A start in elucidating SOSEKI function from *Arabidopsis thaliana* lateral root primordia

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Abstract

Plants are sessile organisms and they develop organs post-embryogenesis to adapt to the environment and accommodate for their nutrient needs. With the formation of new organs, new organismal axes are established. Organismal axes are essential for proper cell pattern formation. How cell polarity is linked to the establishment of organismal axes and translated to proper cell pattern formation is elusive for plants. One type of organ that is formed post-embryonically are lateral roots. Lateral root development starts with the formation of lateral root primordia. Previously SOSEKI proteins were discovered. There are five of them that localize to polar cell corners in a tissue-independent manner. Studying SOSEKIs in lateral root primordia may give new insight into how cell polarity, organismal axes, and pattern formation are linked. Here SOSEKI1, SOSEKI2, and SOSEKI5 expression domains are determined in lateral root primordia. SOSEKI3 and SOSEKI4 were found to not be active in lateral root primordia. SOSEKI1, SOSEKI2 and SOSEKI5 have distinct expression domains where SOSEKI1 localization starts at the basal side and changes to apical side during lateral root primordia development. SOSEKI2 remains localized toward the apical side. And SOSEKI5 first had an inconsistent expression domain with cytoplasmic expression and later localized to the apical side. It is assumed that organismal axes are not fully established in lateral root primordia because SOSEKI localization between primary and emerged lateral roots is identical but not similar to lateral root primordia. To elucidate SOSEKI function a cloning process was started. In this process the generation of plant lines containing a transgenic construct with an artificial microRNA targeting SOSEKI for silencing was started. Separate plant lines that silence SOSEKI1, SOSEKI2, or SOSEKI3 in a tissue-specific constitutive manner were made. In parallel to elucidate SOSEKI function, laser ablation of single lateral root primordia cells was done and SOSEKI1 expression disappeared shortly post-ablation whereas the lateral root primordia cells surrounding the ablated area expanded.

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Introduction

Plants have the capability to derive new organs post-embryogenesis. This is so they can continuously adapt to a changing environment. One of these organs are the lateral roots. Lateral roots are important to anchor the plant and take up nutrients from the soil. Their development starts from the primary root where two adjacent xylem pericycle pole cells are primed into founder cells. These founder cells swell asymmetrically, their nuclei migrate toward their common cell wall and then an asymmetric division occurs. A larger outer cell and a smaller inner cell are created[1]. This marks stage I of lateral root primordia (LRP) development. The stage one LRP continues to divide periclinaly and anticlinaly (with respect to the primary root axes). Each periclinal division that generates another cell layer on top of an already existing cell layer(s) marks a next stage in the LRP growth (Figure 1). Eventually, after sufficient growth and cell wall remodeling, the LRP is able to penetrate through the overlaying tissues (endodermis, cortex and epidermis)[2].

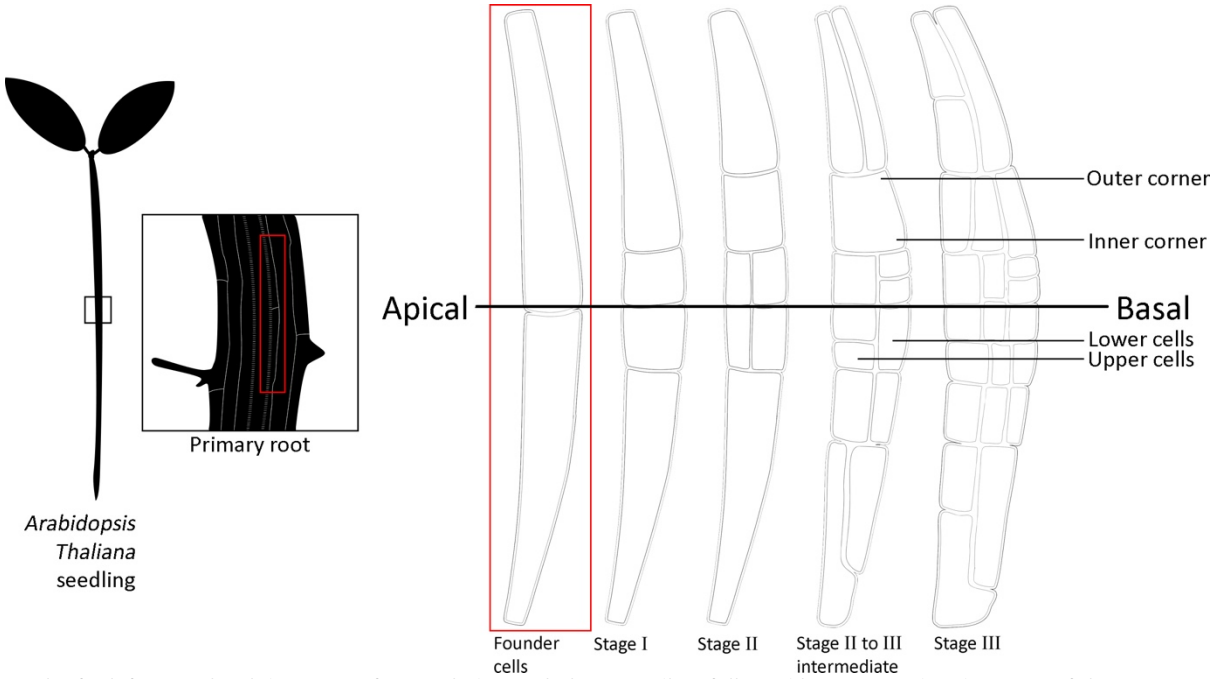

Figure 1: On the far left a graphical depiction of an *Arabidopsis thaliana* seedling followed by a zoomed in depiction of the primary root tissue on the right. On the far right the initiation site of a LRP starting with founder cells and each consecutive cell division marks another stage of LRP development up until stage III. In this figure, the apical-basal axis is drawn and the difference between upper and lower cells is indicated. To later understand where SOSEKIs (*SOK*s) are localized an outer and inner corner are indicated which will be referred to in the results section. It should be said that the apical basal axis functions as a mirror plane. Thus, on the other side of the apical-basal axis the outer and inner corners are mirrored.

With the development of the LRP, new organismal axes are formed (See apical-basal axis in Figure 1). In plants it is unknown how organismal axes are established from cell polarity and how this is translated to proper patterning of cells during the formation of new organs (Figure 2). So far the polar localization of auxin transporters is known to allow for proper LRP development[3–6]. Besides, recently SOSEKI (*SOK*) proteins that localize tissue-independently to specific edges and/or corners of cells were discovered[7]. These *SOK* proteins interpret global polarity cues and can influence cell division orientation. Studying the expression of *SOK* proteins during the LRP development may give more insight into how polarity is established in plants and what their role is during LRP formation.

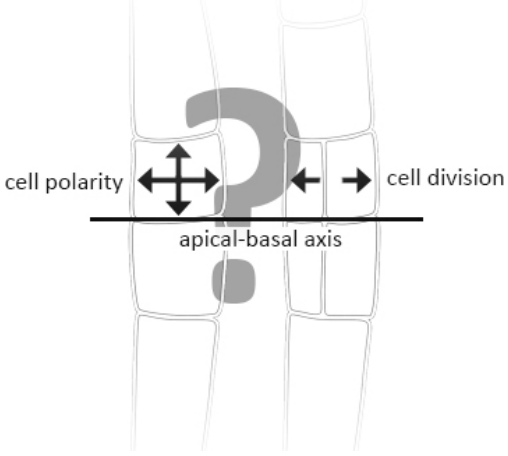

Figure 2. Illustration of what cell polarity, an organismal axis, and cell division is. In plants it is unknown how all three are connected, hence the question mark.

There are five *SOK* proteins codified from the genes: *SOK1*(*At1g05577*), *SOK2*(*At5g10150*), *SOK3*(*At2g28150*), *SOK4*(*At3g46110*) *and SOK5*(*At5g59790*). *SOK1* and *SOK5* were discovered in a transcriptome analysis of globular stage embryos where the gene *MONOPTEROS* (*MP*), which is important for proper cell division, was inhibited[8]. Next to target of *MONOPTEROS 7*, *SOK1*(7.4x) was the second most downregulated gene and *SOK5* was 2.4x downregulated. *SOK2*, *SOK3*, and *SOK4* are paralogous of the former two SOKs.

Plant lines with an extra copy of the *SOK* gene fused to a yellow fluorescent protein (YFP) tag were already available. Previously these lines were used to describe *SOK* expression domains in primary root and emerged lateral root tissues (Figure S1)[7]. However, the expression domain was not yet described in detail for LRP development.

Here this work describes the atlas of expression for each *SOK* up until stage III in LRP development using live-imaging. In a next step to elucidate *SOK* function here constructs that contain an artificial microRNA (amiR) which target *SOKs* for silencing are generated. This was done with the intention to in the future observe the resulting mutant LRP phenotype. To generate these specific amiRs, a sequence that target specifically each *SOK* protein weas designed. When this sequence is transcribed the resulting RNA forms a hairpin loop by binding with complementary sequences that are present within itself. The formed hairpin contains two 21-nucleotide-long sequences that are targeted by a protein called Dicer for cleavage. These 21-nucleotide-long regions are named amiR* and amir. Of these two the amir sequence is incorporated into a protein complex named RNA-induced silencing complex (RISC) where it functions as the specificity component of the complex. RISC is then able to target a *SOK* gene's mRNA transcript for silencing (Figure 3)[9].

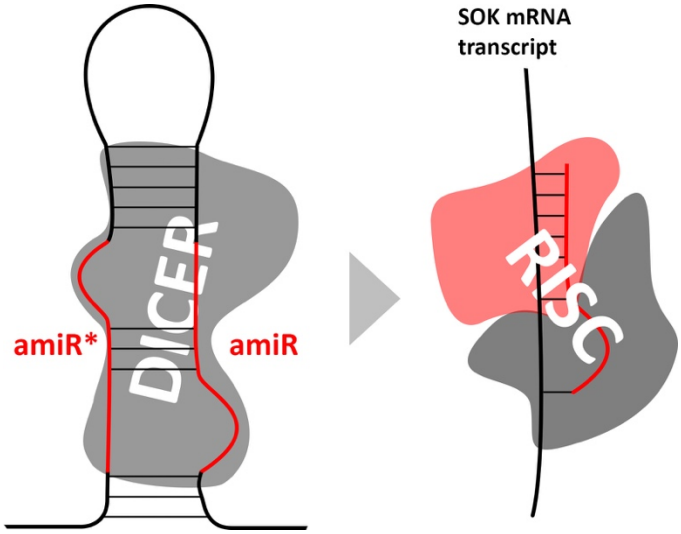

Figure 3. On the left, a hairpin loop of amiR transgene with amiR* and amiR sequences present. On the right, the amiR part is incorporated into the RISC complex that targets a *SOK* mRNA transcript.

To be able to have artificlas microRNAs (amiRs) function in plants, the amiRs have to be cloned in a vector that will be expressed in *Agrobacterium tumefaciens*. *Agrobacterium tumefaciens* can then be used to transform the plants and insert the AmiR sequence in the plants genome. One way to make such a vector is by GreenGate cloning. GreenGate uses a set of premade vectors that contain building blocks (named module A to Z). These vectors are combined in a single reaction where the building blocks are cut from each vector using restriction enzymes. Because the 5'- and 3'-end of these building blocks have an overhang that is complementary with an overhang of another building block, these building blocks can then be ligated in the right order in a final destination vector[10].

The GreenGate method, together with the live-imaging are described in more detail in the next chapter. In the chapter after that *SOK* expression domains in LRP are described. Next progress and results for the GreenGate cloning are described. In parallel with cloning, laser ablation of single LRP cells was done and which will be described. Lastly, a discussion about *SOK* localization in comparison with *SOK* localization in primary and emerged lateral roots is made.

Materials & Methods

Seedling growth

A solution with BactoAgar (8g/L; 4g for 500ml), Sucrose (1%; 5g for 500ml), Murashige and Skoog (MS) salts (2.15 g/L; 1.07g for 500ml) in a 10% 2-(*N*-morpholino)ethanesulfonic acid (MES) pH5.7 0.5 g/L and 90% ultrapure water was made. The solution was autoclaved and poured into square petri dishes to make MS plates. Seeds were sterilized in Triton-X100 for 2min, washed with 99% ethanol and air-dried in a flow cabinet. Sterilized seeds were plated on MS plates, put O/N in a cold room(-4°C) for stratification and then transferred to light in a growth chamber (16 hours of light and 8 hours of darkness) for 4 days.

Live-imaging

Four to six four days old (gravistimulated six to eight hours) seedlings were transferred to a slice of MS medium inside a one-chambered Lab-Tek borosilicate coverglass system. The coverglass system was mounted on the automated Z-galvo stage of a Leica SP5 (confocal) microscope. In bright field (BF) positions of the seedling were marked based on where a LRP could arise. LAS AF software allowed for imaging of multiple marked positions. For live-imaging multiphoton excitation was used to excite (*SOK*1 to *SOK*5)-YFP fusions (970nm, 1.227W output; transmission 30-50%; gain 100%; offset 50%). A hybrid detector captured emission light from YFP (150% HyD gain). For each position a z-stack was generated (40µm in size; 1µm step-size) where the xylem pole was clearly visible in the middle of the z-stack. The positions were live-imaging for at least twelve hours with a 30 min interval (this interval included a 10min rest period with no laser excitation) (Figure 3). The generated time-lapses were 1024x1024 in resolution, bidirectional X was used to lower imaging time, and line averaging of four to six times was used depending on the time left before imaging of the next z-stack started. In Fiji/ImageJ snapshots from time-lapses were selected based on the z-position where most of the LRP was visible. Brightness and contrast was lowered with minimum set to 0 and maximum ranged between 15 and 90, depending on YFP fluorescent signal intensity. Additionally, a scale-bar was added and the snapshots were saved as JPEGs. For figure creation Adobe Photoshop CS6 was used to put the snapshots side-by-side. The time for each snapshot was added and a new scale-bar was made with the exact size in pixels as the scale bar added in Fiji.

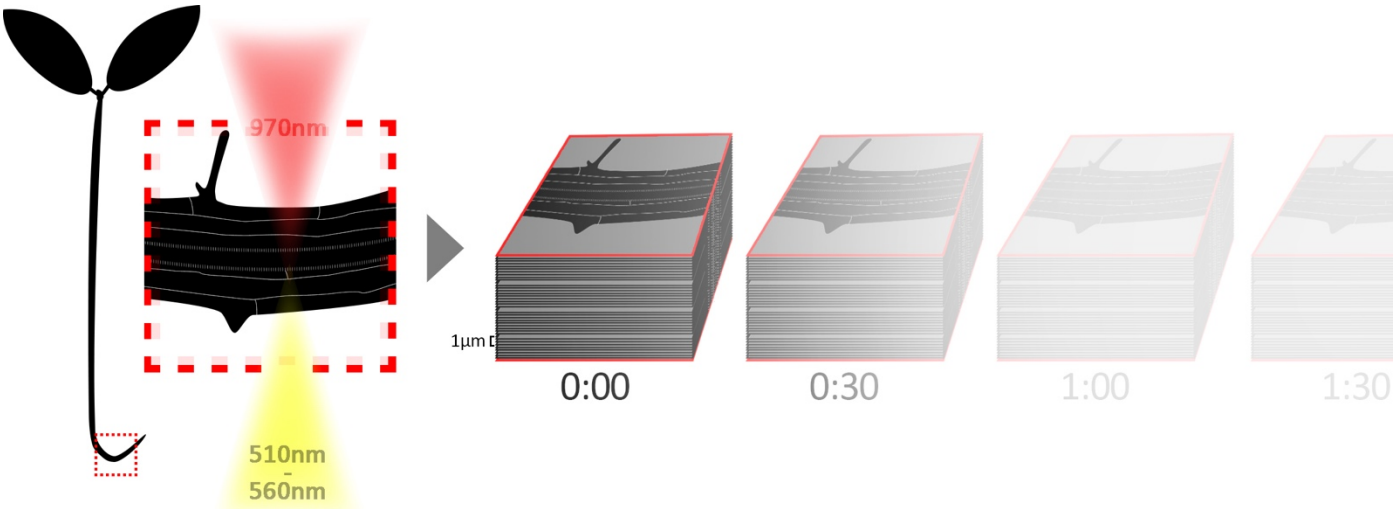

Figure 3. Gravistimulated root is targeted for live-imaging using multi-photon excitation. A z-stack is then generated every 30min of a developing LRP.

Laser ablation

To ablate cells a MP laser (880nm) was used where the emitted light was captured by a photon multiplier tube (PMT) of which the gain was set to maximum. A region of interest was selected the size of a LRP cell nuclei to target for ablation. Single cells were ablated by activating the laser for about five seconds and then the ablated LRP was live-imaged similar to the method described above in Live-imaging.

Light sheet fluorescence microscopy

Seedlings were allowed to grow in a BLAUBRAND intraMARK micropipette (100µl in 20°C, cut to length of 4.5cm) four to six days (gravistimulated for 8h) on similar medium as described in Seedling growth. The only difference is that Phytogel (1.0g/100ml) was used instead of BactoAgar. A Luxendo's light sheet microscope was used. First two laser beams were aligned using Luxendo's MuVi-SPIM software. Time-lapses were recorded using a 561nm laser (20% power) to image mCherry and a 515nm laser (20% power) was used to image YFP. A z-stack (roughly 100µm in size; 0.5µm step-size) was made every 30min and the resulting time-lapse was analyzed and processed in FiJi.

Cloning

amiRs were created using the WMD3 procedure found on wmd3.weigelworld.org. In short this procedure generated a list of possible amiR sequences that are suitable to silence the desired *SOK* gene, *SOK*1 to *SOK*5. Additionally, it gave four primer sequences which were able to assemble three fragments of the desired amiR using polymerase chain reaction (PCR) from a template of AMIR319a (pRS300) (Table S1 & S2). The four primers shared enough overlap with the template to bind to it. They had multiple point mutations to generate the desired amiR instead of copying the amiR on the template. The three fragments were combined in a PCR reaction to assemble the desired amiR (Table S3). The PCR product was run on a 1% agarose gel and a band corresponding to the length of the full-length amiR was cut and purified using GeneJETs column purification kit. All of the purified product was then digested with EcoR31I using ThermoFisher's FastDigest protocol to prepare it for ligation into a destination vector (pGGI) (Figure S2). The digested full-length amiR was purified again using GeneJETs column purification kit using the PCR purification protocol. This was done to remove short-in-length basepair fragments that were cut during the digestion. The purified full-length amiR sequence was inserted into a destination vector (pGGI) using a T4 DNA ligase ligation protocol (Figure S3 & S4). The ligation product was then transformed into electrocompetent *Escherichia coli* (*E. coli*) TOP10 cells for amplification of the plasmid. Transformed bacteria were allowed to recover in Lysogeny Broth (LB) medium for 1h before plating (100µl, 200µl, and the pellet) on LB medium with selection marker ampicillin in a 50 µl/ml final concentration. Grown colonies were used to inoculate 4ml LB medium with selection marker ampicillin. These inoculated cultures were allowed to grow overnight before plasmids were extracted using a miniprep protocol modified at Alexis Maizel laboratory. Extracted plasmids were digested with EcoR31I and both undigested and digested plasmids were run on a 1% agarose gel to check whether the insert was present. When the insert was present, plasmids were sent for sequencing using Eurofinsgenomics's LIGHTrun service (Table S10). This was done to validate that the extracted plasmid had a nucleotide sequence which matches the desired amiR sequence. Validation of the plasmids was done in Geneious version 10.2.3 by aligning the returned sequence to an artificially created desired plasmid.

Correctly assembled entry/pGGI vectors were used for assembly into GreenGate (GG) intermediate (pGGN) and destination vectors(pGGZ) (Table S4)[10]. The assembly into the GG vectors required multiple modules which all have to be aligned in the correct order into the destination/intermediate (pGGZ/pGGN) vector. This was done by combining five GG modules which are stored in vectors (modules are named A to Z, *e.g*. pGGA; Table S5). Vectors containing the modules were combined into one reaction where the modules were cleaved using EcoR31I and ligated into the destination(pGGZ) or intermediate vector(pGGN). To check whether the assembled plasmid had the right order of modules, a digestion and a PCR was performed (Table S1, S9 & S10). Digestion was done using FastDigest's restriction enzyme BglII for pGGZ vectors and two separate digestions using EcoR31I & XbaI for pGGN vectors. Once pGGZ vectors were validated they were transformed into *Agrobacterium tumefaciences* (strain ASE/pSOUP) and plants were transformed with floral dip method[11]. Whereas validated pGGN vectors were used for a second GreenGate reaction with another intermediate vector, pGGM (Figure 4, S14 & S15). Modules from these two intermediate vectors were cut and ligated into a pGGZ destination vector.

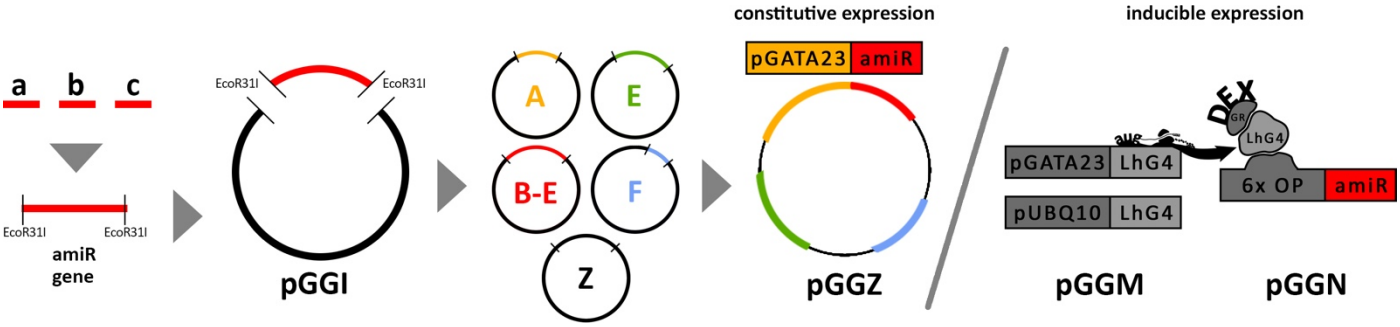

Figure 4. Assembly of abc-fragments and subsequent PCR assembly into the full-length amiR gene. The amiR gene is then inserted in the pGGI vector. This vector is combined in a single GreenGate reaction with four other vectors of which modules are cut and assembled in the destination vector pGGZ. Assembly of pGGN is similar to this apart from that there is one more vector added containing a linker module in the GreenGate reaction. The orange and red part of the pGGZ vector contain the promoter (pGATA23) and amiR gene that is driven constitutively. Next to this the pGGN vector contains a different promoter module that can be induced by DEX with the protein mechanism shown. To do so intermediate vector pGGN has to be combined with another intermediate vector named pGGM in a second GreenGate reaction.

SOSEKI expression domain in lateral root primordia

Previously *SOK* expression domains have been described in primary roots and emerged lateral roots (Figure S1)[7]. However, here we will use LRP as a model to elucidate the function of *SOK*. In order to do so first an atlas of *SOK* expression is made for LRP that can be used later for comparison with mutant and/or ablated phenotypes.

Out of all five *SOK*s only *SOK*1, *SOK*2, and *SOK*5 were active in early stages of LRP (founder cells, stage I and stage II; Figure 3, 4, and 5). *SOK*3 and *SOK*4 activity is absent most of the time in early stages of LRP growth (3 observations for each). In some instances, *SOK*3 or *SOK*4 expression was found (Figure S7). The other 'active' *SOK*s, *SOK*1, *SOK*2, and *SOK*5, have distinct expression patterns from each other. First *SOK*1 expression will be described followed by *SOK*2 expression and lastly *SOK*5 expression. For each description first *SOK* expression in the primary root (PR) was checked and compared with previously observed *SOK* expression domains in primary roots[7]. This was done to ensure the correct *SOK* was being live-imaged (Figure 5A, 6A, 7A).

*SOK*1 activity starts in founder cells where it localizes to the basal side. In stage I *SOK*1 localization changes to the outer basal corner in inner LRP cells (lateral root axes). In stage II *SOK*1 localization changes again and it localizes to where upper inner and lower inner cells are joined when cell division has occurred. Whether *SOK*1 is active in lower and/or upper inner cells of stage II LRPs is unknown because the membranes from upper inner cells cannot be distinguished from lower inner cells. During the transition between stages, *SOK*1 expression fades and returns post-cell division (Figure 5B). Most interestingly the *SOK*1 expression pattern appears to be mirrored around the lateral root apical-basal axis indicating that this axis may already be established at this point (Figure 5).

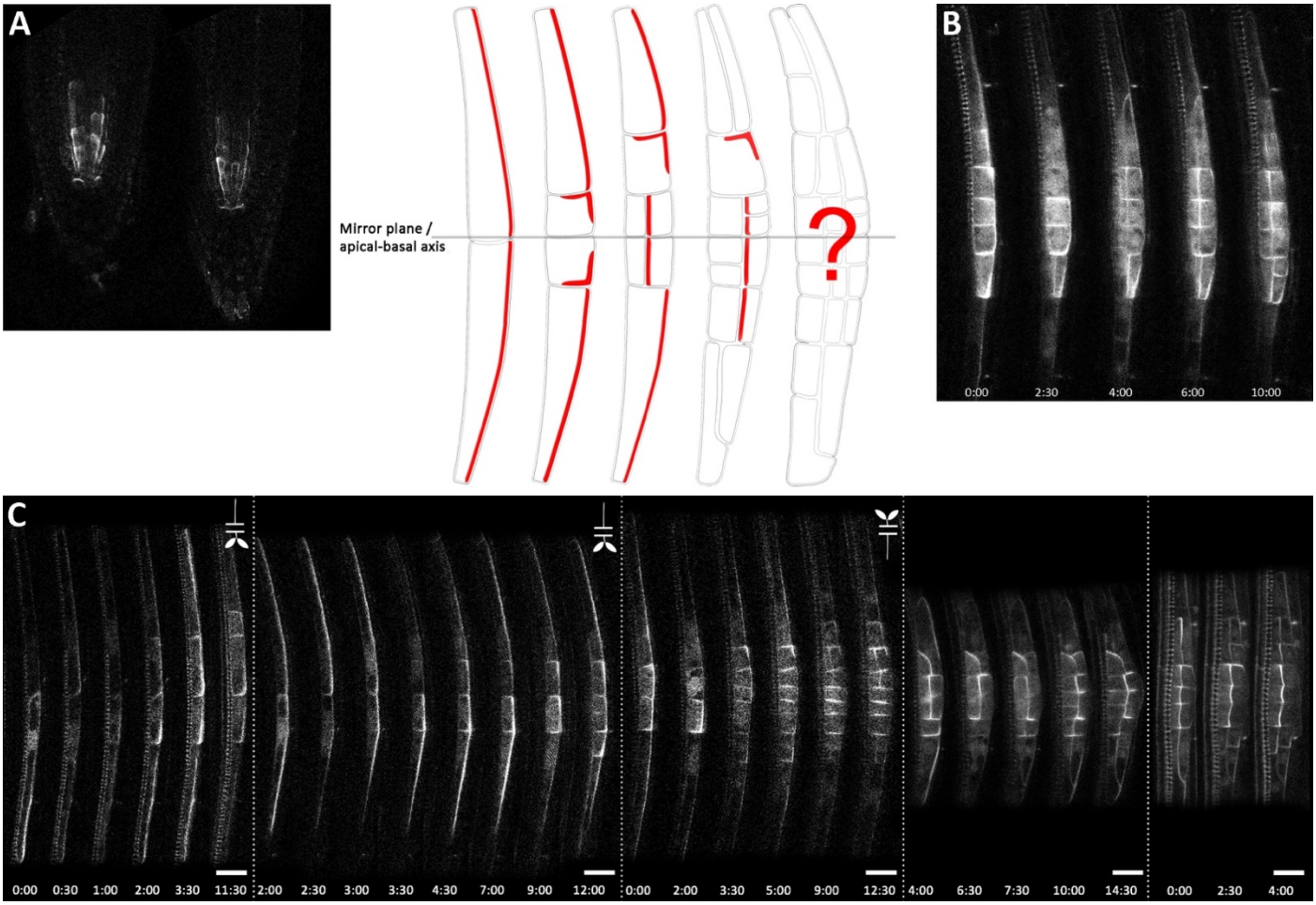

Figure 5. (A) *SOK*1-YFP expression in primary roots. In the middle a graphical depiction of the *SOK*1-YFP expression pattern consensus in lateral roots with time-lapse snapshots around the consensus to support it. The consensus was based on 12 time-lapses (not all are shown in this figure) (B) *SOK*1-YFP in LRP to indicate that *SOK*1 fades between LRP developmental stages. (C) *SOK*1-YFP in LRP to support the graphically depicted consensus (more snapshots can be found in Figure S8 & S9). Indicated time is the time passed after start of live-imaging. Scale bars: 20µm. The image of a small *A. thaliana* plant in the corner indicates where is shootward and where is rootward in the LRP showed.

Whether *SOK*2 is active in founder cells is unknown. However, in this work *SOK*2 expression was seen in stage I where it localizes to the inner apical corner in inner cells. This expression domain is maintained in stage two but only in lower inner cells. Next to the observed *SOK*2 expression in five LRP, in seven LRP no *SOK*2 activity was found up to and including stage II (Figure 6C). This may indicate that *SOK*2 expression can be delayed.

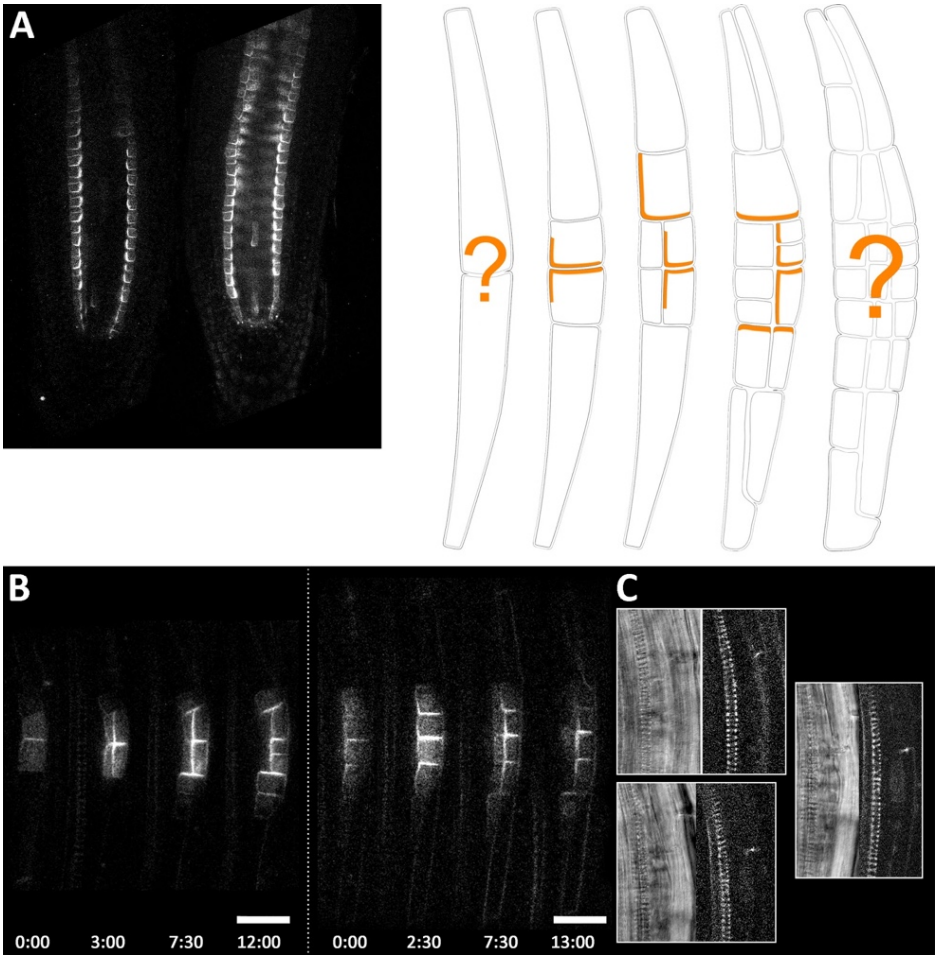

Figure 6. (A) *SOK*2-YFP in primary roots as a reference for lateral roots. In the middle a graphical depiction of the *SOK*2-YFP expression pattern consensus in LRP with time-lapse snapshots. The consensus was based on 5 time-lapses (not all are shown in this figure). (B) *SOK*2-YFP in LRP to support the graphically depicted consensus (more snapshots can be found in Figure S10). Indicated time is the time passed after start of live-imaging. Scale bars: 20µm. (C) Additionally, BF (left) and MP (Right) images are shown to indicate the lack of *SOK*2-YFP expression in stage two LRP.

*SOK*5 was found active in founder cells. Here it had a cytoplasmic expression and with an inconsistent expression domain. Sometimes *SOK*5 was active in the lower founder cell and may switch its activity to the upper founder cell. This inconsistent expression pattern is also present in stage I and II. In stage I there is both membrane and cytoplasmic localized expression. Because of the inconsistent expression, a consensus for *SOK*5 membrane localized expression remains elusive in stage I. However, for cytoplasmic expression, often one of the outer cells had the most cytoplasmic expression after division (Figure 5B; 4:30, 2:00 & 11:00 for the second, third, and last time-lapse, respectively). Later, in stage II *SOK*5 expression follows a more consistent expression pattern and it appears to have stabilized. Here its consensus is that it localizes to the apical side of upper inner cells and later the same localization is present in lower inner cells (Figure 7).

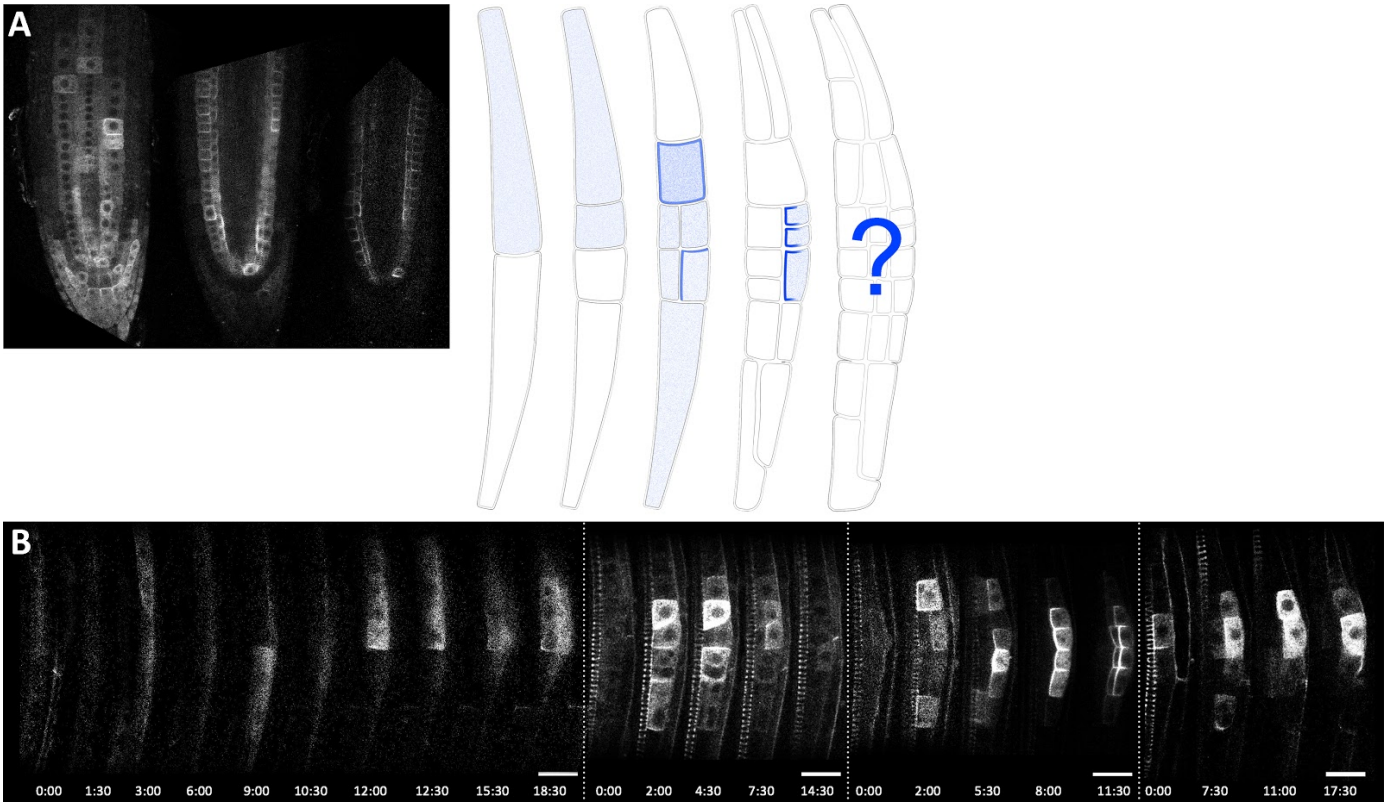

Figure 7. (A) *SOK*5-YFP expression in primary roots. In the middle a graphical depiction of the *SOK*5-YFP expression pattern consensus in LRP with time-lapse snapshots below the consensus to support it. The consensus was based on 15 time-lapses (not all are shown in this figure). (B) *SOK*5-YFP in LRP to support the graphically depicted consensus (more snapshots can be found in Figure S11 & S12). Indicated time is the time passed after start of live-imaging. Indicated time is the time passed after start of live-imaging. Scale bars:20µm.

A start to elucidate SOSEKI function

To elucidate *SOK*s function a process to make transgenic plants that silence *SOK* gene(s) was performed. To do so first genetic codes of amiR that silence *SOK*s were generated (two amiRs per *SOK*). These genetic codes were inserted into an entry vector that was sequenced to validate that the correct amiR sequences were present (Sequencing results, Figure S13).

The validated entry vectors were used to generate a destination vector as was explained in Materials and methods (Cloning, pg.4). These destination vectors contain a transgenic construct in which the amiRs are driven constitutively in the LRP by the promoter of GATA23 (pGATA23). This allowed to make ten final destination vectors, one per amiR per *SOK*, with constitutive expression in the LRP (hereafter these plasmids were named pGGZXX, were the first X is the number of *SOK*, *SOK*1 to *SOK*5, that is targeted, and the second X is the number of amiR, amiR1 or amiR2; Figure S5).

Next to this, a total of 20 destination vectors in which each amiR were driven inducibly by the specific pGATA23 or the ubiquitous pUBQ10 promoter were started to be made. To make these inducible vectors first an intermediate vector per final destination vector had to be assembled (hereafter these vectors are named pGGNXX, were the first X is the number of *SOK*, *SOK*1 to *SOK*5, that is targeted, and the second X is the number of amiR, amiR1 or amiR2; Figure S6). Because of the nature of the GreenGate method (see materials and methods, cloning, pg. 4) a validation that the building constructs are inserted in the complete and right order is required. Here the validation of the generated constructs is described.

Validation for each construct created in this work was done by PCR amplification and restriction enzyme techniques. PCR amplification with specific primers tested whether a sequence in the vector stretching over multiple modules was made (Figure 9, Table 1 & S5). If the PCR product was obtained it was considered that these modules were present in the right order. Besides, if modules were inserted in the complete and right order, after digestion, they also yield fragments of set sizes (Figure 8 & 9). Module Z and N do not need to be validated because they contain the bacteria selection marker.

For pGGZ the PCR amplifies a sequence of 1147 bp that stretches over modules A, B-E, and F (Figure 8) which are responsible for the amiR promoter (pGATA23), the amiR gene, and plant selection marker, respectively. For pGGN the PCR amplifies a nucleotide sequence of 867 bp that stretches over modules B-E and F which contains the amiR gene and the plant selection marker sequence, respectively.

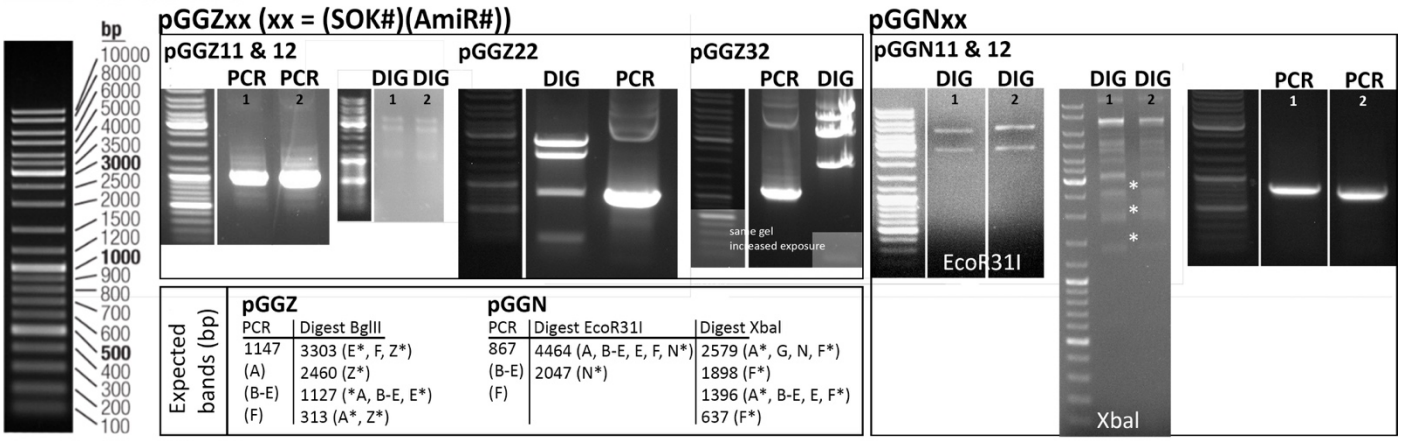

Figure 8. On the left the DNA ladder that was used for each gel. In the middle four validations of pGGZ vectors with amplification of a DNA fragment in the vector using PCR and digestion of the vector. The desired PCR product should be 1147 bp in length. The digest was done with BglII and should yield four fragments of sizes: 3303, 2460, 1127, and 313 bp. On the right a similar validation was done for pGGN vectors. In this case the PCR yields a product of size 867 bp and the digest with EcoRI31 yields two fragments of sizes 4464 and 2047 bp. Behind each expected band size is the modules that are present in the band and for digestion the location of the restriction site is marked with an asterisk (*).

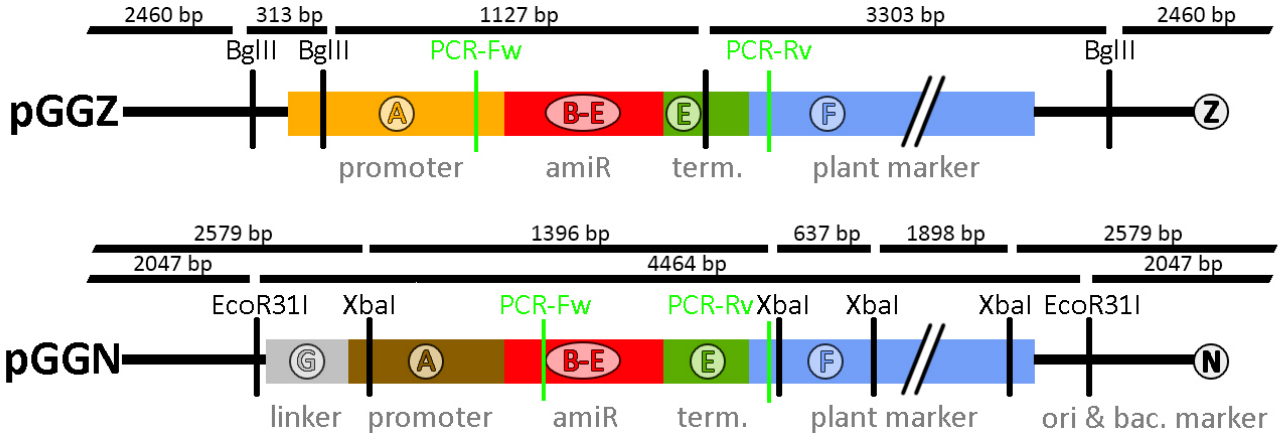

Figure 9. Location of restriction sites on pGGZ and pGGN and the respective band sizes. PCR forward(Fw) and reverse(Rv) bind sites are indicated too. Module letter and function is indicated. Depicted module lengths are relative to each other expect for module F which is longer in reality, hence a break is shown.

First, the validation of the constructed pGGZ vectors will be described followed by the validation of the pGGN vector. On gel all PCR products for pGGZ11, 12, 22, and 32 are present (Figure 8). Thus modules A, B-E, E and F are present. As an extra control a digestion was done. Digestion of pGGZ generated all expected fragments for pGGZ22 and 32. Thus pGGZ22 and 32 are complete. For pGGZ11 and 12 a 313 bp band is missing. The 313 bp band contains part of the amiR promoter sequence. Because all other expected bands are present and the PCR confirmed that the amiR promoter is present, it is assumed that even though the 313 bp band is missing pGGZ11 and 12 contain all modules. Next the validated pGGZ11, 22, and 32 vectors were used to transform *Agrobacterium tumefaciens* and subsequently used to transform the respective p*SOK*x::*SOK*x-YFP plant line.

For pGGN11 and 12 vectors two digestions were done, one digestion with EcoR31I and one with XbaI. For the digestion with EcoR31I two expected bands were present (Figure 6). This meant that all modules were present. For the digestion with XbaI only three out of four of the expected bands were seen, the 637 bp band was missing. The 637 bp band contains a sequence of the plant selection marker. Because both restriction sites for this band are within the plant selection marker and bands of 1898 bp and 1396 bp already verified that the plant selection marker was present the 637 bp band was considered redundant. The PCR amplfication on pGGN11 and 12 generated a band of the expected size which confirmed that B-E and E were present. Combining the results from the digestions and PCR both pGGN11 and 12 are considered complete and were used in two separate secondary GreenGate assemblies to generate destination vectors. The cloning progress made for the remaining *SOK*/amiR combinations in pGGZ and pGGN is also available (Table S6, S7, and S8).

Laser ablation of single lateral root primordia cells

In another attempt to understand *SOK* function a preliminary experiment was started in which single LRP cells expressing *SOK*1 were ablated. The intention was to observe and follow *SOK*1 localization post-ablation and investigate if it is different from what is expected using the atlas (see SOSEKI expression domain in lateral root primordia, pg. 5 to 7) as a reference. The experiment is shortly described below.

Twelve time-lapses were recorded post-ablation. Out of the twelve time-lapses, in two time-lapses, LRP cells and surrounding primary root tissue cells shrank. Whether this was caused by ablation or another cause is unknown. In the remaining ten time-lapses, mostly a cell around the ablated cell expanded (8/10) (Figure 10). However, no cell division could be observed. This may be attributed to not being able to distinguish cell walls and membranes (Figure 7). For all time-lapses *SOK*1 expression disappeared shortly post-ablation (Figure 10). The time at which *SOK*1 expression was not observed no more post-ablation differed between time-lapses (0.5-5h) and may be attributed to how severely each surrounding cell was affected by ablation. For future experiment a smaller region of interest targeting ablation is suggested to see if *SOK*1 remains active longer post-ablation. Additionally, crosses with nuclei and/or membrane markers with all *SOK*-YFP lines have been made that can be used to ensure whether cell division(s) occur post-ablation (Table 1).

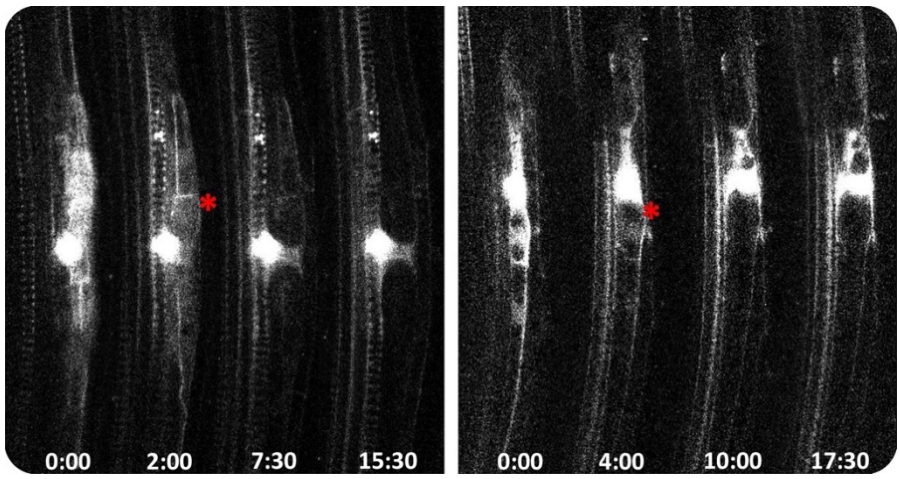

Figure 10: Snapshots from time-lapses in which single LRP cells were ablated. The red asterisk marks where cell growth can be seen post-ablation.

Table 1: Crosses that were made between *SOK*-YFP and membrane and/or nuclei markers.

Available crosses	**p*SOK*(1-5)-*SOK*(1-5)-YFP x pUBQ10-LTi6B-mCherry(2x) (F2)** **p*SOK*(1-5)-*SOK*(1-5)-YFP x pLBD16-SYP122-mCherry(3x) x pSHY-SHY2-mVENUS(3x) (F2)** **p*SOK*(1-5)-*SOK*(1-5)-YFP x pUBQ10-H2B-GFP (F2)**				
Transgenic construct	p*SOK*(1-5)-*SOK*(1-5)-YFP	pUBQ10-LTi6B-mCherry(2x)	pSHY2-SHY2-mVenus(3x)	pLBD16-SYP122-mCherry(3x)	pUBQ10-H2B-GFP
Tissue	LRP	All tissues	Endodermis	LRP	All tissues
Localization	Peripherally membrane bound	Cell membrane	Nucleus	Cell membrane	Nucleus

The cross between *SOK*1-YFP and pUBQ19-LTi6B-mCherry was used to make a time-lapse using LSFM (Figure 10). In the made time-lapse *SOK*1 localizes along the longitudinal axis in each LRP cell whereas previously *SOK*1 also localized along the transverse axis (see SOSEKI expression domain in lateral root primordia, pg. 5). However, this is a single observation in the LSFM and more time-lapses should to be made before before attaching weight to this observation.

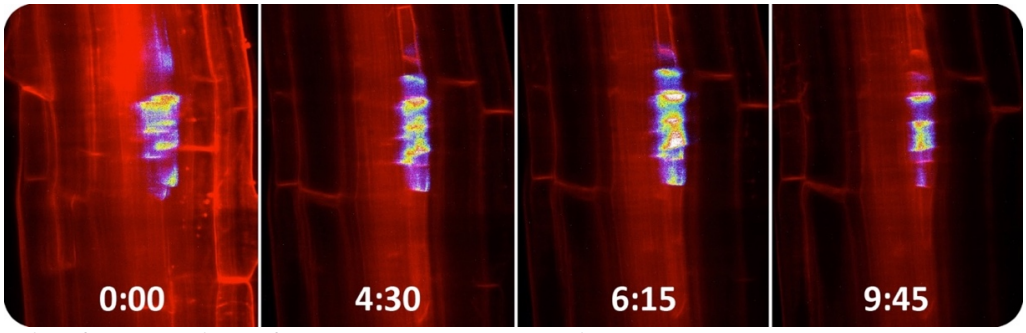

Figure 11: LSFM snapshots from a time-lapse of *SOK*1-YFP x pUBQ10-LTi6B-mCherry.

Discussion

As was seen previously *SOK*3 and *SOK*4 are absent in LRP (see SOSEKI expression domain in lateral root primordia, pg. 5). Because *SOK*3 and *SOK*4 expression has previously been observed in emerged lateral roots it is assumed that these two become active later in LRP development (Figure S1)[7].

In primary roots and emerged lateral roots *SOK*1 localizes to the outer apical corner and *SOK*2 to the basal inner corner. In just primary roots, *SOK*3 localizes to all corners, *SOK*4 expression is absent and *SOK*5 localizes to the basal inner corner (Figure 12)[7]. None of these observations match *SOK* localization in stage I or II LRP, apart from *SOK*4 which is absent in both primary roots and LRP (but becomes active later in emerged lateral roots like mentioned before). Because *SOK*1 and *SOK*2 localization is identical in primary and emerged lateral roots it is expected that the organismal axis in LRP is not fully established yet. As a result of this *SOK* localization may change throughout LRP development and eventually will and may come to a stop to match *SOK* localization in primary and emerged lateral roots (when the organismal axis is fully established). Interestingly in the transition fom stage I to stage II, *SOK*1 localization changes from a basal localization to an apical localization which is more characteristic of *SOK* localization in primary and emerged lateral roots. This may indicate that the establishment of the organismal axis is making progress between stage I and stage II LRP. It is speculated that SOK1 localization (and maybe localization of the other SOKs too) will be match the localization seen in primary and lateral roots when the endodermis is fully established. This is speculated because *SOK*1 always points toward the endodermis and in *scarecrow* mutant, with impaired endodermal identity, *SOK*1 losses its edge localization[7].

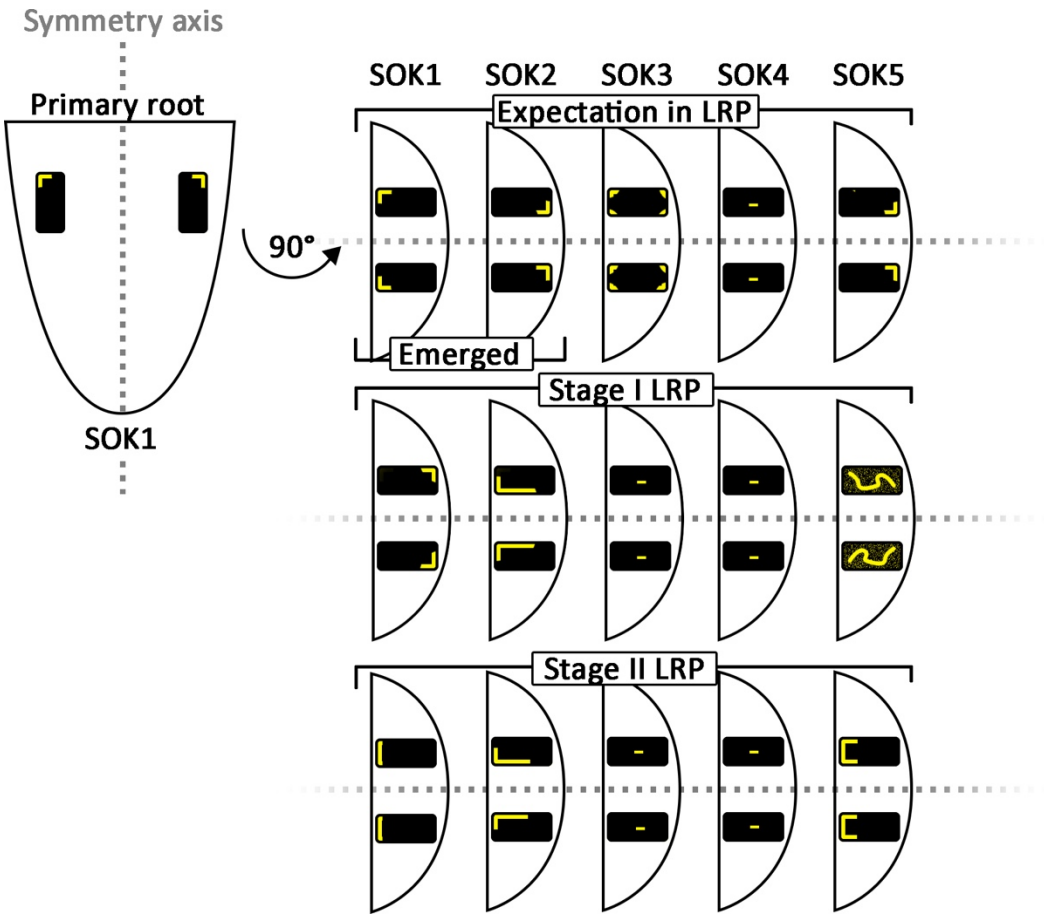

Figure 12: *SOK* localization expectation based on *SOK*'s localization in primary roots. *SOK* localization for *SOK*1 and *SOK*2 in emerged lateral roots is also indicated. The *SOK* localization that was observed in this work is also indicated for stage I and II LRP.

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