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Tightly controlled *WRKY23* expression mediates Arabidopsis embryo development

Wim Grunewald[1,2++], Ive De Smet[1,2,3*++], Bert De Rybel[4], Helene S. Robert[1,2], Brigitte van de Cotte[1,2], Viola Willemsen[5], Godelieve Gheysen[6], Dolf Weijers[4], Jiří Friml[1,2] & Tom Beeckman[1,2]*

[1]Department of Plant Systems Biology, VIB, [2]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium, [3]Plant and Crop Sciences Division, School of Biosciences, University of Nottingham, Loughborough, UK, [4]Laboratory of Biochemistry, Wageningen University, [5]Plant Developmental Biology Wageningen University, Wageningen, The Netherlands, and [6]Department Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

The development of a multicellular embryo from a single zygote is a complex and highly organized process that is far from understood. In higher plants, apical–basal patterning mechanisms are crucial to correctly specify root and shoot stem cell niches that will sustain and drive post-embryonic plant growth and development. The auxin-responsive *AtWRKY23* transcription factor is expressed from early embryogenesis onwards and the timing and localization of its expression overlaps with the root stem cell niche. Knocking down *WRKY23* transcript levels or expression of a dominant-negative WRKY23 version via a translational fusion with the SRDX repressor domain affected both apical–basal axis formation as well as installation of the root stem cell niche. *WRKY23* expression is affected by two well-known root stem cell specification mechanisms, that is, SHORTROOT and MONOPTEROS–BODENLOS signalling and can partially rescue the root-forming inability of *mp* embryos. On the basis of these results, we postulate that a tightly controlled *WRKY23* expression is involved in the regulation of both auxin-dependent and auxin-independent signalling pathways towards stem cell specification.

Keywords: *Arabidopsis*; embryogenesis; WRKY

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[1]Department of Plant Systems Biology, VIB, and
[2]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium
[3]Plant and Crop Sciences Division, School of Biosciences, University of Nottingham, LE12 5RD Loughborough, UK
[4]Laboratory of Biochemistry, Wageningen University, 6703 HA, Wageningen, The Netherlands
[5]Plant Developmental Biology Wageningen University, 6700 AP Wageningen, The Netherlands
[6]Department Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium
*These authors contributed equally to this work.
[+]Corresponding author. Tel: +32 92446611; Fax: +32 92446610; E-mail: wim.grunewald@vib.be
[++]Corresponding author. Tel: +32 93313890; Fax: +32 93313809; E-mail: ive.desmet@psb.vib-ugent.be

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INTRODUCTION

In the *Arabidopsis* root meristem, all cell types originate from stem cells that surround a small group of mitotically less active cells, designated as the quiescent centre (QC). The QC cells function as an organizing centre controlling the stem cell state of its neighbouring cells and derive from a single root founder cell, called the hypophysis, which is established during early embryogenesis [1]. Critical in this event is the action of the plant hormone auxin, which induces a transcriptional cascade through *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*). The *MP* gene encodes a member of the AUXIN RESPONSE FACTOR (ARF) family that can activate auxin-inducible gene expression, whereas BDL, a member of the AUX/IAA family, inhibits MP-mediated transcriptional induction in an auxin-dependent manner [1]. The combined action of MP and BDL promotes the transport of auxin from the embryo to the hypophysis and tightly regulates the expression of auxin-responsive transcription factors during embryogenesis [2,3]. As such, auxin determines the expression domains of the *PLETHORA* (*PLT*) genes, members of the APETALA2 domain transcription factor family, redundantly required for the embryonic specification of the root stem cell niche [4,5]. Accordingly, *mp* mutants, dominant-negative *bdl* mutants as well as multiple *plt* mutants all lack an embryonic root [5–7].

In addition, positioning and maintenance of the root stem cell niche also requires SHORT ROOT (SHR) and SCARECROW (SCR), members of the GRAS family of transcription factors [8,9]. These transcription factors are not controlled by auxin but intriguingly, the combined action of PLT, SHR and SCR contributes to QC and stem cell patterning [4], indicating that both auxin-dependent, as well as -independent programs converge on the specification and maintenance of the root stem cell niche. Despite the identification of above-mentioned master regulators, there is only limited information about their downstream transcriptional networks. Moreover, molecular mechanisms linking auxin-dependent and -independent programs are currently unknown.

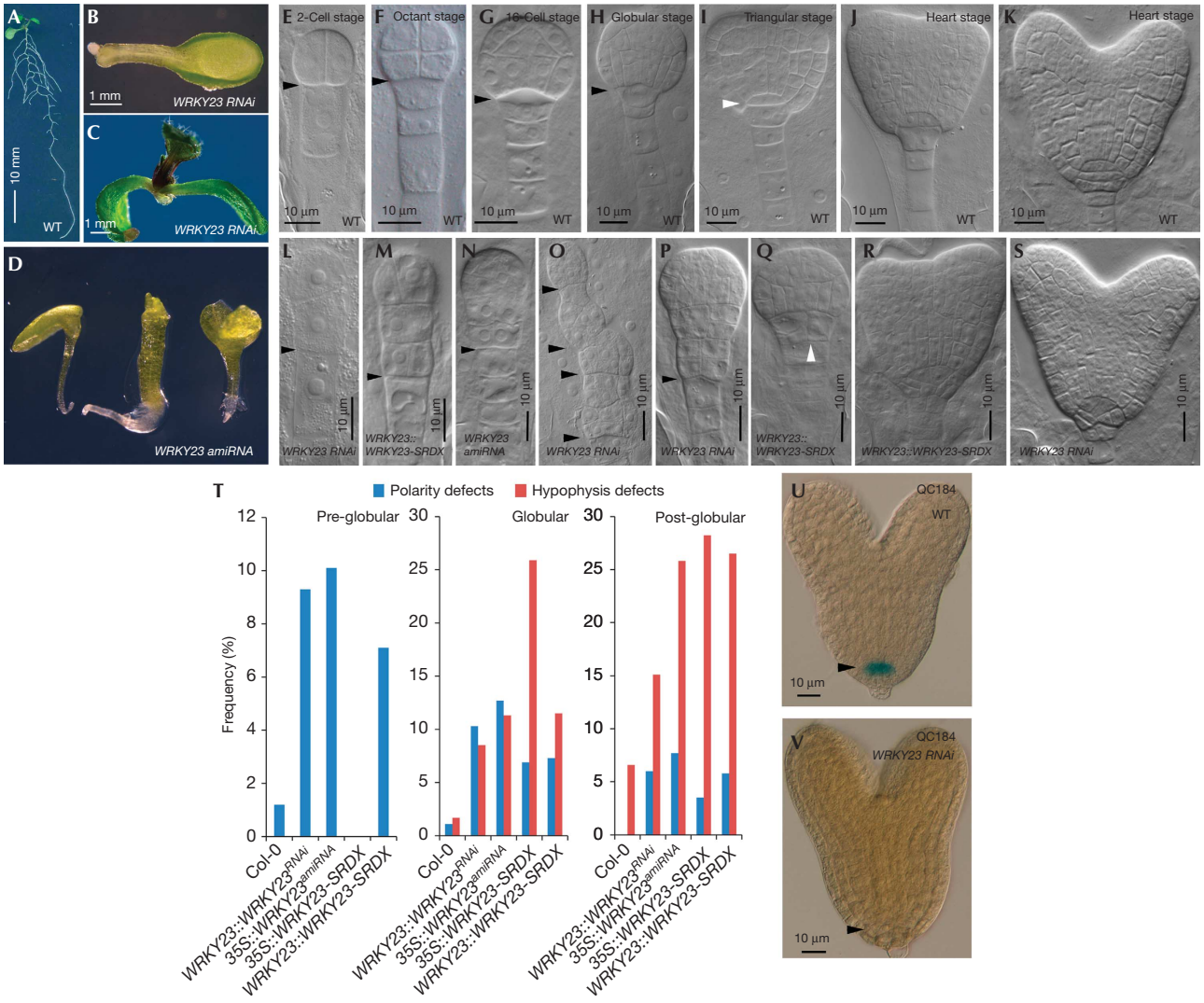


Fig 1 | Analysis of the effect of WRKY23 on embryo patterning and root meristem specification. (**A**) Ten-day-old *Arabidopsis thaliana* seedling. (**B**,**C**) *WRKY23::WRKY23*^{RNAi} seedlings. (**D**) *WRKY23:: WRKY23*^{amiRNA} seedlings. (**E**–**K**) Wild-type embryos. (**L**–**S**) *WRKY23* knockdown and *WRKY23-SRDX* embryos. Black arrowheads mark boundary between apical and basal cell lineages. White arrowheads point to orientation of hypophysis division. (**T**) Quantification of polarity and hypophysis defects in lines with reduced WRKY23 levels or activity. (**U**,**V**) QC184-marker *GUS* expression in wild type (**U**) and in *WRKY23::WRKY23*^{RNAi} embryos (**V**). amiRNA, artificial microRNA; WT, wild type.

In this study, we identify AtWRKY23 as a novel player in the molecular pathway leading to root stem cell niche specification. We furthermore show that its spatiotemporal expression is fine-tuned by a positive auxin-dependent (BDL–MP) regulation and a repressing auxin-independent (SHR–SCR) control.

RESULTS AND DISCUSSION

Reduced WRKY23 activity and embryo development

Recently, we showed that the WRKY23 transcriptional activator is involved in maintenance of root meristematic activity of *Arabidopsis* seedlings by regulating the local production of flavonols [10]. However, in the progeny of plants with a sub-optimal *WRKY23* transcript level (*WRKY23::WRKY23*^{RNAi}) or with a dominant repression of WRKY23's targets (*35S::WRKY23-SRDX*), a significant number of rootless and/or malformed seedlings could be observed shortly after germination [7.7% for *WRKY23::WRKY23*^{RNAi} ($n = 504$) and 77.6% for *WRKY23::WRKY23-SRDX* ($n = 165$)] [10] (Fig 1A–C), suggesting a role for WRKY23 in meristem establishment during embryogenesis. Therefore, we evaluated our transgenic lines (at least two independent lines for each construct) for developmental defects during embryogenesis and generated new transgenic lines expressing the previously used *WRKY23-SRDX* construct and three different artificial microRNA constructs, all from the endogenous WRKY23 promoter (*WRKY23::WRKY23-SRDX* and *WRKY23:: WRKY23*^{amiRNA}).

During *Arabidopsis* embryogenesis, at the globular to early heart stage transition, the wild-type hypophysis divides horizontally in

an upper lens-shaped cell and a lower tier cell. Both cells will subsequently divide vertically giving rise to the QC and the columella root cap, respectively [11] (Fig 1H–K). However, both *WRKY23* knockdown and WRKY23-SRDX embryos fail to organize these essential divisions resulting in an abnormal cellular organization of the root meristem (referred to as hypophysis defects; Fig 1Q–T; supplementary Fig S1 and supplementary Table S1 online). To assess cell identity in the embryonic root stem cell niche of these aberrant embryos, we examined quiescent centre-specific QC184-marker expression. In wild-type embryos, this marker shows GUS activity only in the four QC cells present in the root stem cell niche (Fig 1U) [12]. In *WRKY23::WRKY23^{RNAi}* embryos, however, no QC184 activity could be detected (100% of *WRKY23::WRKY23^{RNAi}* embryos with a phenotype, $n=7$) (Fig 1V).

In about 9–10% of the WRKY23 knockdown embryos ($n=183$ for *WRKY23::WRKY23^{RNAi}* and $n=119$ for *WRKY23::WRKY23^{amiRNA}*) and about 7% of the *WRKY23::WRKY23-SRDX* embryos ($n=267$), defects could be observed in younger (pre-globular) embryos (compared with 1.2% in wild type, $n=171$) (Fig 1L–P, T, supplementary Fig S1 online and supplementary Table S1 online). These embryos failed to establish the proembryo (referred to as polarity defects; Fig 1E–G,L–P). In most cases, the defects in apical–basal patterning were confined to the lower region of the proembryo and frequently, two or more proembryos developed on top of each other (referred to as polarity defects; Fig 1O, supplementary Fig S1 and supplementary Table S1 online). Consistent with the above-described embryo phenotypes, the *WRKY23::WRKY23^{amiRNA}* and *WRKY23::WRKY23-SRDX* lines produced malformed seedlings (Fig 1D, supplementary Fig S1 online), which is also in line with our previous observations of *WRKY23::WRKY23^{RNAi}* and *35S::WRKY23-SRDX* seedlings [10].

Together, these data support a role for WRKY23 in defining the apical–basal boundary and the proper specification of the root pole during early embryonic patterning.

Auxin response in *WRKY23^{RNAi}* embryos

Determination of the apical–basal axis during embryogenesis is preceded by the active organization of auxin response maxima [13]. In wild type, the auxin response maximum, visualized by activity of the synthetic auxin-responsive promoter DR5, is first confined to the apical cell and the derived proembryo and later to the hypophysis (Fig 2A,B). However, in *WRKY23::WRKY23^{RNAi}* globular embryos, DR5::GUS activity was diffuse and could be detected throughout the entire embryo (100% of *WRKY23::WRKY23^{RNAi}* embryos with a phenotype, $n=5$) (Fig 2D,E). Also at later stages during which strong auxin response can be seen at the wild-type root stem cell niche (Fig 2C), *WRKY23::WRKY23^{RNAi}* embryos showed aberrant *DR5* expression patterns (100% of *WRKY23::WRKY23^{RNAi}* embryos with a phenotype, $n=12$) (Fig 2F; supplementary Fig S2 online). These results indicate that defects seen in *WRKY23* knockdown embryos might be due to defects in auxin distribution. This is further supported by analyses of PIN FORMED1 (PIN1) protein localization in *35S::WRKY23-SRDX* embryos (33%, $n=18$), which deviated from the wild type ($n=7$) (supplementary Fig S2 online). For example, ectopic PIN1 below the QC could explain the *DR5* expression pattern, namely more diffuse and in the suspensor. Therefore, intact *WRKY23* expression and WRKY23 activity appear to be important to maintain correct auxin flow and response.

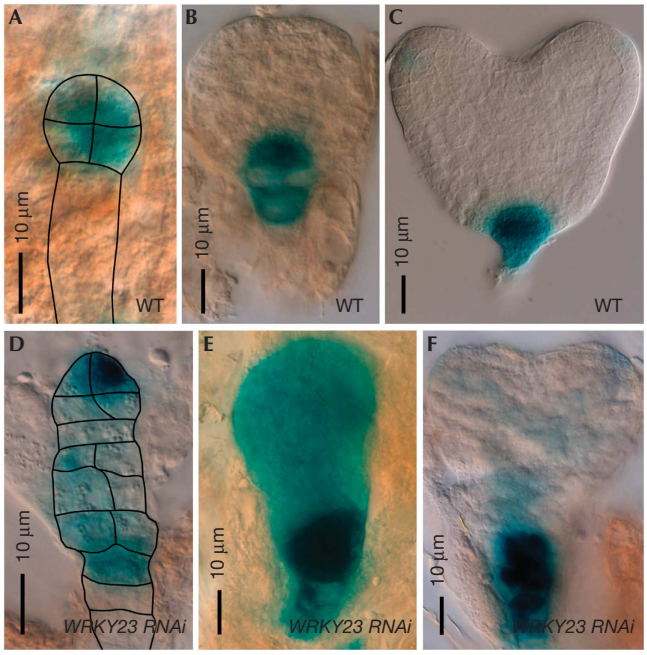


Fig 2 | Auxin response maxima in embryos with reduced *WRKY23* levels. **(A–F)** DR5-visualized auxin response in wild type (WT) **(A–C)** and *WRKY23::WRKY23^{RNAi}* embryos **(D,F)**.

We previously showed that WRKY23 regulates the biosynthesis of quercetin, a flavonol known to act as an endogenous auxin transport inhibitor [10,14]. To analyse whether the observed *WRKY23* knockdown and WRKY23-SRDX embryo phenotypes could be attributed to a lack of flavonols, the flavonol-deficient *tt4-8* mutant ($n=176$) and the quercetin-deficient *tt7-5* ($n=316$) and *tt7-6* mutants ($n=327$) were analysed during embryogenesis. However, no difference could be observed between wild type ($n=203$ for Ws and $n=155$ for Col-0) and the *tt* mutant embryos (supplementary Table S2 online). This suggests that dependent on the developmental stage, WRKY23 might regulate different transcriptional networks resulting in disrupted auxin flow and response: a flavonol-dependent control on root meristem maintenance and a flavonol-independent mechanism during *Arabidopsis* embryo development.

WRKY23 expression overlaps with root stem cell niche

Detailed *WRKY23* expression analysis, using *WRKY23::GUS* and whole-mount *mRNA in situ* hybridization revealed specific expression patterns during embryo development. *WRKY23* expression could be detected in the apical cells of a two-cell stage embryo (Fig 3A) and as embryogenesis proceeded, *WRKY23* transcripts were detected in the proembryo (with lower expression in the protoderm layer) until the globular stage (Fig 3B–C; supplementary Fig S3 online). During the transition from globular to heart stage, *WRKY23* expression became restricted to the developing shoot apical meristem and root stem cell niche (Fig 3D). These experiments show that *WRKY23* has a dynamic spatiotemporal expression pattern, which overlaps with

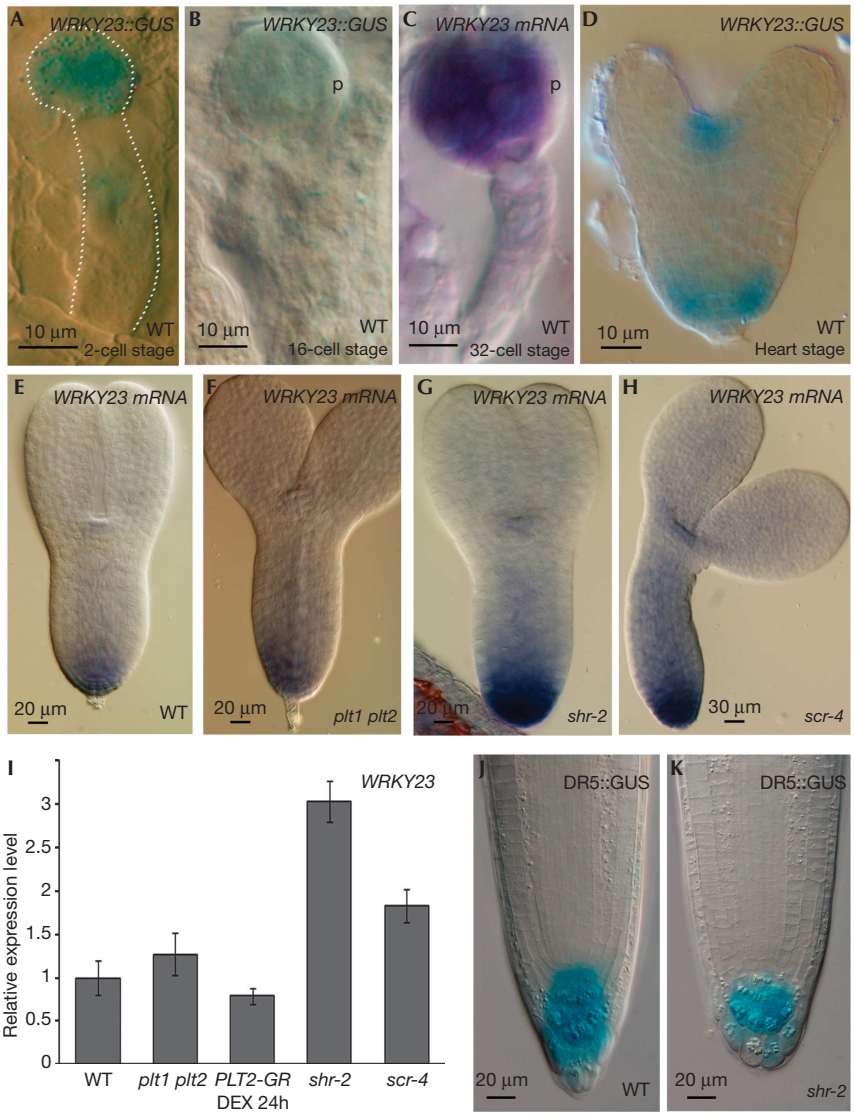
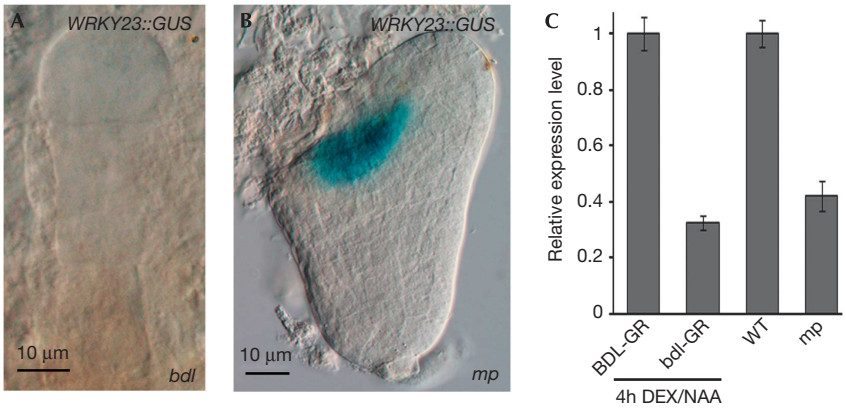
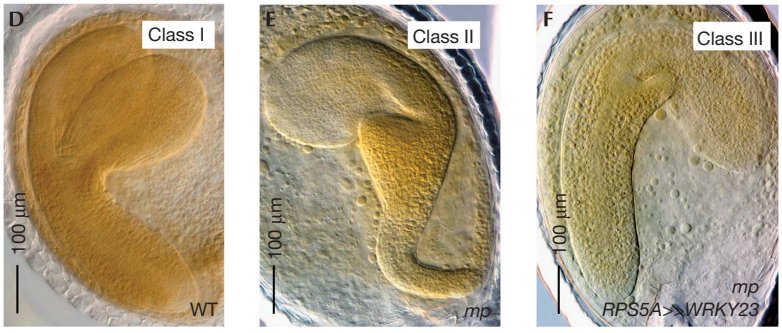


Fig 3 | *WRKY23* expression in embryo and primary root tips. (**A–H**) *WRKY23* expression in wild type (**A–E**), *plt1plt2* (**F**), *shr-2* (**G**) and *scr-4* (**H**) embryos visualized through *WRKY23::GUS* (**A,B,D**) and whole-mount *mRNA in situ* hybridization (**C,E–H**). (**I**) qRT–PCR on *WRKY23* in *plt1plt2*, DEX treated *PLT2::PLT2-GR*, *shr-2* and *scr-4* 3-day-old seedlings. (**J,K**) DR5::GUS activity in 3-day-old wild type (**J**) and *shr-2* root tips (**K**). DEX, dexamethasone; mRNA, messenger RNA; PLT, PLETHORA; qRT–PCR, quantitative real-time polymerase chain reaction; SCR, SCARECROW; SHR, SHORT ROOT; WT, wild type.

the root stem cell niche. This pattern of *WRKY23* expression is maintained during post-embryonic development, both in root and shoot apical meristems [10] (supplementary Fig S3 online).

Previous studies have highlighted SHR, SCR and PLT transcription factors as essential players mediating root pole establishment during embryogenesis and maintenance of the post-embryonic root meristem [4,9]. Therefore, we investigated to what extent *WRKY23* might be influenced by or interact with these pathways. In *plt1plt2* double mutants as well as in the inducible 35S::PLT2-GR lines, *WRKY23* expression did not change significantly compared with the corresponding controls pointing towards a PLT-independent mechanism (Fig 3E,F,I). Using *mRNA in situ* hybridization in embryos and qRT–PCR analysis on primary root tips revealed higher levels of *WRKY23* transcript in *shr* and *scr* mutants (Fig 3E,G–I), suggesting a repressive effect of the SHR–SCR pathway on *WRKY23* expression. However, this effect is likely not direct, as *WRKY23* is absent from the transcriptomics-flagged SHR-regulated genes [15,16] (data not shown). To check if the upregulation of *WRKY23* in the *shr* background could be due to altered auxin distribution or auxin response, expression of auxin-responsive genes and DR5::GUS activity in *shr-2* root tips were analysed. Although no obvious difference could be observed in the DR5-visualized auxin response between *shr* and wild-type roots (Fig 3J,K), the strong upregulation of *WRKY23* in *shr-2* (~10-fold) was associated with a mild increase in the expression level of a selection of auxin-responsive genes (~three to fivefold) (supplementary Fig S4 online). However, although auxin strongly induced *WRKY23* in a wild-type root tip, this auxin-mediated





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	Class I	Class II	Class III
mp/+ UAS::WRKY23 x *mp*/+ (*n*=437)	76.7	23.3	–
mp/+ RPS5A::GAL4 x *mp*/+ (*n*=336)	73.5	26.5	–
mp/+ UAS::WRKY23 x *mp*/+ RPS5A::GAL4 (*n*=282)	74.5	8.5	17.0

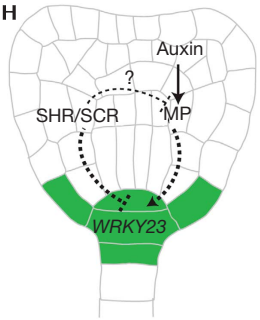

Fig 4 | WRKY23 expression and activity upon perturbation of the MP–BDL module. (**A**,**B**) WRKY23::GUS in *bdl* (**A**) and mp^{B4149}embryo (**B**). (**C**) qRT–PCR on auxin-treated, inducible *bdl* roots as well as in untreated *mp* seedlings compared with their respective controls. Plants were grown for 5 days after germination and then treated for 4 h with DEX and NAA in liquid medium. (**D**–**G**) Activation of UAS::WRKY23 by RPS5A::GAL4 driver line in mp^{B4149} background The embryos observed in the progeny of *mp*/+ UAS::WRKY23 × *mp*/+ RPS5A::GAL4 fall into three classes: class I resembles wild type (**D**), class II resembles *mp* (**E**) and class III appears a partial rescue of *mp* (**F**). (**G**) Quantitative data in percentage of the partial rescue of *mp* by WRKY23. (**H**) Graphical summary of control on *WRKY23* expression during embryogenesis. BDL, BODENLOS; DEX, dexamethasone; MP, MONOPTEROS; qRT–PCR, quantitative real-time polymerase chain reaction; SCR, SCARECROW; SHR, SHORT ROOT; WT, wild type.

upregulation of *WRKY23* occurred in cell layers outside the *SHR*-expression domain (supplementary Fig S5 online). In addition, the SHR–SCR-mediated effect on *WRKY23* is not mediated by auxin, as neither *SHR* nor *SCR* appear to be auxin inducible (supplementary Fig S6 online). On the basis of these results, we cannot fully exclude that the repressing action of the SHR–SCR pathway on *WRKY23* expression is an indirect effect of affected auxin signalling; but, our data support that this is likely not the case. Further genetic analyses will be required to convincingly conclude that WRKY23 functions downstream of SHR–SCR in the same pathway or that SHR–SCR function upstream of WRKY23 in the same pathway.

Genetic interaction between MP–BDL and WRKY23

We previously showed that WRKY23 acts downstream of SLR–ARF7–ARF19, key auxin signalling components during lateral root development [10,17]. However, the establishment of a functional root meristem during embryogenesis largely depends on the transcriptional activator ARF5/MP and its inhibitor IAA12/BDL, as reflected in the rootless phenotypes of their respective mutants [6,7,18]. Remarkably, expression patterns of *MP* and *BDL* in young embryos overlap with *WRKY23* expression, namely in the proembryo excluding the protoderm [2,7,19]. Moreover, the morphological defects observed in the *WRKY23* knockdown and WRKY23-SRDX lines are very similar to those seen in *mp* and *bdl*

mutants. Therefore, we checked *WRKY23* expression in mp^{B4149} and *bdl* mutant backgrounds. We found that the typical WRKY23::GUS activity in the central cells of a 16-cell stage proembryo could not be detected in *bdl* embryos (Fig 4A, compared with Fig 3B). To independently confirm the BDL control on *WRKY23* expression, we made use of seedlings expressing a dexamethasone-inducible, stabilized *bdl* version [3]. After a 4-h dexamethasone and auxin co-treatment of 35S::*bdl*-GR seedlings (using 35S::BDL-GR as a control), it was observed that the auxin inducibility of *WRKY23* is affected in the absence of a functional BDL pathway (Fig 4C).

In the progeny of heterozygous *mp* plants, homozygous *mp* embryos can only be morphologically distinguished at early heart stage by the defective division of the hypophysis [2]. At this stage, no *WRKY23* expression could be detected in the basal pole of mp^{B4149}mutant embryos (Fig 4B, compared with Fig 3D), which might be in line with a repression of *WRKY23* in *mp* seedlings (Fig 4C). However, the observed loss of *WRKY23* expression could be an indirect effect of the absence of root identity in *mp* embryos. Therefore, to genetically analyse whether WRKY23 is part of the MP–BDL signalling pathway defining the root pole during embryogenesis, a UAS::WRKY23 construct was transactivated by the RPS5A::GAL4 driver line in the mp^{B4149} background. Following a Mendelian segregation, mp^{B4149} heterozygous plants produced 76.2% (*mp*/+ *UAS::WRKY23* × *mp*/+; $n=437$) or 73.5% (*mp*/+ *RPS5A::GAL4* × *mp*/+; $n=336$) wild-type looking embryos (class I) and 23.3% (*mp*/+ *UAS::WRKY23* × *mp*/+) or 26.5% (*mp*/+ *RPS5A::GAL4* × *mp*/+) mutant embryos (class II, lacking a proper root and hypocotyl and often displaying 1 cotyledon). Interestingly, although the 1:4 segregation was maintained in RPS5A»WRKY23 × mp^{B4149} embryos, we could morphologically divide the mutant embryos into two classes: characteristic homozygous mp^{B4149} embryos lacking a root and hypocotyl (class II, 8.5%, $n=282$) and embryos that are less affected in their basal parts (class III, seeming restoration of the root, 17.0%, $n=282$) (Fig 4D–G). These embryos still show defects at the root pole but clearly have a more root-like structure compared with the control crosses (Fig 4F). As WRKY23 can partially rescue mp^{B4149} embryos, it is likely that WRKY23 acts downstream of MP. However, given the absence of unambiguous, genetic experimental evidence that can discriminate between epistasis and bypass suppression, WRKY23 can also function in a pathway parallel to that in which MP functions.

CONCLUSION

Our findings suggest a central role for WRKY23 at the onset of plant development independent of its previous reported regulation of flavonol biosynthesis. Correct expression of *WRKY23* is required for defining the apical–basal boundary and for the proper specification of the root pole during early embryonic patterning. Given the strong effect of altered *WRKY23* expression on plant development, we hypothesize that the expression of *WRKY23* should be under stringent control. Auxin (likely through the MP–BDL module) activates *WRKY23* expression but another control mechanism executed by the non-cell autonomous SHR–SCR pathway negatively affects—indirectly—*WRKY23* expression (Fig. 4G). As dosage control of *WRKY23* is important for root stem cell niche maintenance [10], this is likely also the case in the embryo and it is possible that SHR modulates *WRKY23* expression in a constant level instead of just repressing its expression. Recently another WRKY protein, WRKY2, was shown to regulate the expression of *WOX8* and *WOX9* that are necessary during early embryogenesis for the specification of the basal cell lineage [20]. Interestingly, *WRKY23* and *WRKY2* have contrasting expression patterns during embryogenesis. As WRKY proteins have been shown to interact with each other and to form transcriptional networks during plant defence responses (also referred to as the 'WRKY-web') [21], it will be interesting to investigate the existence of a similar WRKY-web during plant development.

METHODS

Plant material and growth conditions. *Arabidopsis thaliana* (L.) Heynh. seeds were sterilized with chloral gas, plated on half-strength Murashige and Skoog medium (1% sucrose; 0.01% myo-inositol; 0.05% 2-(*N*-morpholino)ethanesulfonic acid, 0.8% agar; pH 5.7), stored for 2 days at 4 °C, and grown vertically at 21 °C under continuous light. Previously described plant materials were *plt1plt2* [4], RPS5A::GAL4 activator line [4], 35S::PLT2-GR (*plt1plt2*) [5], mp^{B4149} [6], *bdl* [7], *shr-2* [22], *scr-4* [22], DR5::GUS [23], QC184 [9], WRKY23::GUS [17], WRKY23::$WRKY23^{RNAi}$ [10], 35S::WRKY23-SRDX [10]; RPS5A::BDL-GR [3], RPS5A::bdl-GR [3].

Cloning. WRKY23-related constructs are described in the supplementary Information online. All obtained constructs were mobilized to the *Agrobacterium tumefaciens* strain $C58C1^{RifR}$ and introduced into *A. thaliana* ecotype Col-0 using the floral dip method [24].

Histological analyses and microscopy. GUS staining of embryos is described in the supplementary Information online. Embryos were transferred to slides and mounted with 10% glycerol and analysed with a DIC fluorescence microscope (Olympus). Whole-mount *in situ* hybridization of embryos was done as previously described [25] using a full-length *WRKY23* RNA probe. Embryos were analysed by clearing the ovules in Hoyer medium (for 50 ml: dissolve 30 g Arabic gum in 30 ml distilled water, add 200 g chloralhydrate and finish with 20 ml glycerol [26]).

RNA extraction, cDNA synthesis and qRT–PCR analysis. Total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's instructions. Poly(dT) cDNA was prepared from 2 μg total RNA with Superscript III reverse transcriptase (Invitrogen) and quantified on an LightCycler 480 apparatus (Roche Diagnostics) with the SYBR Green I Master kit (Roche Diagnostics) according to the manufacturer's instructions using gene-specific primers (supplementary Table S4 online). Unless stated otherwise, we performed three biological repeats on a pool of seedlings and all individual reactions were done in triplicate. Graphs show one representative analysis. Data were analysed with qBase [27] and normalized to *EEF1α4, CDKA,* and/or *ACT7* (supplementary Table S4 online).

PIN1 immunolocalization. PIN1 immunolocalization was performed as previously described [28].

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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