Plasma membrane to nucleus trafficking of the transcriptional co-activator BRX – characterization of a novel hormone signaling pathway in plants (BRXtraffic).

Proposal nr. 236220 Scientist in charge : Prof. Christian Hardtke
Final report Stephen Depuydt

The focus of the proposed research was to unravel how BRX, a higly conserved plant-specific transcriptional regulator, resides at the plasma membrane and how it is subsequently translocated to the nucleus upon auxin treatment. To investigate this potential novel auxin signaling pathway, we elaborated on the hypothesis that PRAF proteins would be instrumental for BRX localisation and translocation. Nevertheless, by microscopically analyzing transgenic lines, that were established during the first term of the fellowship, localisation of PRAF1 could be clearly demonstrated at the plasma-membrane and in the nucleus thus overlapping with BRX localization. In addition, Bimolecular Fluorescence Complementation (BiFC) assays also indicated that interactions occur between some PRAF family members and some BRX family members, mainly at the level of the plasma-membrane, supporting our initial hypothesis. PRAF proteins however did not follow BRX behavior : attempts to change the localisation by auxin and/or MG132 treatment were unsuccessful. PRAF proteins possess FYVE and PH domains which are presumably important for membrane trafficking as they are lipid binding domains. However, our experiments could not confirm the importance of these domains for membrane binding as a dominant negative approach in which we mutagenised the PH domain did not change the localisation pattern of the PRAF protein : it was still observed at the PM and occasionally in the nucleus as the WT PRAF protein, in the Col-0 background. Interestingly, in the *brx-2* background however, a minor change in expression window (i.e. expression appears earlier than compared with the WT) and localisation (i.e. more cytosolic) was noted. Overexpression of PRAF proteins yielded roots with an increased length, but in the *brx-2* background this effect is no longer there. These results encouraged us to proceed to the functional analysis of PRAF knock out mutants to unequivocally demonstrate that PRAF proteins are the mediators of BRX localisation and translocation. We made use of knock out lines in several PRAF family members. Although redundancy might be at play and it might still be worthwile to examine higher order mutants however with the current results at hand we need to reject this hypothesis based on the following observations. The PRAF knock out lines (null alleles) do not display a clear root phenotype, except for *praf8* mutants that have a small but significant reduction in main root length and *praf3* mutants that have fewer but larger cells in the meristem. However, attempts to prove interaction between PRAF8/PRAF3 and BRX remained unsuccesful. No phloem discontinuity or shoot phenotypes that have previously been associated with BRX were observed in these PRAF mutants. In addition, no changes in expression pattern of BRX could be noted in these PRAF mutants. Also BRX protein behavior and localisation was not altered in these mutants which was clearly demonstrated by transforming PRAF knock outs with BRX-GFP constructs and constructs with its truncated forms. It was also hypothesised that the RCC domain of the PRAF proteins was potentially regulating the nuclear import of the protein. Nevertheless, targeted mutagenesis of the AA sequence of this domain of the protein also did not alter PRAF protein localization so we can not confirm this hypothesis.

The originally proposed project was built on the hypothesis that PRAFs were crucial for BRX function. Because of the extensive redundancy issues described above, we cannot yet unequivocally confirm this hypothesis. Nevertheless, our observations (of which the most important ones are listed) yielded some insight into PRAF biology and we are finalizing these data for publication.

Given the above described difficulties, in the second term more attention was given to the genetic screen that was initiated in the first term (see midterm report). There it was described that a suppressor screen was performed and that after analyzing 40.000 M2 seeds a total of 209 putants were isolated. We rescreened those mutants to finally confirm 33 mutants (i.e. mutants that display a longer main root when compared with the *brx-2* short root phenotype, but are still in *brx-2* background). To map the mutations we applied a novel and very powerful technique with great succes : whole genome sequencing was used. To this end, we back-crossed the mutants with the *brx-2* mother lines. Of the segregating F2 lines we made root cultures of the

suppressed and the non suppressed lines and extracted DNA that was then used for whole genome sequencing.
We developed a pipeline to analze the massive amount of data (in collaboration with L. Santuari) and after comparison of the suppressed and non suppressed lines lists with possible SNP candidates were made. Based mainly on biological relevance (i.e. location of the SNP, affected gene, AA change, etc) possible mutations were ranked and then subsequently checked by amplifying the surrounding region and Sanger sequencing. In a first step, out of four mutants, two mutations could be confirmed by sequencing. Later on a third one could also be confirmed and very recently (july 2011) two other mutants were sequenced and their mutations identified. We focused on two mutants (i.e. the 1.19.2 and 1.77.1 mutants) and some very promising results were obtained which will be discussed briefly in the next sections.
The 1.19.2 mutant (that shows a partial rescue of the root lenght) has a stop codon in a gene of unknown function (At5g47690) that has an armadillo like fold domain that has also been found in beta-catenins, importins, karyopherin and exportins and can bind DNA and proteins. Seggregation analysis (due to the presence of a CAPS marker) confirmed that the suppression of the *brx-2* phenotype is caused by this mutation. This was also elegantly shown by a cross between brx-2 and an independant knock out line in At5g47690. Although there is no clear shoot phenotype, nor a different response towards auxin and/or cytokinin treatment, the phloem discontinuity of the *brx-2* mutant was rescued. Interestingly, playing with the brassinosteroid content (by exogeneous application of brassinolide or BRZ) showed that the mutant is less sensitive, which might indicate that the gene is linked with brassinosteroid content. This will be investigated further and a functional analysis (overexpression, GFP and GUS reporter lines, …) is undertaken.
The 77.1 mutant was confirmed to have a mutation in an uncharacterized LRR-receptor like kinase. This mutant rescues the *brx-2* root growth phenotype as well as other *brx-2* phenotypes (i.e. hormonal response, phloem discontinuity, size and number of cells in the meristem) completely. Transgenic lines have been created to analyze expression pattern and cell biological behavior of the LRR-receptor like kinase. Additionally, by means of an extensive complementation cross assay, a second allele was picked up that is characterized further. Importantly, the mutation was confirmed by crossing a corresponding T-DNA insertion line with *brx-2* and double mutants have been isolated. Having established that the LRR-receptor like kinase mutation suppresses *brx*, we next wondered what is its ligand. Through educated guesses and extensive assays (for instance, we tested all CLE peptides) we have found the ligand for our LRR-receptor like kinase. Concerning this receptor-ligand pair, experiments are in a final phase and a manuscript will be submitted in the coming weeks. Also worth mentioning is that during the screen we may have found an important regulator of lateral root growth, and on this matter a collaboration with expert labs has been initiated. Finally, the screen also yielded two suppressors that clearly must be involved in the intra-cellular trafficking of BRX, closing the loop towards the initial goal of the project.
In conclusion the EMS mutagenesis yielded an interesting collection of mutants to the lab, of which undoubtedly some papers will follow in the coming months and years and which provide the lab with novel resources to conduct highly innovative science.
On a personal matter, I feel that this postdoc fellowship has not only considerably broadened my technical skills (genetics, cell biology) as a scientist but also opened my way of thinking and tackling scientific problems from another point of view than the one obtained during my PhD training. The initiation of the EMS mutagenesis and the data treatment and the genetics associated are in addition extra competences that have enabled me to take up a sustained position in my home countr, clearly I believe that this Marie Curie fellowship was an asset to make the jump to this next stage.