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Joanna Boruc

Final Report

Full Name	Joanna Boruc
Selection Year	2012
Host institution	Ghent University, VIB
Supervisor	Dirk Inzé, Daniël Van Damme
Period covered by this report	from 01/06/2013 to 01/06/2015
Title	Unraveling the function of Arabidopsis thaliana α Aurora kinases in control of the orientation of formative cell divisions through their substrate, MAP65-1.

1. Objectives (max. 1/2 page)

The aim of this project was to elucidate the role of alpha Auroras in plant development through the identification of downstream targets of plant Aurora kinases. Down-regulation of α Auroras in Arabidopsis leads to multiple phenotypic aberrations, likely due to hypo-phosphorylation of their substrates. Namely, the double α aurora knockdown mutant *aur1/aur2* exhibits defects in orienting formative cell divisions (Van Damme et al., 2011), while RNAi silencing displays impaired cytokinesis with defective cell plate formation. The preferential phosphorylation of TPX2 by Arabidopsis Aurora 1 rather than Aurora 3 (Tomaštíková et al., 2015) and the function of Aurora 1 in MT polymerization (Petrovska et al., 2013), hints towards a role of α Aurora kinases in the regulation of microtubule-related processes. To search for downstream targets of Aurora that might be involved in orienting initial formative divisions, we identified the putative Aurora phosphorylation motif which we used to search the TAIR database. The extensive list of candidate Aurora substrates was then limited to the cell cycle-regulated hits. Among many interesting candidates, we found MAP65-1, a microtubule bundling protein, as a target of Arabidopsis Auroras and we mapped its two putative phosphorylated sites. In the course of this work we used genetic, cell biological and biochemical tools to test the functional interaction of plant α Auroras with this microtubule binding protein. We showed that MAP65-1 interacts with Aurora1 *in vivo*, is phosphorylated by Aurora kinases on two residues (S532 and T552) in its C-terminal tail domain, and over-expression and down-regulation antagonistically affect the α Aurora double mutant phenotypes. Phospho-mutant analysis demonstrated that Aurora kinases contribute to the microtubule bundling capacity of MAP65-1 in concert with other mitotic kinases and that this phosphorylation also affects its antiparallel microtubule bundling preference.

2. Methodology in a nutshell (1/2 page)

The following methods have been used in this study:

- Substrate identification:

Analysis of the TAIR protein database using the Aurora consensus phosphorylation motif determined for budding yeast Ipl1p (Cheeseman et al., 2002) and human Aurora A (Ferrari et al., 2005) to identify putative Aurora phosphorylation sites in the Arabidopsis proteome.

- Co-immunoprecipitation assay:

The functional promAurora1:Aurora1-GFP in the complemented aurora double knockdown background was pulled down from Arabidopsis seedlings to detect the *in vivo* interacting substrate (endogenous MAP65-1).

- Phosphorylation assay:

Kinase assays were performed using a modified protocol from Hans et al. (2009). Recombinant Arabidopsis His-Aurora1 and His-Aurora2 were incubated with recombinant substrates (MAP65-1 protein and its truncated versions). Identification of phosphorylated residues of MAP65-1 by mass spectroscopy analysis was done by EDyP-Service (CEA – iRTSV-LEDyP, Grenoble, France).

- Functionality assays of phosphorylated residues:
  - Determination of the apparent Kd (microtubule affinity) of the substrate and its phospho-mutant forms
  - Turbidimetry bundling assays
  - Microtubule dynamics by TIRF microscopy
  - *In vivo* confocal and spinning disc imaging of the subcellular localization of the substrate and its phospho-mutant forms.

3. Results (6-8 pages)

Substrate identification

Based on *in vitro* kinase assay results, followed by mass spectrometry analysis, evidence showed that the Aurora phosphorylation motif is largely conserved between animal and plant cells. A motif-based search at TAIR (http://www.arabidopsis.org), using the Aurora kinase consensus pattern identified a large list of

candidate Arabidopsis targets (over 6000). This initial list was challenged by coupling it to root and shoot proliferation specific expression patterns of the candidate proteins and conservation of the putative Aurora phosphorylation sites in several orthologous genes identified through the PLAZA tool (Proost et al., 2009). Since the α Aurora double mutant exhibits hypersensitivity to oryzalin, defects in orienting formative cell divisions and the silencing line displays impaired cytokinesis with defective cell plate formation, implying a function of Aurora in microtubule (MT)-related processes, and one of its putative targets is MAP65-1, this protein was a suitable first candidate for further testing.

Analysis of all Arabidopsis MAP65 proteins using the Aurora consensus phosphorylation motif determined for budding yeast Ipl1p (Cheeseman et al., 2002) and human Aurora A (Ferrari et al., 2005) identified putative Aurora phosphorylation sites in all AtMAP65 proteins positioned downstream of their dimerization domain. This motif also identified a third putative Aurora phosphorylation site in MAP65-1, which was not mentioned before (Smertenko et al., 2006). For MAP65-1, MAP65-2, MAP65-6 and MAP65-8, several of these putative sites were also reported to be phosphorylated *in vivo* in large-scale mass spectrometry experiments (PhosPhAt 4.0 Database). This includes the three identified Aurora phospho-sites in MAP65-1: Ser532: (Umezawa et al., 2013), and Ser408 and Thr552 (Roitinger et al., 2015). Although the large-scale phospho-proteomics analyses did not specify the kinases responsible for the observed phosphorylation of the three MAP65-1 residues, they do prove that they are genuine phosphorylation targets *in planta*. One of the three putative Aurora phosphorylation sites is very well conserved in plants (Ser532), one is conserved in several monocot species but not in other dicots (Ser408), and the third one (Thr552) is not conserved among plant species. Alignment of MAP65-1 from various plant species (ClustalW (McWilliam et al., 2013)), verifying evolutionary conservation of these putative sites between Arabidopsis, 14 other plant species, yeast (Ase1) and vertebrates (PRC1), revealed that the putative Aurora phosphorylation sites are not conserved between the different kingdoms.

Co-immunoprecipitation assay

To test whether MAP65-1 interacts with plant Aurora kinases, we performed an *in vivo* co-immunoprecipitation assay, where endogenous MAP65-1 was found to specifically co-purify along with functional Aurora1-GFP in the complemented aurora double knockdown background.

Phosphorylation assay

The *in vivo* interaction was confirmed by *in vitro* phosphorylation assays using recombinant full length MAP65-1 and truncated forms. Recombinant Arabidopsis Aurora 1 and 2 phosphorylated MAP65-1 and the analysis of truncated forms showed that phosphorylation was restricted to its C-terminal domain. Mass spectrometry analysis of the *in vitro* phosphorylated peptides revealed that the phosphorylated residues overlapped with those that were predicted (Smertenko et al., 2006). Although Ser408 matches the consensus phosphorylation motif and was identified before as an *in vivo* phospho-peptide (Roitinger et al., 2015), our *in vitro* phosphorylation assays did not detect it as a target for α Aurora kinases.

Functionality assays of the phosphorylated residues

Since microtubule bundling is the canonical activity of MAP65s, we aimed to verify whether Aurora-dependent phosphorylation of MAP65-1 affected its bundling capacity. For this purpose, phospho-mutant recombinant forms of MAP65-1 were created, carrying a substitution of Ser532 and Thr552 to alanine (constituting a double phosphorylation-dead "AA" form) or to aspartic acid (a phospho-mimicking "DD" form). Co-sedimentation assays showed that the *in vitro* phosphorylated and the MAP65-1(DD) forms had a slightly lower affinity for MTs than the wild type (WT) and MAP65-1(AA) forms. *In vitro* TIRF experiments determining the bundling of MTs by either the WT MAP65-1 or the phospho-mutant forms showed that phospho-mimicking the α Aurora phosphorylation sites does not abolish MAP65-1 MT bundling *in vitro* and *in vitro* MT dynamics were not significantly different between MAP65-1 and both phospho-mutant forms. Thus, Aurora-dependent phosphorylation alone does not strongly affect the *in vitro* bundling capacity of MAP65-1. However, we found that MAP65-1(DD), mimicking the Aurora-dependent phosphorylation, altered the bundling polarity preference of MAP65-1. Whereas the vast majority of microtubules bundled by recombinant MAP65-1 or MAP65-1(AA) and imaged via TIRF microscopy showed antiparallel orientations (87% and 71% respectively), equal proportions of parallel and antiparallel bundled microtubules were observed in the presence of recombinant MAP65-1(DD). Aurora-dependent phosphorylation of the C-terminal unfolded domain of MAP65-1 may therefore cause a conformational change of the protein dimer, altering its MT bundling properties.

As the next functional assay of these phospho-sites, the subcellular localization of MAP65-1 and its phospho-mutants was analyzed *in vivo*. For this, additional phospho-mutant forms were created: a phospho-mimicking form in all 9 putative phospho-sites (called thereafter 9D, reported previously (Smertenko et al., 2006)) and a phospho-mimicking form in 7 putative phospho-sites (all non-Aurora sites, called 7D). Tobacco BY-2 cells have been used extensively to study the dynamic localization of MAP65 proteins (Van Damme et al., 2004b; Smertenko et al., 2006; Gaillard et al., 2008; Fache et al., 2010; Sasabe et al., 2011). We therefore used this system to analyze the effect of Aurora-phospho-mutations on the localization of MAP65-1 throughout mitosis.

In vivo imaging of BY-2 cells demonstrated that the microtubule bundling regulation (decrease in metaphase and increase in anaphase) of MAP65-1 was comparable between C-terminal and N-terminal GFP fusions. However in cells expressing the C-terminal fusion there were some cytoplasmic aggregates visible and the internal MTs were more prominently labeled in the C-terminal fusion. Also, the timing of cell division duration did not differ. Based on our previous work, we continued our analyses with the C-terminal fusion. Compared to WT MAP65-1, MAP65-1(AA) ectopically bundled prophase, metaphase and phragmoplast MTs. MAP65-1(DD), on the other hand, localized similarly to the WT MAP65-1. Quantification of the timing of each cell division phase revealed that MAP65-1(AA) delayed the anaphase onset (prolonged metaphase, defined from the PPB disappearance until just before chromosome separation), in agreement with previous data (Smertenko et al., 2006). Surprisingly, MAP65-1(DD) delayed this phase even more, although

the protein was not visibly associated with the spindle MTs. It is thus likely that the delay caused by MAP65-1(DD) expression is MT-independent.
In late cytokinesis, BY-2 cells expressing MAP65-1(AA) showed remnant MT bundling in the center of the phragmoplast, in contrast to cells expressing WT MAP65-1 and MAP65-1(DD), where the highest fluorescence signal was associated with the outer rim of the expanding cell plate. These observations are indicative of a function of Aurora-controlled MAP65-1 phosphorylation during the transition of cell plate formation to phragmoplast expansion.

To distinguish between the effect of Aurora phosphorylation and the phosphorylation on the other 7 putative phosphorylation sites in MAP65-1, MAP65-1(7D) and MAP65-1(9D), were localized during cell division in BY-2 cells. Phospho-mimicries in all 9 and 7 phospho-sites did not significantly affect the timing of cell division, although MAP65-1(7D)-GFP did prolong metaphase duration. However, their binding to MTs was altered. MAP65-1(7D) only faintly decorated the cortical MTs, even though it was clearly present at the PPB, and failed to reappear on the anaphase and phragmoplast MTs. MAP65-1(9D) lost all visible MT association capacity with the exception of the PPB. The recruitment to the cortical MTs of MAP65-1(7D), in contrast to MAP65-1(9D), showed that phospho-mimicking both Aurora-dependent residues has an additional negative effect on the MT-binding capacity of MAP65-1, while the absence of recruitment to metaphase and anaphase spindle and phragmoplast MTs suggests that MAP65-1(7D) is phosphorylated on its Aurora residues during these cell cycle stages. PPB recruitment of MAP65-1, independent of its phosphorylation status, points to additional factors controlling its recruitment compared to cortical or other mitotic MT arrays.

To confirm the effect of the Aurora-dependent phosphorylation of MAP65-1 in Arabidopsis cells, MAP65-1 was localized *in vivo* in WT (Col-0) and *aur1/aur2* double knockdown mutant seedlings. Confocal and spinning disc imaging revealed no significant change in MAP65-1 prophase spindle intensity or metaphase duration (WT Col-0 vs *aur1/aur2* double mutant) when MAP65-1 was driven by either the MAP65-1 endogenous promoter or when ectopically expressed under the strong mitotic AUR1 promoter. However, when WT MAP65-1, MAP65-1(AA) and MAP65-1(DD) localization patterns were compared in Arabidopsis, the metaphase to anaphase spindle signal ratio for MAP65-1 increased in the *aur1/aur2* mutant background and this was similar to the effect of MAP65-1(AA) in Col-0 when compared to the WT MAP65-1 in the Col-0 background. A ratio increase was also observed for MAP65-1(DD), although statistically less significant. Dynamic Aurora-dependent phosphorylation and de-phosphorylation of MAP65-1 is therefore required for the differential accumulation of this protein between the metaphase and anaphase spindle in Arabidopsis.
The calculation of the timing of the cell division phases disclosed that metaphase was slightly longer in Col-0 cells expressing MAP65-1(AA) than MAP65-1 WT or MAP65-1(DD) (T test P=0.00974). Lacking the capacity to phosphorylate MAP65-1 by Aurora might thus delay the anaphase onset also in Arabidopsis due

to ectopic spindle MT bundling and remnant expression in the *aur1/aur2* double knockdown likely softens this effect compared to MAP65-1(AA) expression.

Similar to the observations in BY-2, cytokinesis in Col-0 cells expressing MAP65-1(AA) or WT MAP65-1 in *aur1/aur2* was also affected: MTs in the center of the phragmoplast were not depolymerized as efficiently compared to the control situation. This was also the case for MAP65-1(DD) in Col-0, although this was not observed in BY-2. Consequently, the duration of cytokinesis (measured from the beginning of the phragmoplast expansion through the disappearance of the MTs as the phragmoplast rim reached the plasma membrane) was significantly (around 50%) longer in cells expressing MAP65-1 in *aur1/aur2*, MAP65-1(AA) in Col-0 and MAP65-1(DD) in Col-0 when compared to the control. Hence, similar to the metaphase to anaphase transition, MAP65-1 might need to undergo dynamic, Aurora-dependent phosphorylation changes in cytokinesis to allow efficient phragmoplast MT dynamics.

If Aurora-dependent phosphorylation restricts MAP65-1 MT bundling capacity throughout the cell cycle, then ectopic (mitotic) expression of MAP65-1 in the *aur1/aur2* double knockdown mutant background should aggravate the *aur1/aur2* developmental phenotype due to its reduced Aurora activity. To verify this hypothesis, MAP65-1 was expressed under a strong mitotic promoter (prAUR1) in WT Col-0 and in the *aur1/aur2* mutant. In contrast to Col-0, MAP65-1 mitotic over expression aggravated the primary root length, lateral root (LR) density, rosette size and bushy shoot phenotypes of the *aur1/aur2* mutant. Mitotic over-expression of MAP65-1 did not significantly reduce the number of non-emerged primordia up until the first emerged primordium, indicating that the LR density reduction is likely caused by LR outgrowth rather than initiation defects.

As over expression of MAP65-1 aggravated the *aur1/aur2* double knockdown mutant phenotype, we anticipated that removing this substrate might cause a partial rescue. To test this, we crossed a *map65-1* (GABI-Kat_198A01; *map65-1-3*) mutant into the *aur1/aur2* mutant. Western blotting using a MAP65-1 antibody (Smertenko et al., 2004) confirmed the strong down-regulation of MAP65-1 in this mutant, as the remaining signal is likely a cross-reaction with other MAP65 isoforms (Lucas et al., 2011).

The rosette size of the triple *aur1/aur2 map65-1-3* mutant did not statistically differ from the *aur1/aur2* mutant and the single *map65-1-3* mutant was morphologically indistinguishable from Col-0 at this developmental stage (25 day-old). However, although mature *map65-1-3* mutant plants were slightly taller than Col-0 WT plants, depletion of MAP65-1 in the *aur1/aur2* double mutant background (*aur1/aur2 map65-1-3* triple mutant) had a stronger and statistically significant effect on the overall plant height compared to the control (43% length increase compared to 12%; P=3.7E-11;) and partially rescued the bushy *aur1/aur2* mutant phenotype. Additionally, *aur1/aur2 map65-1-3* triple mutants partially rescued the LR density phenotype of the *aur1/aur2* mutant, which was also confirmed using an independent mutant allele of *map65-1* (*map65-1-2* SALK_118225; (Lucas et al., 2011)). The antagonistic effects of over-expression versus knockdown further indicates that MAP65-1 phosphorylation by Aurora regulates its function. Taken together, our biochemical, imaging and genetic interaction data reveal a regulatory system in which the activity of MAP65-1 is controlled by α Aurora phosphorylation in Arabidopsis.

To test whether the enhanced LR mutant phenotypes were Aurora phosphorylation-dependent, MAP65-1(AA) and MAP65-1(DD) forms were introduced into the Col-0 and *aur1/aur2* mutant backgrounds. If MAP65-1 hypo-phosphorylation by Aurora is the underlying cause of the phenotype enhancement in the *aur1/aur2* background, over expression of the phospho-mimicking MAP65-1(DD) form should not aggravate the mutant phenotype. However, primary root length and LR density analyses showed that both MAP65-1(AA) and MAP65-1(DD) fusions affected the phenotype of the *aur1/aur2* mutant similarly to the WT MAP65-1 over expression.

Phospho-mimicry of all 9 phospho-sites in MAP65-1 (9D), as well as in the putative cyclin-dependent kinase (CDK) sites (MAP65-1(2D)) did however not aggravate the mutant phenotypes. Immunoblot analyses demonstrated that the differential effect between the WT and aurora double knockdown mutant backgrounds cannot be attributed to differential over expression levels. The aggravation of the LR phenotype in the *aur1/aur2* mutant is therefore dependent on the phosphorylation status of MAP65-1, rather than on its mere over expression, but not causal to direct Aurora-dependent phosphorylation of MAP65-1. Hence, the phenotype is probably due to indirect phosphorylation events which differ between the WT and the *aur1/aur2* mutant backgrounds.

As the aurora double mutant is mostly affected in lateral root outgrowth by altered orientation of the first divisions and mitotic over expression of MAP65-1 aggravates the LR phenotype even more, this represents the most likely system to identify the causal effect of the aggravated mutant phenotype. The severe alterations in LR primordia patterning in the aurora double knockdown mutant, however, hinder proper characterization of the effect of mitotic over expression of MAP65-1 (Van Damme et al., 2011). Nevertheless, time lapse imaging of lateral root primordia divisions of mitotically expressed MAP65-1-TagRFP did reveal stronger metaphase spindle signals in the *aur1/aur2* double mutant and ectopic bundling in the center of the expanding phragmoplast compared to the WT control, similar to the effects observed in the root epidermal cells.

We therefore examined the effect on cell divisions in the main root apical meristem (RAM). Quantification of the number of meristematic cells in the primary roots revealed that mitotic over expression of MAP65-1 reduced the amount of meristematic cells in the *aur1/aur2* double mutant. Moreover, this RAM phenotype enhancement in *aur1/aur2* is dependent on the phosphorylation status of MAP65-1, but not directly controlled by Aurora kinase, as this decrease of the number of cells in the RAM is reverted to *aur1/aur2*-like values by the expression of the phospho-mimicry of all MAP65-1 9 phospho-sites (9D), as well as the putative cyclin-dependent kinase (CDK) sites (2D). It is therefore likely that the enhanced lateral root outgrowth defects observed in the *aur1/aur2* mutant upon mitotic over expression of MAP65-1 are also caused by a decreased number of divisions.

4. Perspectives (max. 1/2 page)

Aurora kinases exert multiple roles in diverse cell cycle-related processes by phosphorylating a wide variety of substrates. We have identified Arabidopsis MAP65-1 as the first microtubule-associated substrate of plant α Aurora kinases and we confirmed the functionality of its phosphorylation sites. We showed that two sites located in the C-terminal part of MAP65-1are phosphorylated by α Aurora kinases. Moreover, Aurora-dependent phosphorylation of MAP65-1 at its C-terminus likely evolved specifically in plants, since PRC1 (the animal homologue of MAP65-1) is an Aurora binding partner, but likely not a substrate, while Ase1 (the MAP65-1 counterpart in yeast) is phosphorylated *in vitro* by Ipl1 (the Aurora homologue in *S.cerevisiae*) on completely different residues. Therefore, the presence of diverged phosphorylation sites hinted towards a plant-specific MAP65-1 control mechanism by Aurora kinases, which might be linked to the functional expansion of this protein family in plants compared to the single PRC1 and Ase1 proteins in animals and yeasts.

We aimed to identify the function(s) of Aurora phosphotylation sites in plants. Our *in vitro* experiments argue against Aurora-dependent phosphorylation of MAP65-1 directly controlling its MT bundling or dimerization capacity. The observed slight reduction in MT binding and bundling properties of the phospho-mimicry version compared to the WT form rather fits with a combined role of Aurora and other mitotic kinases controlling the MT binding capacities of MAP65-1, in agreement with previous data (Smertenko et al., 2006). The reduced bundle orientation specificity of Aurora-phospho-mimicked MAP65-1 points, on the other hand, to a specific function for this phosphorylation. Whether the orientation preference control is specific for Aurora or reflects merely an intermediate phosphorylated state of MAP65-1, and whether there is a function for parallel bundling by MAP65-1 throughout plant development remains to be addressed.

Through this and previous works it became more apparent that the affinity of MAP65-1 for MTs is unlikely controlled by mere protein phosphorylation, since a complete phospho-mimicry in all 9 MAP65-1 sites at its C-terminus still retained partial MT association *in vitro* (Smertenko et al., 2006) and associated with the PPB MTs *in vivo* in BY-2 cells (our results). Therefore, it is reasonable to envisage additional, plausibly cell cycle-dependent mechanisms regulating MAP65-1 properties and activity. Future work, leading to a spatio-temporal resolution of Aurora activity in plant cells with respect to the other mitotic kinases, will allow to test this model of progressive MAP65-1 phosphorylation.

5. Valorisation/Diffusion (incl. Publications, Conferences, Seminars, Missions abroad, max. 1/2 page)

In the past 2 years I have published the following articles:

- Emission spectra profiling of fluorescent proteins in living plant cells. Evelien Mylle, Mirela-Corina Codreanu, Joanna Boruc, Eugenia Russinova, Plant Methods 04/2013; 9(1):10.
- Ectopic expression of Kip-related proteins restrains root-knot nematode-feeding site expansion. Paulo Vieira, Carmen Escudero, Natalia Rodiuc, Joanna Boruc, Eugenia Russinova, Nathalie Glab,

Manuel Mota, Lieven de Veylder, Pierre Abad, Gilbert Engler, Janice de Almeida Engler, New Phytol. 2013 Jul;199(2):505-19.

- GAP Activity, but Not Subcellular Targeting, Is Required for Arabidopsis RanGAP Cellular and Developmental Functions. Joanna Boruc, Anna H.N. Griffis, Thushani Rodrigo-Peiris, Xiao Zhou, Bailey Tilford, Daniël Van Damme and Iris Meier. Plant Cell. 2015 Jul;27(7):1985-98.

- Endomembrane trafficking overarching cell plate formation. Joanna Boruc and Daniel Van Damme. Curr Opinion Plant Biol. 2015 Dec;28:92-8.

Manuscripts in preparation:

- Dynamic phosphorylation of Arabidopsis MAP65-1 by alpha Aurora kinases is required for proper plant development. Joanna Boruc, Annika K. Weimer, Virginie Stoppin-Mellet, Evelien Mylle, Maria Njo, Liesbeth De Milde, Nathalie Gonzalez, Dirk Inzé, Tom Beeckman, Marylin Vantard and Daniël Van Damme.

I also contributed to a book chapter: "Cell division plane determination in plant development" in The Plant Sciences - Cell Biology; 9 June 2014. David Bouchez , Daniël Van Damme, Joanna Boruc, Estelle Schaefer, Martine Pastuglia, edited by Sarah Assmann and Bo Liu.

I have participated in the following conferences:

- The Society for Experimental Biology (SEB) Annual Main Meeting, 3rd-6th July 2013, Valencia, Spain (poster presentation)

- EMBO Conference: Microtubules: Structure, Regulation and Functions, 28th-31st May 2014, Heidelberg, Germany (poster presentation).