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Abstract

Background: In dicot Arabidopsis thaliana embryos two cotyledons develop largely autonomously from the shoot apical meristem (SAM). Recessive mutations in the Arabidopsis receptor-like kinase RPK1 lead to monocotyledonous seedlings, with low (10%) penetrance due to complex functional redundancy. In strong rpk1 alleles, about 10% of these (i.e., 1% of all homozygotes) did not develop a SAM. We wondered whether RPK1 might also control SAM gene expression and SAM generation in addition to its known stochastic impact on cell division and PINFORMED1 (PIN1) polarity in the epidermis.

Results: SAM-less seedlings developed a simple morphology with a straight and continuous hypocotyl-cotyledon structure lacking a recognizable epicotyl. According to rpk1’s auxin-related PIN1 defect, the seedlings displayed defects in the vascular tissue. Surprisingly, SAM-less seedlings variably expressed essential SAM specific genes along the hypocotyl-cotyledon structure up into the cotyledon lamina. Few were even capable of developing an ectopic shoot meristem (eSM) on top of the cotyledon.

Conclusions: The results highlight the developmental autonomy of the SAM vs. cotyledons and suggest that the primary rpk1 defect does not lie in the seedling’s ability to express SAM genes or to develop a shoot meristem. Rather, rpk1’s known defects in cell division and auxin homeostasis, by disturbed PIN1 polarity, impact on SAM and organ generation. In early embryo stages this failure generates a simplified monocotyledonous morphology. Once generated, this likely entails a loss of positional information that in turn affects the spatiotemporal development of the SAM. SAM-bearing and SAM-less monocotyledonous phenotypes show morphological similarities either to real monocots or to dicot species, which only develop one cotyledon. The specific cotyledon defect in rpk1 mutants thus sheds light upon the developmental implications of the transition from two cotyledons to one.

Keywords: RPK1, Arabidopsis, Shoot meristem, SAM, Cotyledon, Monocot, Dicot, Plant embryo, Angiosperm evolution

Background

As typical representatives of dicot angiosperms, Arabidopsis thaliana seedlings display a body plan beginning with an epicotyl region harbouring the shoot apical meristem (SAM), flanked by two cotyledons and followed by the hypocotyl, which ends in a root tip carrying the root apical meristem (RAM) [1]. The initiation of cotyledons vs. SAM is largely independent, as evidenced by mutations that delete the SAM but not the cotyledons [2, 3] and vice versa [4, 5].

Although exceptions from normal cotyledon number in angiosperms are known in several genera [6] cotyledon number is a relatively constant pattern element. Modern taxonomy recognizes eudicots with two cotyledons and monocots with one cotyledon, as monophyletic groups [7, 8]. However, the mechanisms of “counting” and arranging these organs together with the SAM in order to establish the apical region are poorly understood.
The use of Arabidopsis thaliana mutants with cotyledon defects helps to get a deeper insight into this developmental process. Careful categorization of known mutants displaying cotyledon defects reveals a group, which obviously reflects more fundamental perturbations such as cell differentiation in altered meristem program [9, 10], control of meristem cell fate and lateral organ development in dornröschen [11] and division plane orientation in fass [12]. This leaves a number of seedling mutants whose defects are cotyledon specific. These mutants are regularly linked to defects in auxin synthesis and transport by the polar auxin efflux carrier PIN1, which generates auxin maxima required to induce cotyledon primordia [13, 14]. For instance, mutants of the AGC kinase PINOID (PID) and D-myo-inositol-3-phosphate synthase (MIPS) frequently produce abnormal supernumerary cotyledon numbers [15, 16] whereas combinations of pinoid (pid) with mutants of related kinases, auxin-synthesis genes and the NPH3-like gene ENHANCER OF PINOID (ENP/enp) result in cotyledon-less seedlings which retain a functional SAM [4, 5, 17–19]. In contrast, mutants specifically segregating a monocotyledonous phenotype are relatively rare and known from sic mutants in pea and mutations in the Arabidopsis receptor-like kinase RPK1 [20, 21]. The reason for this sparsity is possibly due to redundant gene functions encoded in the Arabidopsis genome. In fact, the monocotyledonous phenotype of rpk1 mutants has a maximum penetrance of ca. 10 % [21, 22], which could be elevated by adding mutations in the related TOAD2/RPK2. However, this combination simultaneously resulted in additional severe pattern effects and high frequency of embryo lethality because TOAD2/RPK2 has adopted additional functions in radial pattern formation [21, 23] and as regulator of meristem development [24].

Avoiding such pleiotropic effects rpk1-7 and rpk1-6 single mutants were recently analysed. This revealed that the primary rpk1 defect stochastically compromises epidermal cell division and PIN1 polarity during embryogenesis [22]. The defect is stochastic because the accuracy of every new cell division depends on whether the redundant RPK1-like genes achieve the required threshold of RPK1 function or not. This implies that the rpk1 defect can become manifest in different stages (time dependence) and in different regions (spatial dependence). The perturbation of epidermal cell division and PIN1 polarity in a cotyledon anlage might disturb or eliminate the establishment of an auxin maximum and lead to monocotyledonous seedlings (henceforth named monocot seedlings for convenience). The existence of SAM-less monocot seedlings suggested an interference with both cotyledon and SAM development during the early globular stage in the strong rpk1 alleles.

Here we show that SAM-less monocot seedlings retain basic SAM functions. However, they develop a simple morphology with a continuous hypocotyl-cotyledon organization that lacks a clear separation between these structures. The well-developed lamina is sometimes larger than in the wild-type. Although these monocot seedlings have initially no SAM, they have not lost the capacity to generate one. Some develop a delayed SAM or even an ectopic shoot meristem (eSM) on the adaxial side of the cotyledon. Our analyses suggest that the topological peculiarity of these monocot seedlings is linked to the loss of a spatially and timely coordinated expression of SAM specific genes during early embryogenesis, indicating a loss of positional information by altered morphology.

**Results**

**Strong rpk1 alleles generate SAM-less monocot seedlings**

The allele rpk1-7 was induced in a gl1 Columbia background and generates ca. 10 % seedlings with cotyledon abnormalities most of them lacking one cotyledon [22]. We detected that, five days after germination, some of the monocot seedlings did not possess developed SAMs in comparison to their monocot siblings (Fig. 1a-c). The cotyledon of these seedlings varied in shape and size and had a well-developed lamina with recognizable adaxial and abaxial sides (Fig. 1). The SAM-less monocots regularly occurred in the pedigree of crosses with plants of different genetic backgrounds with a frequency ranging between 0.5 % and 1.8 % of all seedlings (Table 1). Upon further growing, part of the SAM-less seedlings developed SAMs at some distance from the cotyledon lamina, suggesting that meristem development lagged behind that of SAM-bearing monocots. We considered that the SAM-less phenotype could be a specific character of the rpk1-7 allele, which is a fast neutron-induced inversion [22]. Therefore, we searched this phenotype in the independently generated rpk1-6 allele, which is a T-DNA insertion in the RPK1 coding region [22] and found SAM-less seedlings with similar frequencies as in rpk1-7 (Table 1). The other SAM-less seedlings did never develop a normal SAM but necrotic cotyledons and green, continuously growing roots as long as cultured in sterile 1/2MS medium (Fig. 1d). Notably, in these seedlings the hypocotyl and cotyledon petiolo formed a continuous structure without recognizable separation of a SAM region (Fig. 1c, e and f). This was true for both alleles (compare Fig. 1c, e, g) and showed that cell differentiation in these tissues had been fundamentally altered. Whole mount preparations of rpk1-7 seedlings displayed vascular defects stressing RPK1's link to PIN1 polarity and auxin transport [22]. In rpk1 monocots, the wild-type diarchic vascular system, which branches into both cotyledons, was variably organized. Either both strands intruded into the remaining cotyledon, or one strand ended in the “hypo cotyl”. In other cases supernumerary
vascular cell files were formed (Fig. 1f; Additional file 1: Figure S1).

**SAM-less monocot seedlings are capable of developing ectopic meristems on the cotyledon**

During the analyses of rpk1-7 monocots we repeatedly found SAM-less seedlings, which could enter another rare developmental route by developing an eSM on the adaxial surface of the cotyledon (Fig. 2). The eSMs did not develop on any other SAM-bearing dicot or monocot rpk1 seedling and displayed some specific characteristics. Firstly, the eSM was positioned on the recognizable adaxial not on the abaxial site of the cotyledon (Fig. 2a-e). Secondly, the eSM appeared in median position on the cotyledon i.e. near the mid-rip (Fig. 2a, b, e1-e5). Thirdly, the eSM generated primary leaves with irregular phyllotactic patterns not additional cotyledons (Fig. 2a, b). Primary leaves of the original line carrying the glabra1 mutation did not form the trichomes. However, back-crossing to GLABRA1 background (Table 1) demonstrated that these developed the leaf specific trichomes (Fig. 2c, d). The eSMs generated single leaf organs or (in the other extreme) even rosettes with fertile shoots (Fig. 2e6). The resulting pedigree exhibited a similar range of cotyledon

![Morphology in SAM-less rpk1 monocot seedlings.](image-url)

**Fig. 1** Morphology in SAM-less rpk1 monocot seedlings. a Magnifications of parts of monocot rpk1-7 seedlings (gl1/gl1 background) with SAM and primary leaves (top) and without SAM (bottom). b and c Whole plants with long roots (indicated by arrowheads) illustrate the continuous root growth. d A shoot-less monocot seedling from long-term cultivation shows a necrotic cotyledon while the root has continued growth and turned green. e A SAM-less monocot seedling with a homozygous rpk1-7 GL1 background (carrying a PIN1:GFP reporter). f Seedlings cleared with Hoyers mount visualize the vascular system in the contiguous hypocotyl-cotyledon structure with interruptions (white arrowheads) and supernumerary and/or blindly terminating vascular elements (small arrows). There is no bend recognizable, which in the wild-type separates apically the SAM/epicotyl from the laterally placed cotyledon. g A SAM-less monocot seedling originating from the rpk1-6 allele. Cotyledons (c), normal leaf (lf) indicated. Scale bars: 1 mm a-e, 0.5 mm g, 100 μM f
The eSM displays organizational similarities to wild-type SAMs

A plant with an eSM was histologically compared with a “normal” monocot seedling (Fig 3). The latter developed a SAM at the base of the cotyledon, which harboured regular cell files belonging to epidermis, palisade, mesophyll and xylem/phloem tissue, very much like a SAM of a dicot seedling. Within all tissues, the cells showed regular cell size proportions and vacuolation. Stomata were found above small cavities and were well separated from each other by epidermal cells (Fig. 3a). The SAM was positioned at the base of the remaining cotyledon where it would be normally expected. Its organization consisted of a group of small densely stained cells, which laterally gave rise to leaf primordia (Fig. 3a). As seen from the vascular system, the origin of the cotyledon is lateral and not terminal.

The cotyledons of SAM-less monocots always displayed an adaxial/abaxial orientation as evidenced by well-developed laminae, their bending, the form of the continuous hypocotyl-cotyledon structure, lacking a real petiole, and the position of the developed SAM (Figs. 1 and 2). However, the tissues and cells were significantly disproportionate in shapes and sizes (Fig. 3b). Abnormal shapes of epidermal cells indicated abnormal (not anticlinal) divisions. Stomata were sometimes neighboured to each other (Fig. 3b, top inset) and inner cells could be extremely large (> > 100 \(\mu\)m in length) and loosely attached to each other. In contrast, the regular (cellular) organization of the eSM was reminiscent of a wild-type SAM or the SAM in monocot siblings (compare Fig. 3a and b). A series of leaf primordia emerged from a cluster of small, plasma rich (densely stained) cells in the centre. The emerging eSM possibly caused a tension along the proximo-distal axis such that the cotyledon bent to form a buckle, which in turn produced a cavity beneath (Fig. 3b, compare with Fig. 2d).

Next, we addressed the question whether the loss of SAM in monocot \(rpk1-7\) is the extreme of a gradual reduction of meristem size. Due to the abundance of plasma, shoot apical meristem cells of DAPI-stained seedlings show intensive fluorescence, which can be taken as an approximation to meristem size [25]. SAMs of seedling phenotypes of \(rpk1-7\) (i. e. dicots, monocots, seedlings with irregular e. g. fused cotyledons) were compared with wild-type SAMs (Col-0 ecotype) as well as with mutant \(clavata3\) SAMs (Fig. 3c, d). The latter have been shown to be significantly larger than wild-type SAMs [26]. SAM-less monocot seedlings did not show densely stained SAM cell clusters (not shown).
The distribution of SAM sizes of rpk1-7 seedlings significantly overlapped with the sizes of wild-type SAMs. In contrast, the control clavata3 mutant exhibited significantly larger SAMs (Fig. 3c, d). We conclude that the representatives of the different rpk1-7 cotyledon variants are not members of a continuum of gradual decrease of SAM size. This suggests that the SAM-less monocot phenotype results from the incapability to reach a threshold required to establish a SAM (e.g. a critical amount or activity of coordinated SAM gene expression).
Table 2 Frequency of ectopic meristems (eSMs)

| Linea | Dicots & othersb | Monocots + SAM [%]c | Monocots - SAM [%]d | Monocots + eSM [%]c | Monocots (+SAM, −SAM, +eSM) [%]c |
|-------|------------------|---------------------|---------------------|---------------------|-----------------------------------|
| rpk1-7 X KNAT2:GUS A | 1246 | 52 [4] | 12 [0.9] | 0 [0] | 4.9 |
| rpk1-7 X KNAT2:GUS B | 1902 | 165 [7.7] | 62 [3] | 1 [4.5x10^-4] | 10.7 |
| rpk1-7 X KNAT2:GUS C | 1995 | 187 [8.3] | 62 [2.8] | 1 [4.4x10^-4] | 11.1 |
| rpk1-7 X KNAT2:GUS D | 421 | 49 [10.1] | 12 [2.7] | 1 [2.0x10^-4] | 12.8 |
| rpk1-7 X KNAT2:GUS E | 332 | 50 [12.9] | 5 [1.5] | 1 [2.5x10^-4] | 14.4 |
| rpk1-7 X KNAT2:GUS F | 3202 | 262 [7.4] | 78 [2.3] | 2 [5.6x10^-4] | 9.7 |

aOutcrosses to marker line KNAT2p:GUS, repeatedly selfed and with gl1/gl1 and non-KNAT2p:GUS background
bOnly monocots vs. others were considered, seedlings with irregular cotyledons, e.g. unequally sized (= anisocots), were not separately counted
cPercentage of all seedlings counted
dIn three randomly selected batches tested, between 15-66 % of initial −SAM seedlings developed a late SAM

Fig. 3 Meristem structure and size of monocot rpk1-7 seedlings. a Median section of a seedling with SAM and insets showing a magnified series of sections through the SAM (that of the median section is framed). Stomata are separated by other epidermal cells (arrowheads). Note the seemingly terminal position of the cotyledon, while the vascular elements demonstrate a lateral origin. b An eSM seedling (left). Insets show magnifications with details (right): irregularly spaced stomata (top, arrowheads); a regularly shaped meristem with leaf primordia (middle); a further section few microns apart from the former (bottom). Arrows point towards the root. Scale bars: 100 μm (left parts of a and b) and 20 μm (insets). c Means and SDs of rpk1-7 seedlings with one, two irregularly sized and two normal cotyledons and of wild-type and the clv3 mutant respectively (brackets: numbers of seedlings analysed). d Representatives of the different seedlings (except irregular seedlings)
Cotyledons of SAM-less monocot *rpk1-7* seedlings display SAM-specific gene expression

Next we analysed expression of SAM-specific genes such as *WUS, STM, KNAT1* and *KNAT2* (Fig. 4a) by semi-quantitative RT-PCR (see Methods). In this and other experiments care was taken that SAM-less seedlings were in fact devoid of a recognizable (late) SAM and that experiments with separated cotyledon tissue were not contaminated with hypocotyl and root tissue (see Methods). The cotyledon and leaf specific *ASI* [27, 28] was included as control (in addition to *ACT2*). In one experiment, two seedlings of the SAM-less and two of the SAM-bearing group were separately analysed (including those shown in Fig. 1a to 1c). SAM-less seedlings expressed three of the four SAM-specific genes together with *ASI*, which was strongly expressed (Fig. 4a). While *WUS* was not found in these SAM-less seedlings, *STM, KNAT1* and *KNAT2* appeared to be aberrantly expressed in comparison to monocot seedlings with SAMs (Fig. 4a). The aliquots of both *ASI* and *ACT2* displayed significantly stronger expression since these genes have an overall expression in the cotyledon and the rest of the seedling respectively. Testing *STM* and *ASI* (and *AS2*, not shown) in pools of cotyledons separated from the rest of the body, showed *STM* expression in cotyledons of SAM-less seedlings but not in those of controls (Fig. 4b). In addition, *STM* expression was also found in the rest of SAM-less monocots and as expected in the two controls (Fig. 4b). All bands had the expected sizes (as derived from the known transcripts). Additionally, representative bands were sequence verified. The expression of *STM* in both groups of monocot seedlings was comparable. A similar result was obtained using material of single seedlings (Additional file 1: Figure S2).

**Fig. 4** RT-PCR analysis of monocot *rpk1-7* seedlings with and without SAM. *a* Analysis of complete seedlings with (+ SAM) and without (− SAM) shoot meristem. RT-PCR amplification products after 40 cycles with primer pairs of genes as indicated. Note, that the expression of *KNAT1* and 2 was present but very weak in seedlings with SAM. *b* Analysis of *rpk1-7* monocot (− SAM) and *rpk1-7* wild-type dicot seedlings (+ SAM) separated into cotyledon tissue (Cot) and (epi- and) hypocotyl and root tissue respectively (Rest).

In situ hybridization analysis of late monocot *rpk1-7* embryos detects a rare ectopic *STM* expression

We monitored the expression of SAM-specific (*STM, CLV3*) and cotyledon-specific (*PID, ENP*) genes, which starts at very early embryo stages. However, in contrast to our former study [22] we concentrated on late embryo stages for two reasons. First, in late embryogenesis, *PID* and *ENP* show an additional expression in the SAM (e.g. [5]). Second, we wanted to increase the probability to find the expectedly rare ectopic expression of one of these genes in the monocot embryos, which have themselves a rare penetrance.

Late monocot *rpk1* embryos displayed a "banana"-like appearance with a more or less recognizable notch harbouring the presumptive SAM region. As expected, we mostly detected correct expression patterns. *STM* showed a larger while *CLV3* exhibited a small expression domain as known (Fig. 5a-e6, Additional file 1: Figures S3 and S4 for comparison). Similarly, *ENP* and *PID* showed normal late expression in cotyledons and the SAM (Fig. 5f, g1-g4; Additional file 1: Figures S5 and S6 for comparison). Although any of these probes could have potentially detected an abnormal expression pattern, we found only one among 30 monocots (out of 328 *rpk1-7* torpedo embryos). Considering the 10 % frequency of SAM-less seedlings among monocot *rpk1-7* seedlings, this is in the same range. Surprisingly, in the identified monocot embryo the hybridization with the *STM* probe extended almost along the complete embryonic hypocotyl but not into the cotyledon tissue, with the strongest concentration being at the normal SAM position (Fig. 5b1-b5; stippled line in B2 and B3). The size of the domain expressing *STM* in this specimen clearly exceeded 15-20 μm in apical-basal axis, which is the size displayed in dicot and monocot SAM-bearing *rpk1-7* torpedo embryos (Fig. 5a, d1-d6, e1-e6; brackets). This result coincides with one of the subsequently observed *KNAT2p:GUS* expression pattern variants in SAM-less monocot *rpk1-7* seedlings (see below).

The SAM-specific *KNAT2p-GUS* activity is variable and abnormally distributed in SAM-less *rpk1-7* monocot seedlings

In order to obtain a larger number of specimen with informative ectopic expression patterns of a SAM-related gene, we analysed *Arabidopsis* seedlings carrying a *KNAT2p:GUS* reporter [29]. *KNAT2* is a *STM*-dependent transcription factor whose expression is localised in the SAM [30] (Fig. 6a). The monocot pedi-gree of a *rpk1-7 X KNAT2p:GUS* cross contained normal dicot, SAM-bearing monocot and SAM-less monocot seedlings. The former two exhibited GUS stain as expected at the apex next to the base of the cotyledon(s) (Fig. 6a, b). The SAM-less monocots displayed a spectrum of variants with respect to *KNAT2*
expression. Many seedlings showed very weak (Fig. 6c) to more intensive GUS expression in the central (vascular) tissue in the fused hypocotyl-cotyledon structure. This could extend either in direction towards the cotyledon tip or towards the root tip (Fig. 6d-h). The variability was further increased by some seedlings, which displayed smaller or larger patches of GUS staining in the cotyledon lamina (Fig. 6f-h). Monocot seedlings generating an eSM showed a strong GUS staining in the cotyledon (Fig. 6j). The variable KNAT2 expression in the cotyledon coincided with the results of the foregoing experiments. Thus, all expression data together suggest that SAM-less seedlings display an aberrant SAM gene expression pattern causing the generation of an eSM to be a rare event because it requires the concerted and precise coordination of several SAM genes.

Discussion

The timely and spatially stochastic alteration of cell division and PIN1 polarity in the embryo epidermis of rpk1 mutants causes a variable development of the cotyledon primordia, in particular the complete loss of one cotyledon indicating an early developmental accident during globular embryo stages [22]. Later we detected that among monocots of different rpk1 alleles the loss of the SAM had a low but consistent frequency and seemed to occur together with the generation of a continuous hypocotyl-cotyledon organ lacking a discernable epicotyl region. In this study we have systematically analysed this particular phenotype. Since the SAM-less phenotype is not a specialty of a single allele, we have focussed on rpk1-7 when analysing the cellular morphology and gene expression patterns.

SAM-less rpk1 seedlings lack a recognizable organ separation and display a compromised cell differentiation when developing eSMs

The apex in Arabidopsis is formed through antagonistic activities of SAM-specific versus cotyledon/leaf specific genes [31]. Essentially, in the apex STM activates KNAT1/KNAT2 (and KNAT6) directly or indirectly through repression of AS1 and AS2 [32, 33]. Conversely, a complex of the proteins AS1 (a MYB protein) and AS2 (a LOB
domain protein), which recruits chromatin-remodeling factors, excludes the activity of SAM specific class I KNOX genes, in particular KNAT1/BP and KNAT2 in leaf and cotyledon tissue [27, 28, 34, 35]. Thus, with the exception of plants, which have exploited the reactivation of SAM-related genes in order to generate compound leaves [36], SAM gene activities are excluded from leaf tissue.

In cotyledon tissue of SAM-less rpk1-7 seedlings, we detected ectopic expression of the SAM-related STM, KNAT1 and KNAT2 genes together with cotyledon specific expression of AS1. This means that, antagonistic gene activities were detected within close neighbourhood in the same tissue and likely compromised cotyledon organization by generating tissues and cells with altered position, size and shape as evidenced from histological analysis. Similar profound changes in cell morphology have been observed in leaf tissue ectopically expressing single SAM specific genes (e. g. [37]). In accordance with the defect in PIN1 polarity, the disturbed vascular tissue pattern pointed to an auxin defect. Interestingly, eSMs generated rosettes with irregular phyllotactic patterns. In this context it is worth mentioning, that a balanced homeostasis of auxin and cytokinin impact on shoot development and phyllotaxis [38–40]. The development of a fused hypocotyl-cotyledon organ, at the expense of a petiole connecting hypocotyl and cotyledon, indicated severe perturbations of normal cell differentiation. In spite of these cellular disruptions, the morphology of this fused hypocotyl-cotyledon organ clearly retained the wild-type ab- and adaxial polarity in both rpk1-6 and rpk1-7 SAM-less monocots. No radialisation as reported for mutants of adaxial vs. abaxial identity genes was observed [41].

**SAM loss and eSM gain in monocot rpk1-7 seedlings is likely due to timely and spatially non co-ordinated expression of SAM specific genes**

Previous studies showed that, although ectopic (over-) expression of (single) KNOX genes could lead to ectopic SAMs, their stabilization required the balanced and concerted activity of stem cell identity and other SAM genes [30, 37, 42, 43]. Our study shows that this is a main problem in SAM-less rpk1-7 mutants since the analyzed genes often exhibited a non-coordinated and unbalanced activity. For instance, in one case WUS was not expressed in cotyledons of SAM-less monocots while STM, KNAT1 and KNAT2 were. The latter also seemed to be even more strongly expressed in the mutant than in the wild-type. Since WUS expression is required for SAM generation on first place [44], this explains why these seedlings lacked a shoot meristem in spite of expressing other SAM related genes. Additionally, we detected inconsistencies of expression with respect to space and timing. Seedlings with late SAMs indicated a time-delayed co-ordination. This was also corroborated by SAM-less seedlings, which revealed ectopic KNAT2 p:GUS signals while others were almost devoid of this activity. The former also showed a spatial defect since GUS staining could occur in quite different positions and with variable extension. These observations explain why eSMs are rare and have no predictable frequency. They only develop by coincidence when all required SAM related genes are active in a concerted fashion and surpass critical values. Similarly, SAMs in “normal” monocot seedlings overlapped in size with wild-type SAMs instead of showing a continuum of gradually decreasing sizes until reaching a SAM-less seedling.

**SAM-less rpk1 seedlings are caused rather by lack of positional information than suppression of SAM specific gene activity**

The rpk1 phenotypes raise the question whether RPK1 induces the initiation of cotyledon primordia and the SAM through direct control of the corresponding genes. Both possibilities can be excluded. First, in case of the
former, rpk1 mutants should provide seedlings precisely lacking both cotyledons like pid enp double mutants [4]. This has not been the case among all analysed rpk1 homozygous progenies (> ≥ 10,000). Interestingly, monocot rpk1 embryos develop only one primordium but establish both cotyledon anlagen [22]. This is compatible with former fate-mapping experiments, which suggest a sequential generation of cotyledons [45]. Second, our data also exclude the possibility that RPK1 directly controls SAM gene expression and development because SAM-less rpk1-7 seedlings retain the capacity to express a variety of SAM-specific genes and even to generate eSMs. This corroborates the notion that cotyledons and SAM are largely developmentally independent.

However, what then causes ectopic SAM gene expression and eSM development? Homozygous rpk1 mutants differ from previous examples where ectopic shoot meristems were induced in transgenic and complex dominant mutation backgrounds respectively [30, 37, 42, 43, 46]. In contrast, rpk1 mutants represent a loss-of-function state and form late SAMs at correct positions or eSMs ectopically on top of cotyledons. The rpk1-7 ectopic shoots, although larger, are reminiscent of epiphyllous inflorescences on foliage leaves in fil-5 yab3-1 mutants [47] and of ectopic leaf buds in as1 mutants [27]. However, none of these genes is mutated in rpk1 plants. The only link to ectopic SAM gene expression (and eSMs) in these mutants is the altered hypocotyl-cotyledon fusion morphology. The probability that eSMs occurred exclusively in morphologically altered SAM-less monocots (6 in 10,000; Table 2) just by chance is extremely low (≤ 10⁻¹²). This leads us to a model, which integrates the primary defects of rpk1 mutants, i.e. disturbance of epidermal PIN1 polarity and cell division, and their phenotypes (Fig. 7). In fact, disturbance of PIN1 polarity and auxin homeostasis respectively have been demonstrated to affect initiation of shoot regeneration [39, 48, 49]. Our model takes into account, that due to functional redundancy these defects stochastically scatter along the complete embryo development (Fig. 7). The earlier the rpk1 defects manifest the more severe are the consequences. The extreme is a fused hypocotyl-cotyledon morphology with the loss of the SAM, which is one of the earliest cell commitments in the embryo (Fig. 7). Apparently, the continuous hypocotyl-cotyledon morphology is accompanied by a loss of positional information because post-embryonically a shoot meristem can form at different positions (late SAMs, eSMs). This circumstance is also reflected in variable ectopic SAM gene expression patterns in those SAM-less monocots, which fail to form a shoot meristem (Fig. 7).

**Conclusions**

This study shows that RPK1 does not primarily control SAM genes, even the extreme rpk1-7 phenotype retains the capacity to resume shoot meristem development (eSM) and to generate a fully functional plant. However, RPK1 does well impact through its primary defects on the generation of shoots and (cotyledon) organs demonstrating a significant extent of morphological plasticity. This plasticity leads to intriguing similarities with extant angiosperms in particular real monocots and monocotyledonous dicots of the genera Monophyllea [50] and Streptocarpus [51] respectively. RPK1 mutants are also instructive in a way that sheds light on an aspect that has received less attention. This is the penetrance problem. In contrast to full penetrance of cotyledon-loss in pid...
enp [4], known single or combined mutations in Arabidopsis, do not stably produce 100 % monocots [22, 23]. This phenomenon has been previously addressed by studying modifier genes of cotyledon number in Antirrhinum majus (e. g. [52]). More recently, an association study using A. thaliana ecotypes has identified RPK1 as an essential (but not the only) gene for shoot organ regeneration [53]. Thus, the rpk1 monocot phenotype furthers our understanding of angiosperm development in two ways. First, it points to the organizational and genetic peculiarities required to generate a monocotyledonous plant from a dicot. Second, it shows, that it might be promising to search for those genes whose functions have to be altered in concert to obtain full penetrance of monocotylly.

**Methods**

**Plant strains and growth conditions**

The Col-0 ecotype was used as wild-type reference. The strong rpk1-7 allele originated from the seling of a fast neutron mutagenized seed of Col/gl-1 background and represents an inversion mutation [22]. Monocot rpk1-6 and rpk1-7 seedlings were analysed in the original line and in different backgrounds resulting after crossing with different (reporter) lines. In rpk1-7, the gl-1 background results in loss of trichomes characteristic for post-embryonic leaves. Therefore, rpk1-7 was crossed to GL-1 background (harbouring the PIN1p:PIN1::GFP reporter). The KNAT2p::GUS reporter [29] was crossed with rpk1-7 in order to detect ectopic SAM gene-related expression patterns. Segregating gl1/gl1 pedigree of this cross lacking the KNAT2p::GUS reporter was used for assessing eSM frequency. The rpk1-6 allele is a T-DNA insertion 357 bp from the ATG in the ecotype WS-2 obtained from NASC (Nottingham Arabidopsis Stock Center; for further details see [22]). This allele was either analysed as original line or as line harbouring the PIN1p:PIN1::GFP reporter. Growing of seedlings on soil (2015) 15:171

**Microscopy**

Semi-thin sections and whole analysis of embryos and seedlings were carried out as previously described [4, 12, 54]. Photographs were taken using a ZEISS AXIophot microscope equipped with a Digital Nikon camera (FSSLR) and corresponding software (Nikon Camera Control Pro). Epifluorescence microscopy on the same Axiohot used a HBO50 UV/Light-source with a DAPI filter system (Zeiss filter set 01, BP365/FT395/LP397).

**GUS-Staining**

Staining of seedlings carrying the GUS reporter construct was carried out after fixation by vacuum infiltrating a solution of NaH₂PO₄ (pH 7.0) and 1 % Formaldehyde for 10 min in an Eppendorf tube. After placing the tube for 20 min on ice, the fixative was washed off with 50 mM NaH₂PO₄ (pH 7.0) and staining was performed as previously described [55]. SAM-less monocot seedlings showing GUS staining were taken to estimate the proportion of SAM-less monocots with ectopic expression in the cotyledon vs. those with expression exclusively in the hypocotyl.

**RT-PCR and PCR**

Plant DNA was isolated following conventional protocols. RNA isolation, reverse transcription and PCR were performed according to the supplier’s instructions using a NucleoSpin®-RNA Plant (Machery-Nagel) or PolyATract-System IV kit (Promega) respectively. Reverse transcription of total RNA with a TaqMan® kit (Applied Biosystems, Roche) included the following steps: 20 min 25 °C followed by 45 min 48 °C and stopped with 5 min at 95 °C. RT-PCR analysis was semi-quantitative; i. e. for probes to be compared the same amount of RNA material was used in the RT reaction and/or amounts of PCR products loaded were adjusted with respect to the ACT2 reactions. Fig. 4a, Fig. 4b and Additional file 1: Figure S2 show independent experiments because three different seedling batches were used. Special care was taken using isolated cotyledon tissue by locating the section at safe distance to the hypocotyl-cotyledon fusion region.

The following forward and reverse primer pairs were used (gene and fragment size in parentheses):

- 5’-GCCCATCATGACATCACATC-3’ and 5’- CTTT AAGCTCTCTATCCTCAGCTTG-3’ (STM; 701 bp fragment); 5’-GGCACCGAGCTTGGGCGGAC-3’ and 5’- GAGACGGTTCAGGGGCGGTC-3’ (AS1; 322 bp); 5’-TCAGAAAGAGAGATTCAAC-3’ and 5’-AGGGCCGAA CTTCGGATTGG-3’ (WUS; 562 bp); 5’-CACCCTCT GTCTCTGCTCCTCTCTA-3’ and 5’-ATTCGCGCAAG CTACCTTCTCT-3’ (KNAT1; 534 bp); GGAGCTGATC CTGAGCTTGTAG-3’ and 5’-CACAACGGAGACG TTCGTGC-3 (KNAT2; 380 bp); 5’-TTGTTCCAGCCC TCGTTTTGT-3’ and 5’-CCTGGACCTGCTCATTACA CT-3’ (ACT2; 323 bp). PCR cycles were: 3 min 93 °C, 40X (45 s 93 °C, 60 s 60 °C and 60 s 72 °C), 3 min 72 °C, 3 min 4 °C.

In order to assess correct gene identities some RT-PCR products were sequenced through EUROFINS/MWG services.

**In situ hybridisation analyses**

In situ hybridization, assessment of anti- and sense probes and wild-type expression patterns were as previously reported and had been previously confirmed.
respectively [4, 22]. In contrast to the study of Luicht et al. [22], we focused on embryos from early torpedo stage onwards.

Additional file

Additional file 1: Figure S1. Progeny of an eSM of monocot rpk1-7 plants. Figure S2. RT-PCR analysis of single rpk1-7 monocot seedlings. Figure S3: In situ hybridization of dicot rpk1-7 embryos with a STM probe. Figure S4: In situ hybridization of dicot and monocot rpk1-7 embryos with a CLV3 probe. Figure S5: In situ hybridization of dicot rpk1-7 embryos with an ENP probe. Figure S6: In situ hybridization of dicot and monocot rpk1-7 embryos with a PID probe.

Abbreviations

ACT2: ACTIN2; AS1: ASYMMETRIC LEAVES1; AS2: ASYMMETRIC LEAVES2; BP: BREVIPEDICELLUS; CLV3: CLAVATA3; cv3: clavata3; CoD: Columbia-O; ENP: ENHANCER OF PINOID; expn: enhancer of pinoid; eSM: ectopic Shoot Meristem; gl1: glabra1; GUS: beta-Glucuronidase; KNAT1: KNOTTED1-LIKE ARABIDOPSIS THALIANA1; KNAT2: KNOTTED1-LIKE ARABIDOPSIS THALIANA2; LOC: Lateral Organ Boundary; MIPS: D-myo-inositol-3-phosphate synthase; Ms: Murashige Skoog; NASC: Nottingham Arabidopsis Stock Center; NPH3: NON-PHOTOTROPIC HYPOCOTYL3; PID: PINOID; pid: pinoid; PIN1: P1NFORMED1; RAM: Root Apical Meristem; RP1K: RECEPTOR-LIKE PROTEIN KINASE1; RP2K: RECEPTOR-LIKE PROTEIN KINASE2; SAM: Shoot Apical Meristem; sC: single cotyledon; STM: SHOOT MERISTEM-LESS; wS-2: Wassilewskija-2; wUS: WUSCHEL.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

BSF and ML performed mutant characterization and in situ analyses, XY, MM and OP participated in further molecular analyses and characterization of lines. RATR designed the project, participated in molecular, phenotyping and genetic work and wrote the paper. All authors read and approved the final manuscript.

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