Regulation of the production of reactive oxygen species by the plant NADPH oxidase and its role in pathogen response and in response to other environmental or developmental factors

The production of Reactive Oxygen Species (ROS) is one of the earlier responses observed after pathogen infection in plants. In plant defence, ROS are not direct killers but signals that mediate the activation of the defences. In addition, plants use ROS-derived signals in a variety of developmental contexts and in abiotic stress. It is hence important to understand the signalling controls that allow plant cells to interpret ROS-dependent signals.

Our goal is to decipher, using functional genomics tools, the functions of the plant NADPH oxidase gene family. Different members of the *rboh (respiratory burst oxidase homologues*) family, components of the plant NADPH oxidase, control production of ROS during defence and other responses. We are performing this research in *Arabidopsis*, model organism for studies in plants, where many genomic tools are available.

We propose the following ***2 Specific Aims:***

Specific Aim 1: To Investigate the regulation of Rboh-dependent NADPH oxidase activity and its function in control of ROS production, Pathogen Response, and other responses.

We and others have shown that members of the Rboh-NADPH oxidase family are responsible for the production of ROS in many of contexts. Interestingly, whereas *AtrbohC* regulate specifically root hair formation, *AtrbohD* and *AtrbohF* seem to be pleiotropic, functioning in defense response, cell death control and several stress responses. In addition, *AtrbohD* and *AtrbohF* display functional overlap, since the double mutant always displays stronger phenotypes than either individual mutant. But they also clearly perform independent functions. Thus, ROS produced by different Atrboh may have a qualitative (location, time …) difference that may serve diverse signalling functions in different situations. To unravel the specificity of the function of ROS produced by the Atrboh, we generated different constructs with *AtrbohD* and *AtrbohF* alleles driven by a strong promoter or by their own promoters. These alleles carry modifications in the different regulatory domains to analyze or a fluorescent tag to follow its accumulation and cell localization in planta. Its functionality *in planta* is checked by complementation of mutant lines. The assays to test these transgenics are 1) ROS production (nearly completely dependent on *AtrbohD*) and 2) response to pathogen infection o assessing the size of the plant, where *AtrbohF* plays a more important role.

We generated the battery of different *AtrbohD* and *AtrbohF*, alleles both in vectors suitable to transfer the gene by regular cloning and in vector compatible with the GATEWAY system. Alleles have been transferred to binary vectors that will allow their constitutive expression under the 35S promoter or under their own promoters, and some transgenic homozygous lines have been identified. Intriguingly, the over-expression constructs are not working properly. Most of these lines do not show complementation suggesting that over-expression of these genes could have deleterious effects in the plant. We also experienced considerable problems to obtain GFP fused lines in order to monitor cell localization of these proteins, obtaining just a few lines that are currently under evaluation. In the other hand, we are obtaining good complementation with constructs with their own promoter obtained both by regular and GATEWAY cloning strategies.
We got interesting results on the role of the promoter on the specific function of these genes. The analysis of transgenic plants with their promoters fused to the *uidA* gene revealed that the

AtrbohD and *AtrbohF* promoters are active in several tissues. Interestingly the two promoters are induced by pathogens, although with a different pattern of expression. Additionally, complementation studies with their own promoter indicate the importance of the promoter region to obtain complementation of *atrbohD* and *atrbohF* mutants.

Specific Aim 2: To examine the regulation of the plant NADPH oxidase by heterotrimeric G-proteins, especially in the context of defence response and cell death.

Analyses of Rboh function suggest that ROS act in complex signalling networks operational in many pathways in response to developmental cues or to the environment. Particularly relevant are the links between Rboh-dependent ROS production and Ca^{2+}, phosphorylation, and plant regulators like SA and ABA. The response to ozone also induces an oxidative burst that offers similarities to the pathogen-induced oxidative burst, and where Rboh and heterotrimeric G proteins have been implicated (Joo et al., 2005). We wanted to study the interaction between the Rboh and heterotrimeric G proteins in the context of the defence response.

Our analysis of the mutants in the heterotrimeric G proteins reveals that the mutant in the Gβ subunit of heterotrimeric G proteins (AGB1) displays enhanced susceptibility to different strains of *Pseudomonas syringae*, suggesting that this gene is important for basal resistance.

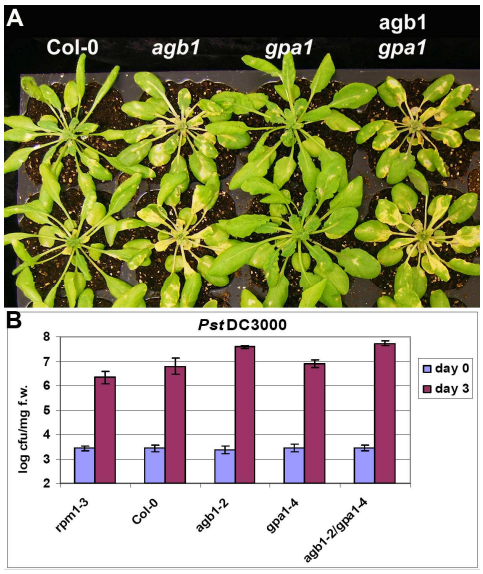

FIGURE 1. The *agb1* mutant in the Gβ subunit of heterotrimeric G proteins displays enhanced susceptibility to *Pseudomonas*. **A** Symptoms in 4 weeks old plants from different genotypes after spray with *Pseudomonas syringae* pv tomato DC3000. **B** Bacterial growth 3 days after injection of *Pseudomonas syringae* pv tomato DC3000.

We performed epistasis studies between the G protein mutants and the *atrbohD* and *atrbohF* mutants. The analysis of the different mutant combinations Indicate that heterotrimeric G proteins and NADPH oxidase mediate the same pathway in response to hemibiotrophic bacteria *Pseudomonas syringae*, whereas they mediate two different pathways in response to the necrotrophic fungus *Plectosphaerella cucumerina*, stressing the fact that these signals play different functions depending on the typo of pathogen. In addition, epistasis studies between the heterotrimeric G proteins and salicylic acid deficient mutants reveal that the resistance pathway mediated by AGB1 is independent of salicylic acid signalling.

Information about this research can be found at:http://www.cbgp.upm.es/ros.php