level exclusively in sperm cells and seem to be paternally inherited to the zygote where the *SSP* protein transiently accumulates [25]. Since *SSP* directly interacts with YDA, the presence of *SSP* in the zygote links YDA activation with the fertilization event [18].

The rather mild defects in zygote polarization and suspensor development seen in *ssp* mutants are accompanied by strikingly slower embryo development possibly due to a malformed suspensor [26]. Therefore, the *Brassicaceae*-specific *SSP* protein might provide an additional, beneficial boost of early YDA activity in parallel to a *BSK1*/*BSK2*-dependent YDA activation [18].

One of the targets of this pathway is the transcription factor WRKY DNA-BINDING PROTEIN 2 (*WRKY2*) that is phosphorylated and activated by *MPK6* [6, 27].

WRKY2 together with *HOMEODOMAIN GLABROUS* (*HDG*) *HDG11* and *HDG12* transcriptionally activates the homeodomain transcription factor gene *WUSCHEL RELATED HOMEODOMAIN 8* (*WOX8*) in the basal cell [6, 27, 28]. This possibly indicates that the embryonic YDA pathway is active at the basal pole of the zygote and/or in the basal daughter cell. Loss of *WOX8* and its close homolog *WOX9* leads to developmental defects in the suspensor. However, expression of the *WOX* gene family member *WOX2* as well as other genes that are normally confined to the apical daughter cell and its descendants is also lost in *wax8 wax9* double mutants, indicating a possible non-cell autonomous function [29].

Additional transcription factor complexes that include the RWP-RK domain-containing (RKD) protein GROUNDED (*GRD*)/*RKD4* may be further targets of the embryonic YDA pathway as suggested by the genetic interaction of these mutants and the strikingly similar loss-of-function phenotype [30]. However, as members of this family seem to play a role in egg-to-zygote transition, a more general role in establishing a zygotic or embryonic transcriptional program and therefore setting the stage for

a functional embryonic YDA pathway might also be possible [30–32].

2.3 Auxin Taking the Stage

The two daughter cells shaped by the first zygotic cell division are characterized by differential gene expression [33]. Among the differentially expressed genes are members that control auxin transport and transcriptional auxin response [33, 34]. Consequently, with the formation of the embryogenic apical cell, auxin becomes one of the central players in the patterning process of the embryo. According to current data, sporophyte-derived auxin is transported by the auxin efflux facilitator PIN-FORMED 7 (PIN7) in the apical membrane of the basal cell to the terminal apical cell [34, 35]. Rising auxin levels in the apical cells lead to transcriptional auxin responses as well as a deviation of the stereotypic horizontal cell divisions by a 90° turn of the division plane [36]. If auxin responses are blocked in the early embryo, a stereotypic horizontal division plane following simple geometric rules of a local minimum in division plane size is formed [36, 37]. The initial apical transport of auxin and the corresponding differential auxin responses in the apical cell therefore seem to initiate the three-dimensional growth of the embryo.

It has been shown that auxin synthesis is coupled to auxin transport and response [38–40] and early in development of the embryo, the suspensor is a prominent source of auxin [39]. With rising auxin concentrations, the embryo proper itself will become the dominant source of auxin around globular stage, initiating a reversal of auxin transport in apical–basal direction [39, 41–43].

The differential auxin response in the apical cell vs. basal cell and their descendants is accomplished by directional auxin flow toward the apical cell and by different auxin response modules in the embryo vs. suspensor [44]. At this early stage, suspensor cells still possess embryogenic potential and cell proliferation seems to be repressed by a suspensor-specific auxin response module [44]. This is highlighted by laser ablation of the embryo or by suspensor-specific expression of certain embryo-specific factors initiating suspensor-derived secondary embryos [45, 46].

This raises the question how polar auxin transport and different auxin response modules are set up early in embryogenesis. As many factors in auxin synthesis, transport and response are expressed in an auxin-dependent manner, this could be in principle a self-organizing system [47]. However, since *max8 max9* double mutants show expanded auxin responses in the suspensor and lack the expression of some components of the embryo-specific auxin response module, a link between MAP kinase signaling in the zygote and differential auxin responses in the zygotic daughter cells seems to exist [6, 27, 29]. How direct this connection is, however, needs to be determined.

3 Patterning Along the Apical–Basal Axis

3.1 *WOX* Gene Expression

Three-dimensional growth of the embryo is initiated by two rounds of vertical divisions in the apical lineage of Arabidopsis. These four cells then divide each horizontally to form an upper and lower tier in the eight-cell preglobular embryo. The order of these cell divisions does not seem to be strictly necessary as other plant species (i.e., in the *Solanaceae* family) form a similar structure by an initial horizontal division of the apical cell followed by two rounds of vertical divisions in the upper and lower tier of the embryo, respectively [7].

At the eight-cell stage, the embryo can be functionally divided along its apical–basal axis. The four cells of the upper tier are the precursors of the embryonic leaves and the shoot apical meristem (SAM). Cells of the lower tier will form the hypocotyl, vasculature, and parts of the root meristem. The uppermost suspensor cell or hypophysis is the founder cell of the quiescent center (QC) of the root meristem as well as the columella. The rest of the suspensor remains extraembryonic. This organizational segmentation along the apical–basal axis is paralleled by overlapping expression domains of three *WOX* family genes [28] (Fig. 1).

The upper tier is characterized by *WOX2* expression, while in the lower tier, *WOX2* expression overlaps with the expression domain of *WOX9*. In the hypophysis, *WOX9* is coexpressed with *WOX8*. In the suspensor, *WOX8* is the only member of these three genes that is expressed [28] (Fig. 1). While the loss of *WOX2* or *WOX8* and *WOX9* lead to patterning defects in the embryo and the suspensor, respectively [29], their exact role during early embryogenesis is not fully understood. In the seedling, *WOX* family members control the proliferation of stem cells in various meristems (reviewed in [48]), a similar role in cell cycle regulation during early embryogenesis seems therefore possible [49].

3.2 Setting Boundaries

As the expression patterns of *WOX* genes illustrate, there seem to be clear boundaries between expression domains in the early Arabidopsis embryo that often are confined to single cell files. How this is achieved mechanistically is not clear. However, the GATA transcription factor HANABA TARANU (HAN) seems to play a critical role in setting up or maintaining the embryo–suspensor boundary [50]. In *han* mutants, genes typically expressed in the suspensor expand their expression domain to the lower tier. How *HAN* restricts the expression of these genes to the suspensor, however, and if/how this relates to the transcriptional network of *WOX* transcription factor genes, still needs to be investigated [50].

4 Radial Patterning

4.1 Protoderm Formation

Radial patterning is initiated with tangential divisions that form the 16-cell or dermatogen stage embryo [51]. This step depends on *WOX* gene function, as indicated by occasional anticlinal cell divisions in the upper tier in *wax2* mutants [29]. Similar incorrect orientation of cell divisions at this stage can be achieved by expression of a nondegradable version of the transcriptional repressor *BODENLOS* (*BDL*)/ *INDOLE-3-ACETIC ACID INDUCIBLE 12* (*IAA12*) [36], a critical component of the transcriptional auxin response module [37, 52]. This indirectly implies that asymmetric divisions forming the protoderm layer involve transcriptional auxin responses. Despite these early defects in division plane orientation, these embryos still form a functional epidermis [44], indicating that protoderm formation might be a rather robust process. Only a few molecular players involved in protoderm initiation are known (reviewed in [53]). However, the involvement of receptor kinases such as RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and RPK2 (TOADSTOOL2 (TOAD2)) would suggest that protoderm formation relies on signaling at the outer cell surface [54, 55].

A key player in protoderm formation is the homeodomain leucine zipper class IV (HD-ZIP IV) transcription factor *A. thaliana* MERISTEM LAYER1 (*AtML1*) that is necessary and sufficient for epidermal cell identity [56–60]. Double mutants of *ATML1* and its closest homolog *PROTODERMAL FACTOR2* (*PDF2*) show strong defects associated with misspecification of the protoderm while overexpression of *AtML1* can induce epidermal cell differentiation in nonepidermal cells [56, 58–60]. *AtML1* is initially expressed in all cells of the embryo but then confined to the outer cells (L1 layer) from the 16-cell stage onward [61].

How L1-specific expression of *AtML1* is controlled on a molecular level is not clear. However, this seems to involve posttranscriptional regulation [62, 63]. *AtML1* and *PDF2* activate the epidermis-specific expression of target genes by binding to a cis-regulatory element termed L1 box. As this element can be found in the *AtML1* and *PDF2* promoter region and is necessary for L1-specific expression of *PDF2*, a feedforward regulation seems to play a central role in controlling *AtML1* expression [56, 57, 61].

4.2 Patterning in the Lower Tier

Radial patterning in the lower tier establishes the initials for the vasculature, ground tissue and epidermis (Fig. 2; reviewed in [64]). By periclinal division, the ground tissue initials will then form endodermis and cortex. Many factors for differentiation and maintenance of different cell identities in the vasculature are known (reviewed in detail in [10, 65]). However, the initial setup in the embryo is less well understood. Radial patterning of the vasculature critically depends on auxin responses mediated by the auxin

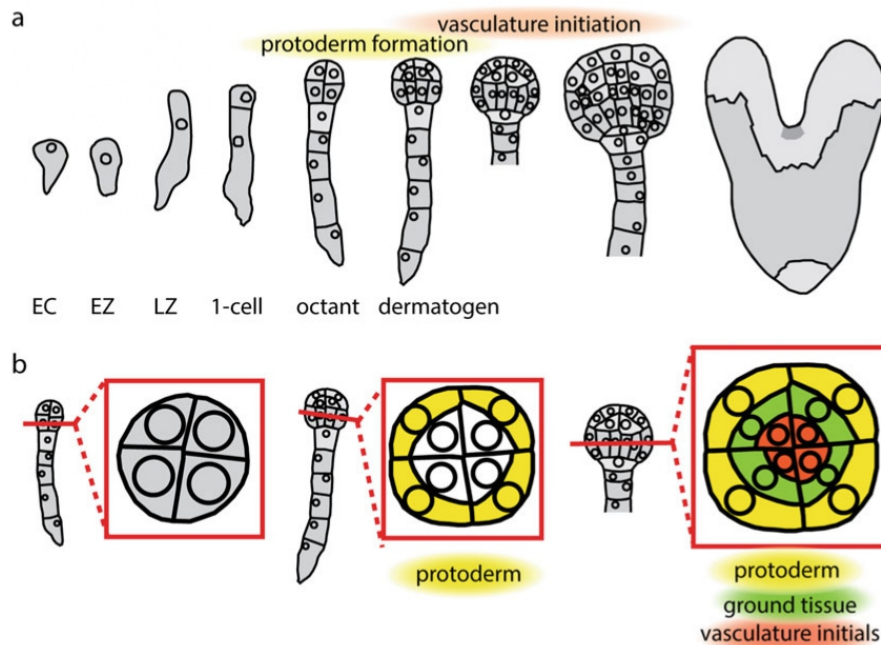


Fig. 2 Radial patterning in the Arabidopsis embryo. (a) Overview of early embryo morphology in Arabidopsis. Developmental stages are indicated below the graphic. EC egg cell, EZ early zygote, LZ late zygote. (b) Patterning along the radial axis in the lower tier of the embryo. Cross sections are illustrated in red squares. Yellow, protoderm; green, ground tissue; red, vasculature precursor cells

response factor (ARF) MONOPTEROS (MP) [66, 67] (Fig. 3). MP transcriptionally activates the bHLH transcription factor gene *TARGET OF MONOPTEROS 5* (*TMO5*) in the vasculature initials. TMO5 forms heterodimers with the bHLH transcription factor LONESOME HIGHWAY (LHW) and stimulates the expression of the cytokinin synthesis genes *LONELY GUY* (*LOG*) 3 and *LOG* 4. Together, this transcriptional complex controls periclinal divisions and therefore the number of cell files in the vasculature bundle (for comprehensive review, see ref. 68).

5 Establishing the Stem Cell Niches

5.1 Root Initiation

The first step of root meristem formation in Arabidopsis is the specification of the hypophysis [69]. This uppermost suspensor cell will form the QC that is responsible for stem cell maintenance [70]. The QC is characterized by expression of *WOX5* that maintains quiescence by controlling the cell cycle [28, 71, 72] (Fig. 3).

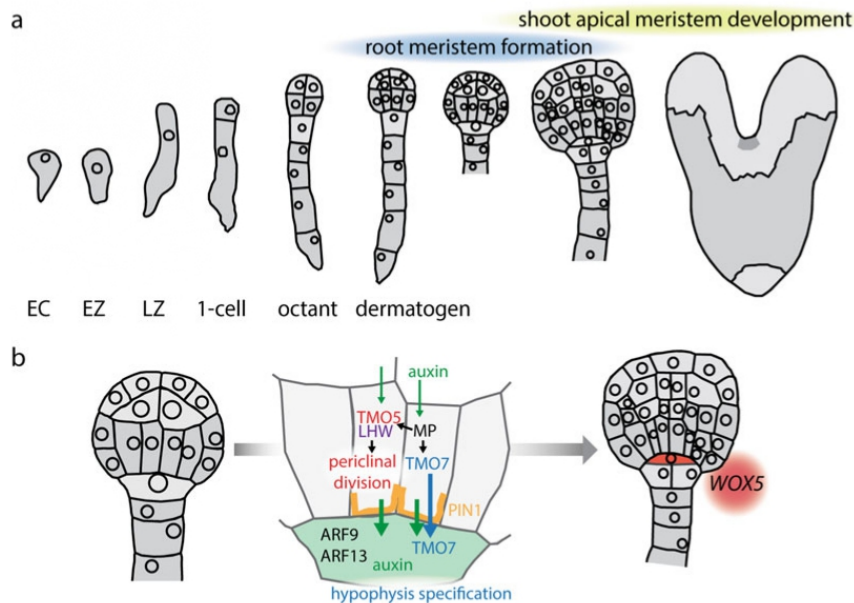


Fig. 3 Auxin signaling in vasculature formation and root meristem initiation. **(a)** Overview of early embryo morphology in Arabidopsis. Developmental stages are indicated below the graphic. *EC* egg cell, *EZ* early zygote, *LZ* late zygote. **(b)** A schematic overview of auxin responses leading to periclinal divisions in prevasculature cells and hypophysis specification. Auxin responses in the central cells of the lower tier and in the future hypophysis require cell-type specific auxin response machineries. After the asymmetric division of the hypophysis, expression of the *WOX* gene family member *WOX5* marks the quiescent center precursor cell

An intricate interaction of cells of the lower tier and the future hypophysis precedes the asymmetric division of the hypophysis that forms the QC. Auxin responses in the central cells of the lower tier lead to MP-dependent expression of *TMO7* [69]. This small bHLH transcription factor moves to the neighboring hypophysis where it plays a critical role in initiating QC formation [69]. This process also depends on the transcription factors NO TRANSMITTING TRACT (NTT)/WIP DOMAIN PROTEIN 2 (WIP2), WIP4, and WIP5 [73] that are expressed in the hypophysis, possibly in an auxin-dependent manner (reviewed in [64]).

Basal transport of auxin via PIN1 in the neighboring proembryo results in high auxin levels in the hypophysis as inferred from strong activity of synthetic auxin response reporters [34, 44, 69, 74]. Accumulation of auxin triggers transcriptional auxin responses mediated by a suspensor-specific auxin response machinery including ARF9, ARF13 and IAA10 [44] (Fig. 3).

Root initiation is also controlled by AP2 domain transcription factors of the PLETHORA (PLT) family [75]. *PLT1* and *PLT2* are expressed in the lower tier from the 8-cell embryo on and in the suspensor [50, 75]. PLT transcription factors can be considered to be master regulators of root identity. Loss of the four *PLT* gene family members *PLT1*, *PLT2*, *PLT3*, and *PLT4/BABY BOOM* (*BBM*) leads to misspecification of the hypophysis and rootless seedlings [76]. Ectopic expression of *PLT1* and *PLT2* in the shoot region or in the apical region of the embryo can induce ectopic root formation [75–77]. Expression of *PLT1* and *PLT2* depends on *MP*-dependent auxin signaling, emphasizing the importance of auxin in root formation [78].

5.2 The Shoot Apical Meristem

In tissue culture and in vitro plant regeneration, it has been known for a long time that the auxin-to-cytokinin ratio is crucial for the induction of shoots or roots, respectively. Under growth conditions where cytokinin is dominating, shoots can be regenerated, indicating an important role of cytokinin in formation of the SAM [79].

In the *Arabidopsis* embryo, this stem cell niche is first recognized at late heart stage as organized cell layers between the cotyledons [1, 80]. The organizing center of the SAM is characterized by the expression of the *WOX* gene family member *WUSCHEL* (*WUS*) [80, 81]. *WUS* moves to the neighboring stem cells where it suppresses differentiation [80–82]. The expression domain of *WUS* is negatively controlled by a receptor kinase pathway including the receptor kinase *CLV1* [83–85]. *WUS* activates the expression of the corresponding peptide ligand *CLV3* in the stem cells [86] and promotes its own expression in the organizing center by modulating cytokinin signaling [87, 88]. These (positive and negative) feedback loops restrict the size of the shoot meristem (reviewed in detail in [89]).

The position of stem cells underneath the shoot apex is controlled by L1-specific *miR394* [90]. This microRNA moves to the L2 and L3 layer and downregulates the expression of the F-box gene *LEAF CURLING RESPONSIVENESS* (*LCR*). *LCR* inhibits *WUS* activity. The *miR394/LCR* pathway therefore promotes *WUS* activity in the subepidermal region and ensures the correct position of the stem cell region in a growing shoot [90].

Although *WUS* expression starts at the 16-cell stage in the inner cells of the upper tier, it is dispensable for SAM formation. Instead, *WOX2* (together with *WOX1*, *WOX3*, and *WOX5*) is a key factor in meristem initiation [91]. In *wox1/2/3/5* quadruple mutant, a correct shoot meristem is not formed and the apical region of the embryo is characterized by increased auxin signaling [91]. Furthermore, the expression of the HD-ZIP III family members *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) [92, 93] is strongly reduced or absent in the shoot meristem region of the embryo [91]. Therefore, *WOX2* seems to

promote HD-ZIP III expression in the shoot meristem initials. The HD-ZIP III genes *PHB*, *PHV*, and *REV* are key players in shoot formation as ectopic accumulation in the basal part of the embryo leads to transformation of the root pole into shoot structures [77]. Since *PHB* and *PHV* control cytokinin production [94], *WOX2* regulates shoot meristem formation in part by promoting cytokinin levels in the upper tier of the embryo [91].

6 Outlook

While many molecular players in tissue differentiation and maintenance of cell types have emerged in recent years, our knowledge about the very first steps of initiating different identities in the early zygotic embryo is still fragmentary. What signals polarize the zygote and set up different auxin transport and response machineries in its daughter cells? How are different cell identities along the radial axis initiated? And how is the origin of the stem cell region for the shoot apical meristem controlled on a molecular level? Many fundamental questions about the early steps of zygotic embryogenesis are still waiting to be answered. Nevertheless, much less information is available about how microspore-derived embryos develop from dividing microspores, and about the genetic basis of some of the anatomical and functional abnormalities found in some cases. The knowledge summarized here may serve as a reference to compare with for the genetic dissection of microspore embryogenesis.

Acknowledgments

We would like to thank Gerd Jürgens for comments on the manuscript and apologize to our colleagues whose work we have not included in the chapter due to our focus on Brassicaceae embryogenesis. Research in our group is supported by the German Science Foundation (Deutsche Forschungsgemeinschaft—DFG: SFB1101/B01 to M.B.), the Chinese Scholarship Council (Fellowship No. 201806320131 to Y.M.), and the Max Planck Society.

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Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Martin Bayer for offering me the opportunity to work in his lab and sincerely appreciate all his support and guidance during my PhD.

I would like to express my thanks to Prof. Dr. Gerd Jürgens for his comments on my progress reports and scientific manuscripts. I would also acknowledge all scientific suggestions from Prof. Dr. Marja Timmermans, Prof. Dr. Detlef Weigel and Dr. Farid El Kasmi as part of my thesis advisory committee. Special thanks to Prof. Dr. Marja Timmermans for evaluating my PhD thesis and to Prof. Dr. Detlef Weigel for hosting me in the last several months.

Many thanks to my colleagues, especially Dr. Kai Wang, Yingjing Miao, Martina Kolb, Agnes Henschen and Franca Schneider, who offered me plenty of help with my projects. Appreciation should also go to Dr. Ancilla Neu, Dr. Marina Ortega-Perez, Dr. Lisa Yasmin Asseck and Dr. Daniel Slane, who shared experience and ideas with me.

In addition, I am also grateful for the kind help from Aurora Panzera and Dr. Christian Feldhaus for technical support in microscopy, Caterina Brancato and Dr. Kenneth Berendzen for protoplast transformations, Irina Droste-Borel and Dr. Manoj Singh for ColP-MS assays, Dr. Feng Xiong and Prof. Thomas Laux for plant materials and yeast assays, Dr. Arvid Herrmann and Prof. Keiko U Torii for offering seeds and plasmids, Prof. Yiting Shi and Prof. Shuhua Yang for providing transgenic lines and Prof. Dr. Ziduan Han for his careful proofreading of my thesis.

Lastly, I would like to show my great gratitude to my grandma for the love and care from my childhood, my parents for consistent support and my girlfriend Yimin Hu for her love and encouragement.