Postdoc Fellowships for non-EU researchers

Final Report

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Selection	2012
Host institution	Department Plant Systems Biology, VIB, UGent
Supervisor	Prof. Dr. Dirk Inzé
Period covered by this report	From 05/08/2013 to 04/02/2015
Title	Characterization of E3 ubiquitin ligases involved in the jasmonate signal transduction cascades that regulate plant growth.

1. Objectives of the Fellowship (1/2 page)

As sessile organisms, plants need to adapt to fluctuations in environmental conditions. A complex hormonal network plays a central role in the plant's response to different types of endogenous and exogenous signals, leading to extensive transcriptional, proteomic and metabolomic changes that determine the specifically required response (Grant and Jones, 2009).

Jasmonate and its derivatives (JA(s)) are essential and ubiquitous fatty acid-derived phytohormones. They are involved in plant growth, development and in the response to biotic and abiotic stresses. Upon wounding or pathogen attack plant cells activate several defence mechanisms and produce specific protective specialised metabolites (Wasternack, 2007).

It has recently been demonstrated that the Ubiquitin (Ub)-26S proteasome system is connected to almost all aspects of plant development, by acting in the centre of most hormone responses. It was also demonstrated that hormonal crosstalk can occur at the proteolysis level (Dreher and Callis, 2007). However, despite the extremely high number of potential E3 ligases encoded by plant genomes, only a few have been specifically characterised and ubiquitination of target proteins has barely been proven.

In the research group of Prof. Dr. Alain Goossens we are studying jasmonate (JA) signalling using a functional genomics platform that allows the discovery of novel genes. This platform integrates genome-wide transcriptomics, proteomics, interactomics and metabolomics, followed by the functional characterization of lead genes and proteins. One of these technologies is Tandem Affinity Purification (TAP), which is used to identify new proteins involved in signalling pathways and cellular processes modulated by JAs (Van Leene et al., 2008).

The aim of this project was to characterise the mode of action of two Arabidopsis E3-ubiquitin ligases (RGLG3 and RGLG4) putatively involved in JA signalling that were selected through *in silico* analysis of genome-wide transcriptome data and used in a TAP screening. During this project we focused on the characterisation of the targets of these E3-ubiquitin ligases: ROX1 which corresponds to an Arabidopsis glutaredoxin and started with the characterization of another target, namely ROX2 which corresponds to the Arabidopsis truncated haemoglobin.

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

All Arabidopsis plants used in this study were in the Columbia 0 ecotype background and all plant transformations were carried out by floral dip using *Agrobacterium tumefaciens* strain C58C1.

Wild type *Nicotiana benthamiana* plants (4-week-old) were used for transient expression of constructs by *A. tumefaciens*-mediated transient transformation of lower epidermal leaf cells. All *Agrobacterium* strains were grown for 2 days, diluted to OD 1 in infiltration buffer and incubated for 3 hours at room temperature

before agroinfiltration. In all cases a P19 expressing *Agrobacterium* strain was added and *Agrobacterium* cultures were mixed in 1:1 ratio before injecting.

Tandem affinity purification (TAP) experiments were carried out in *Arabidopsis thaliana* PSB-D cells to identify new interacting proteins of ROX1 and ROX2. TAP consists in the co-isolation of a specific tagged protein (bait) and its interactive partners (preys) under native conditions through two consecutive affinity-purification steps, followed by the subsequent identification of the interacting proteins by mass spectrometry (MS). We used previously established TAP methods that had already allowed to identify new components of the JA signalling machinery such as the NINJA (Pauwels and Goossens, 2011; Van Leene et al., 2011). Identified protein interactors were further confirmed using yeast two hybrid (Y2H) and/or bimolecular fluorescence complementation (BiFC).

Total protein extracts were prepared by resuspending ground tissue in cold extraction buffer and centrifuged at 4°C. To test protein stability, 50 µM MG132 or 1 % v/v dimethyl sulfoxide as a control was added to the total protein extract. Each reaction was incubated at room temperature, samples were harvested and the reaction stopped by adding SDS sample buffer and boiling for 10 minutes before gel analysis.

To test protein stability in wild type and mutant background, total protein extraction was performed as above but *E. coli* recombinant protein was added to each protein extract just before the room temperature incubation.

3. Results (6-8 pages)

ROX1 and ROX2 are interactors of the E3-ubiquitin ligases RGLG3 and RGLG44

The Arabidopsis E3 Ub-Ligases RGLG3 and RGLG4 were described to play a role in Jasmonate (JA) signalling (Zhang et al., 2012) but their exact role is still unknown. To find their targets and assess their role, RGLG3 and RGLG4 were fused to a TAP-tag and expressed in Arabidopsis cell cultures. After two affinity purifications and MS analysis, we identified two targets, namely ROX1 and ROX2. These interactions were confirmed by yeast two-hybrid (Y2H) and split-GFP assays (Figure 1A and B). Accordingly, all proteins ROX1, ROX2, RGLG3 and RGLG4 presented nucleo-cytoplasmic localization (Figure 2).

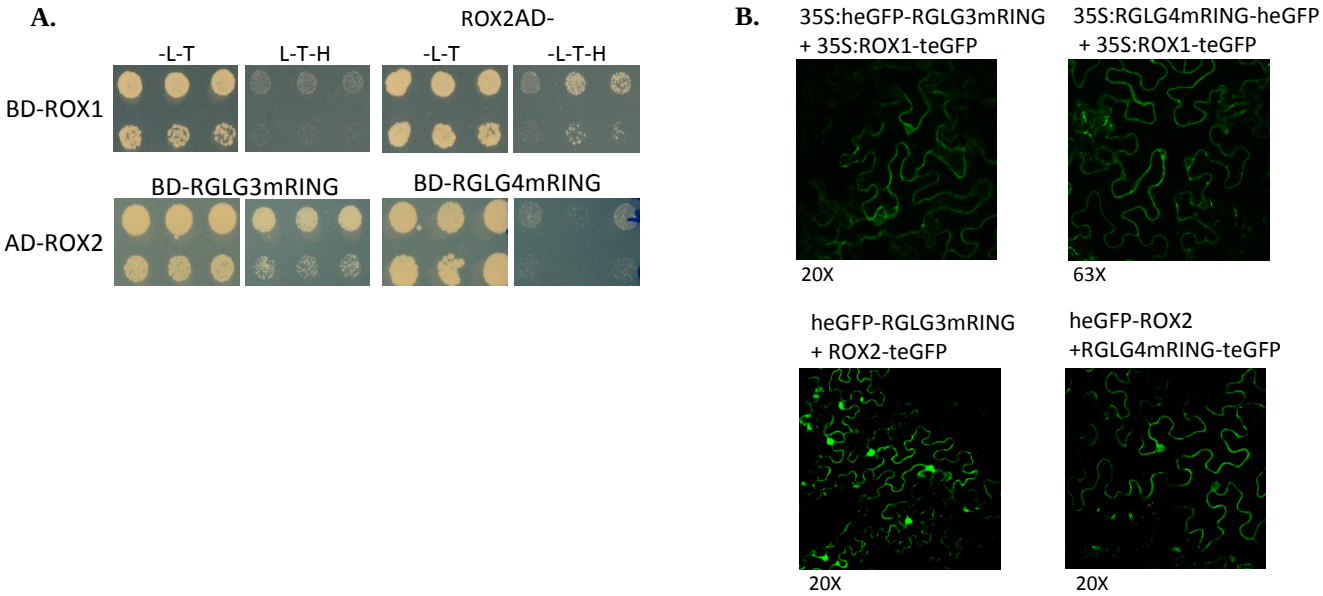

Figure 1. ROX1 and ROX2 are direct interactors of RGLG3/4
(A) Yeast two-hybrid assay between RGLG3mRING/RGLG4mRING and ROX1 and ROX2. Transformed yeast were spotted in 10 fold and 100-fold dilutions on control medium (-L-T) and selective medium (-L-T-H). **(B)** RGLG3mRing/4mRing interaction with ROX1 and ROX2 by bimolecular fluorescence complementation. Head (he) or tail (te) GFP fusion proteins were transiently co-expressed in *N. benthamiana* leaves and analysed by confocal microscopy 3 days after agroinfiltration.

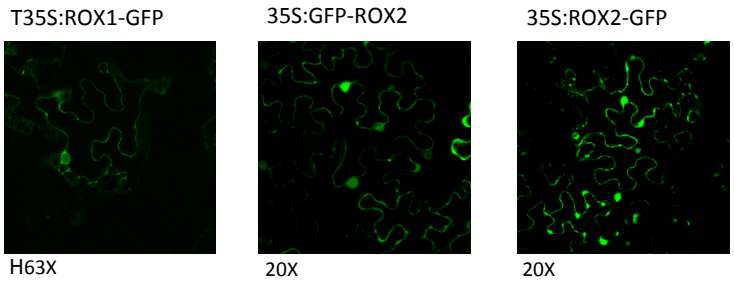

Figure 2. ROX1 and ROX2 are nucleo-cytoplasmic proteins.
Confocal microscopy images of *N. benthamiana* leaves transiently expressing ROX1-GFP, GFP-ROX2 and ROX2-GFP. Images were taken 3 days after agroinfiltration.

RGLG3/4 target ROX1 for protein degradation by proteasome

As ROX1 and ROX2 are interactors of E3-Ub ligases, we first analysed whether these proteins were degraded by the proteasome. We prepared recombinant protein ROX1:V5-His and used it in a cell free degradation assay. In this experiment the recombinant protein was incubated with a total protein extract prepared from Arabidopsis wild type seedlings and protein degradation is tested in the presence or absence of the proteasome inhibitor MG132. Higher levels of the recombinant protein ROX1:V5-His were observed in the presence of MG132 (Figure 3A). Similar results were obtained using transgenic lines expressing a ROX1:HA fusion protein (Figure 3B). These results suggest that ROX1 is degraded by proteasome.

In the same way, we tested ROX2 stability using Arabidopsis transgenic lines (Figure 3C) and cell cultures (Figure 3D) expressing a ROX2-TAP fusion protein. Total proteins were extracted from these materials and ROX2-TAP stability was analysed in the presence or absence of MG132. In this case, no differences in protein stabilities were observed.

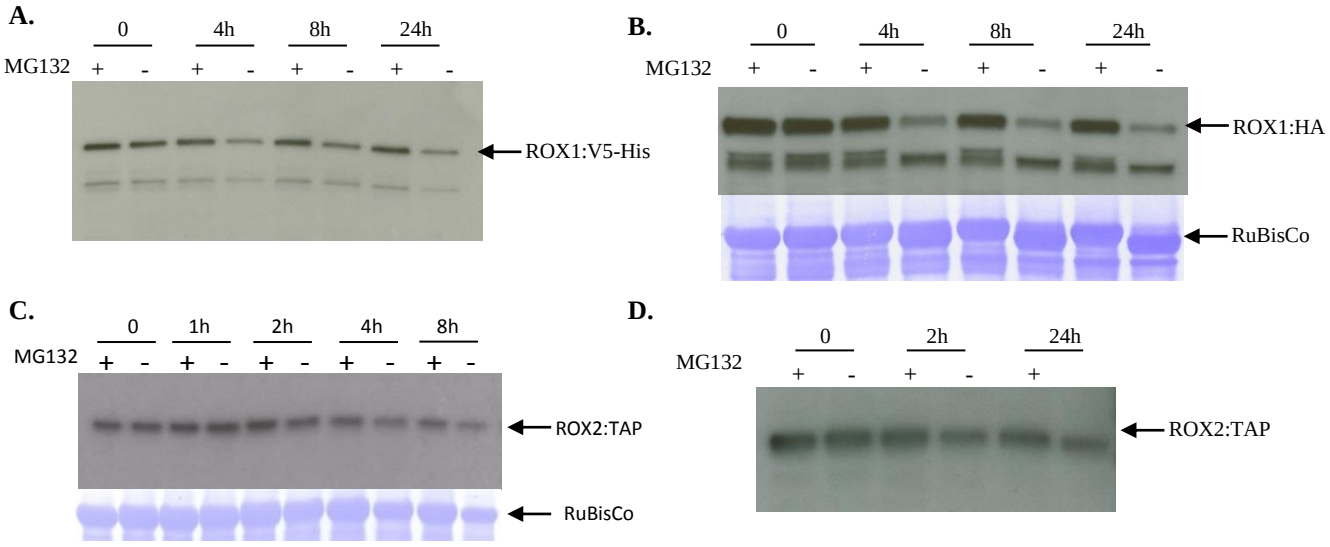

Figure 3.ROX1 is degraded by proteasome whereas ROX2 is stable.
(A) Cell free degradation assay. Recombinant protein ROX1:V5-His was incubated with total protein extract prepared from wild type Arabidopsis seedlings in the presence or absence of MG132 (50 µM). **(B-D)** Total proteins were extracted from *35S:ROX1:HA* **(B)**, 35S:ROX2-TAP **(C)** seedlings or Arabidopsis cell cultures expressing ROX2-TAP **(D)** and incubated in the presence or absence of MG132. Samples were harvested at the indicated times. Coomassie-stained RuBisCo were used as loading control. Proteins were detected using anti-V5 (1/2000), anti-HA (1/1000), or PAP (1/1000) antibodies with their respective HRP-conjugated secondary antibody.

To evaluate whether ROX1 protein degradation is dependent on the E3-Ub ligases RGLG3/4, we used two different approaches. First, we used agroinfiltration to transiently express ROX1 with or without RGLG3 or RGLG4 in *N. benthamiana* leaves. We determined ROX1 protein accumulation in the infiltrated area and found that ROX1 protein levels decreased when RGLG3 or RGLG4 were co-expressed (Figure 4A).

Second, we used the recombinant protein ROX1:V5-His in a cell free degradation assay using total protein extract prepared from seedlings from wild type or double *rglg3/rglg4* knock-out lines that we generated. As expected, in the loss-of-function line *rglg3/rglg4* we observed higher levels of the recombinant protein (Figure 4B). No difference in protein levels were observed when single mutants *rglg3* or *rglg4* were used, thus suggesting that both E3-Ub ligases act redundantly in ROX1 protein degradation.

The same experiments were performed with ROX2. In this case, ROX2 was not degraded when RGLG3 and RGLG4 were co-infiltrated and no difference in ROX2-V5-HIS protein levels were observed in cell free degradation assays with protein extracts prepared from wild type or double *rglg3/rglg4* mutants (Figure 4C).

These results demonstrate that ROX1 is a direct target of RGLG3/4 and that ROX1 is targeted by these E3-Ub ligases for protein degradation by the proteasome. However, ROX2 is a direct interactor of RGLG3/4 but is not a direct target for ubiquitination and degradation.

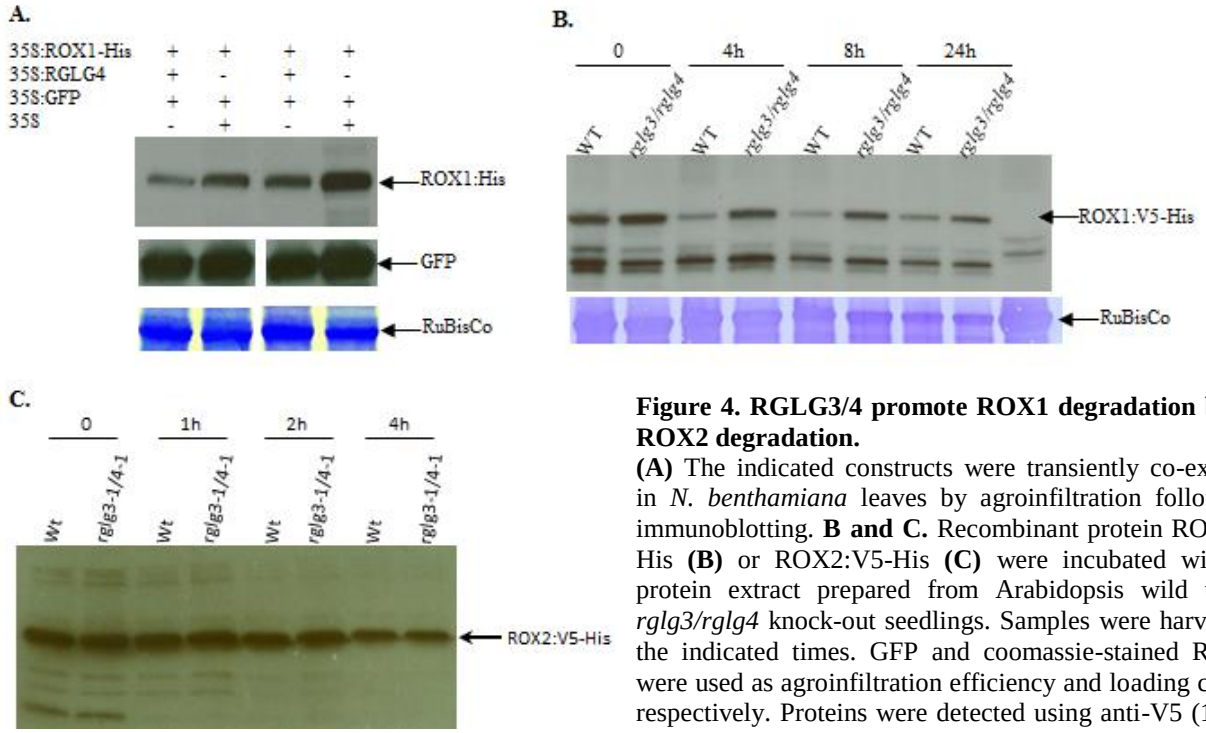

Figure 4. RGLG3/4 promote ROX1 degradation but not ROX2 degradation.
(A) The indicated constructs were transiently co-expressed in *N. benthamiana* leaves by agroinfiltration followed by immunoblotting. **B and C.** Recombinant protein ROX1:V5-His **(B)** or ROX2:V5-His **(C)** were incubated with total protein extract prepared from Arabidopsis wild type or *rglg3/rglg4* knock-out seedlings. Samples were harvested at the indicated times. GFP and coomassie-stained RuBisCo were used as agroinfiltration efficiency and loading controls, respectively. Proteins were detected using anti-V5 (1/2000), anti His (1/2000) or anti-GFP (1/1000) antibodies with their respective HRP-conjugated secondary antibody.

ROX1 is poly-ubiquitinated in vitro and ROX2 is a regulator of RGLG3/4

Zhang *et al.*showed that RGLG3 and RGLG4 have E3 ligase activity using an *in vitro* auto-ubiquitination assay with the human Ubc5b E2 conjugating enzyme (UBC) (Zhang et al., 2012). In our lab, we analysed which Arabidopsis UBC was able to interact with RGLG3 and RGLG4 by testing their physical interaction with the Arabidopsis UBC collection by Y2H and found the Ubc5b homolog AtUBC30 as one of the hits. We expressed and purified the recombinant proteins RGLG3, RGLG4 and AtUBC30 and confirmed the *in vitro* auto-ubiquitination of RGLG3/4 using AtUBC30. Using mutant RGLG3/4 versions with point mutations in the RING-domain (mRING) demonstrated that the E3 ligase activity was dependent on the integrity of the RING-domain (Figure 5A).

Then, we carried out the *in vitro* ubiquitination assay adding the recombinant protein ROX1 and in the presence of RGLG3 an extra band was detected (Figure 5A). We could efficiently detect higher molecular weight bands using anti-V5 antibody, corresponding to ubiquitinated forms of ROX1 (Figure 5B).

Both samples (Figure 5B) were analysed by Q-Exactive mass spectrometry in LC-MS/MS and 12 ubiquitination sites of ROX1 were identified in the sample treated with RGLG3. Furthermore, no

ubiquitination was found in the sample with the mutant RGLG3mRING. These results demonstrate that ROX1 is poly-ubiquitinated by RGLG3.

We also carried out in vitro ubiquitination assays using the recombinant protein ROX2:V5-His, but as expected ROX2 ubiquitination was not observed using RGLG3 and Arabidopsis E2s AtUBC30, AtUBC35, AtUBC36 or human Ubc5b (data not shown). These results confirm that ROX2 is not a target of RGLG3/4 and suggest a role as a regulator of the E3-ub ligases RGLG3/4.

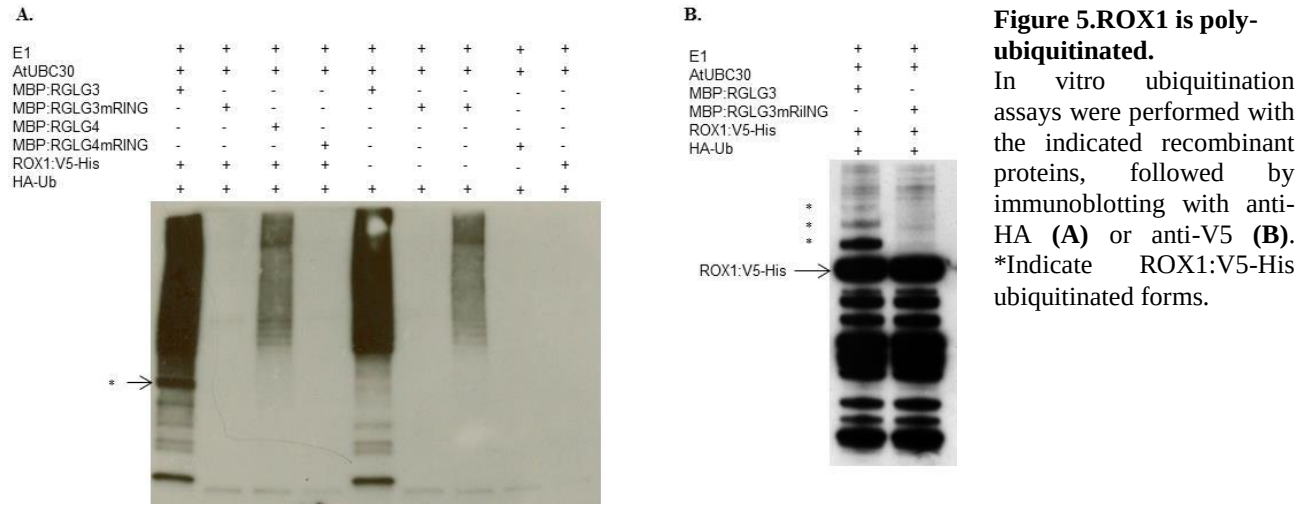

Figure 5.ROX1 is poly-ubiquitinated.
In vitro ubiquitination assays were performed with the indicated recombinant proteins, followed by immunoblotting with anti-HA **(A)** or anti-V5 **(B)**. *Indicate ROX1:V5-His ubiquitinated forms.

ROX1 and ROX2-interacting proteins

To study the function of ROX1 and ROX2 in planta, we performed TAP screenings using ROX1 or ROX2 as bait. Between ROX1-interacting proteins, multiple components of the cytoplasmic iron-sulphur assembly and related pathways were identified (data not shown). Moreover, the F-box protein COI1, the JA receptor, was also identified as a putative interactor. This protein-interaction was confirmed using an *in vitro* pull down assay and BiFC by a group of collaborators.

Several ROX2-interacting proteins were identified (Table 1), including TGA1, nitrate reductase (NIA2), xanthine dehydrogenase 1 (XDH1), cofactor for nitrate reductase and xanthine dehydrogenase 1 (CNX1) and several members of the MAP KINASE family, including MAPK4 and MAPK6. We could confirm ROX2 interactions by Y2H only with TGA1 and XDH1 (Figure 6) but in the near future we will test the other interactions by BiFC.

We also found some common interactors with ROX1 which prompted us to think that there could be a link between ROX1 and ROX2. The interaction between ROX1 and ROX2 was also confirmed by Y2H and BiFC (Figure 7).

TGA1 is a clade I TGA transcription factor that has been shown to regulate basal defence in Arabidopsis (Shearer et al., 2012). It is proposed that in contrast to class II TGAs, TGA1 and TGA4 do not bind to the central regulator of SA signalling NPR1 in unchallenged conditions due to the presence of oxidized cysteines hampering interaction. However, when challenged, TGA1 is reduced by S-nitrosylation, and activated (Lindermayr et al., 2010). The ROX2 interactor NIA2 is known to be involved in SA-mediated NO production (Hao et al., 2010) and nitrite is the major source of NO production in Arabidopsis (Modolo et al., 2005). The interactor MPK6 is a known positive regulator of NIA2 and NO signalling (Wang et al., 2010) whereas MPK4 and MPK6 have been implicated in JA and SA signalling (Brodersen et al., 2006; Takahashi et al., 2007). Therefore, ROX2 could be a modulator of different pathways. During the current Postdoc BELSPO fellowship-2014 we will continue with the characterization of ROX2 and we aim to determine whether ROX2 is the molecular link between these three hormones (JA, SA and NO), and how it may enable plant hormone crosstalk in the decision between growth and survival.

Summary of ROX2 targets identified by TAP	Locus	C-GSrh		N-GSrh		Total
		1h JA	1h mock	1h JA	1h mock	
Protein of unknown function (duplicated DUF1399)	AT2G22660	2	2	2	2	**8**
Molybdopterin biosynthesis CNX1 protein	AT5G20990	2	2	2	1	**7**
NIA2, B29, NIA2-1, CHL3, NR, NR2, ATNR2 | nitrate reductase 2	AT1G37130	1				**1**
SKP1, ASK1, ATSKP1, SKP1A, UIP1 | S phase kinase-associated protein 1	AT1G75950		1			**1**
ATMPK4, MPK4 | MAP kinase 4	AT4G01370			2	2	**4**
ATMPK6, MPK6, MAPK6, ATMAPK6 | MAP kinase 6	AT2G43790			1	2	**3**
ATMPK2, MPK2 | mitogen-activated protein kinase homolog 2	AT1G59580			1	1	**2**
ATMPK7, MPK7 | MAP kinase 7	AT2G18170			1	1	**2**
ATMPK11, MPK11 | MAP kinase 11	AT1G01560		1			**1**
Fe-S biogenesis (DRE2). Member of Arabidopsis CIA complex	AT5G18400	2	2	2	2	**8**
SDH1-2 | succinate dehydrogenase 1-2	AT2G18450			2	1	**3**
SDH2-1 | succinate dehydrogenase 2-1	AT3G27380			1	2	**3**
Glutaredoxin family protein (GLUTAREDOXIN C2, GRXC2)	AT5G40370	2	1			**3**
2-Cys Prx B, 2CPB | 2-cysteine peroxiredoxin B	AT5G06290	2				**2**
ATXDH1, XDH1 | xanthine dehydrogenase 1	AT4G34890			1		**1**
NBP35, ATNBP35 | nucleotide binding protein 35	AT5G50960			1		**1**
ROL5 | repressor of lrx1 (CTU1)	AT2G44270				1	**1**
CXIP1, ATGRXCP | CAX interacting protein 1 (GrxS14)	AT3G54900			1		**1**
ATX1, ATATX1 | homolog of anti-oxidant 1	AT1G66240		2			**1**

Table 1.Putative ROX2-interacting proteins.
Summary of ROX2-interacting proteins obtained by TAP screenings using Arabidopsis cell cultures expressing 35S:ROX2-TAP and 35S:TAP-ROX2. Cell cultures were treated with 50 µM jasmonate (JA) or 50 µl ethanol (mock) for 1 hour before harvesting.

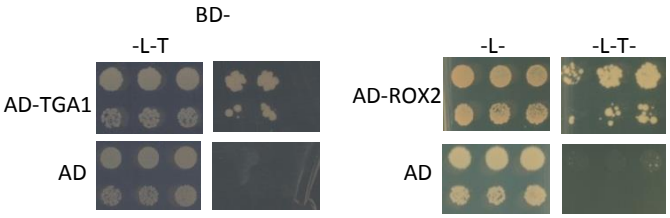

Figure 6. ROX2 interacts with TGA1 and XDH1
Yeast two-hybrid assay between ROX2 and TGA1 or XDH1. Transformed yeast were spotted in 10 fold and 100-fold dilutions on control medium (-L-T) and selective medium (-L-T-H).

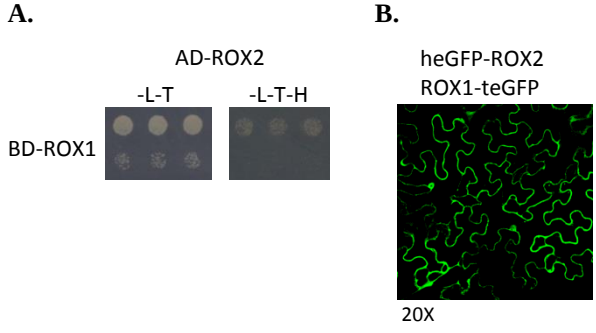

Figure 7. ROX2 interacts with ROX1.
(A) Yeast two-hybrid assay between ROX1 and ROX2. Transformed yeast were spotted in 10 fold and 100-fold dilutions on control medium (-L-T) and selective medium (-L-T-H). **(B)** ROX1 interaction with ROX2 by bimolecular fluorescence complementation. Head (he) or tail (te) GFP fusion proteins were transiently co-expressed in *N. benthamiana* leaves and analysed by confocal microscopy 3 days after agroinfiltration.

Phenotypic characterization of loss-of-function and gain-of function Arabidopsis lines

In the laboratory we obtained loss-of function *rglg3*, *rglg4*, and *rox1* T-DNA insertion lines and after crossing we got different double and triple mutants. Root growth and anthocyanin accumulation have been analysed in *rox1*, *rglg3*, *rglg4* simple and double mutants by growing the seeds in medium lacking or supplemented with JA. In contrast with a previous report by (Zhang et al., 2012), we found only a minor effect of RGLG3 and RGLG4 in JA-signalling regulation. ROX1 may also play a minor role in JA-signalling, as we found that ROX1 positively regulates JA-mediated anthocyanin accumulation but negatively regulates JA-mediated root growth inhibition. However, JA-marker genes showed no altered response in *rox1* mutant.

We obtained T-DNA mutant lines for *ROX2*, *rox2-1* and *rox2-2*. Both T-DNA insertion lines are located in introns and showed different levels of *ROX2* expression (Figure 8A), *rox2-1* is a knock-down line and *rox2-2* is a knock-out line. We also generated microRNA artificial transgenic lines to knock down *ROX2* gene (Figure 8B). On the other hand, we obtained the overexpression *35S:ROX2* transgenic line (Figure 8C) from (Wang et al., 2011) and generated the different transgenic lines overexpressing *TAP-ROX2* or *ROX2-TAP* fusions (Figure 8D).

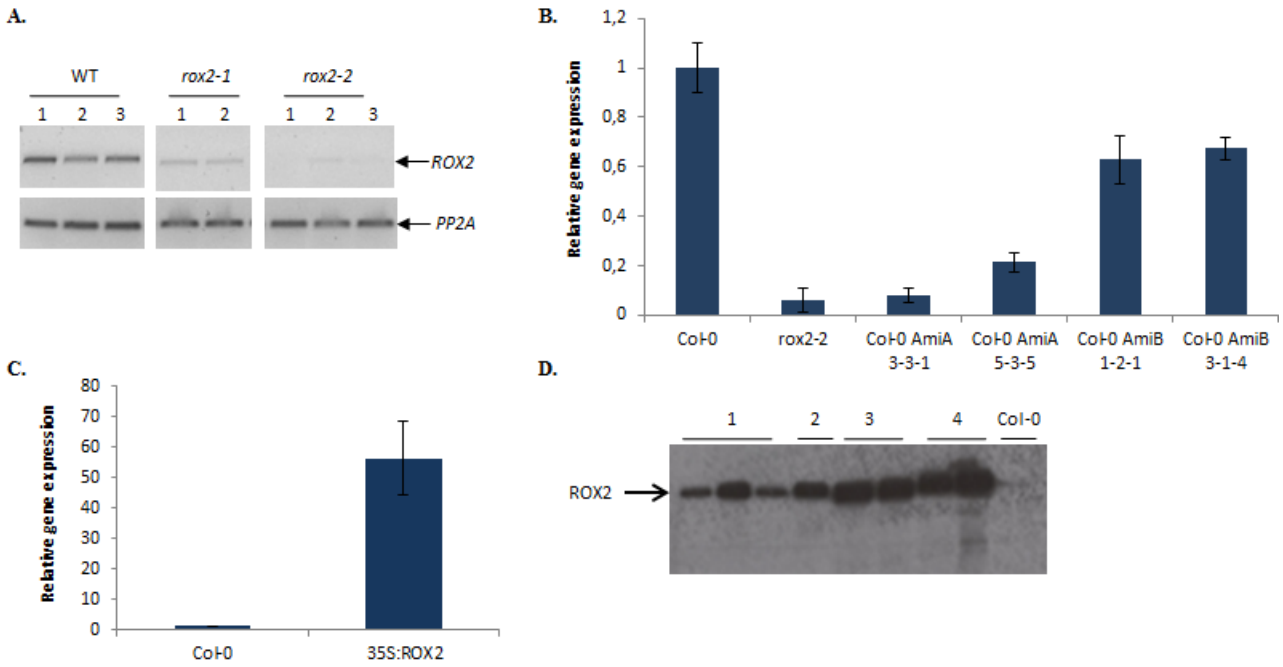

Figure 8. Loss-of-function and gain-of function *ROX2* Arabidopsis lines.
(A-C) RT-PCR analysis of mutants and overexpression lines. WT, *rox2*,*35S:ROX2* and Col-0 transgenic lines expressing AmiRNAs seedlings were grown for 7-days on MS medium under continuous light **(A and C)** or 10 days under long day conditions **(B)**. **(A)** *ROX2* transcript levels were analysed by RT-PCR. *PP2A* was used as an internal control. (**B and C)** qPCR, *ROX2* expression is shown relative to WT, *PP2A* and *UBC10* expression were used as internal controls. Error bars: SE plotted, n=3. D. ROX2 protein levels in transgenic lines expressing ROX2-TAP fusion. (1) Col-0 *35S:ROX2-TAP*, (2) Col-0 *35S:TAP-ROX2*, (3) *rglg3-1 rglg4-1 35S:ROX2-TAP*, (4) *rglg3-1 rglg4-1 35S:TAP-ROX2*. Total proteins were extracted from 10-day-old transgenic lines seedlings and analysed by western-blot. Proteins were detected using PAP (1/1000) antibody.

We used these lines to measure the JA and SA-growth root inhibition and we found only a slight difference in *rox2-2* sensitivity to SA (Figure 9A and B). No difference was found between the *rox2-2* and *ROX2* overexpression lines in the expression of JA- and SA-responsive genes (Figure 9C).

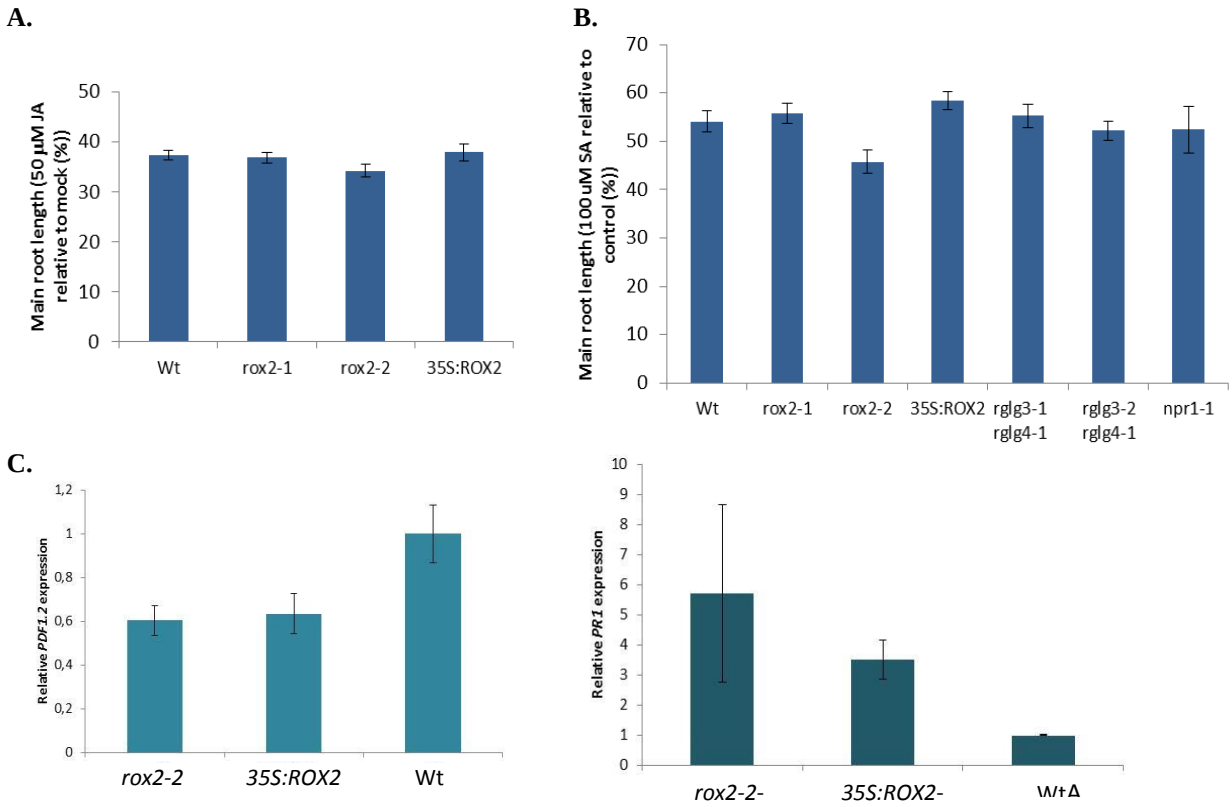

Figure 9. *rox2* mutant and overexpression line-response to JA and SA.

(A and B) Primary root growth of *ROX2* mutants and overexpression lines and *rglg3 rglg4* double mutant. Seedlings were germinated on MS medium supplemented with 50 µM JA or 100 µM SA or ethanol as a control. Primary root length was determined after 11 days. Error bars: SE, n=40 **(A)** or n=25 **(B)**. *PDF1.2* **(C)** and *PR1* **(D)** expression relative to Wt. *PP2A* and *UBC10* expression were used as internal controls. Error bars: SE plotted, n=3. WT, *rox2-2* and *35S:ROX2* seedlings were grown for 7-days on MS medium under continuous light.

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4. Perspectives for future collaboration between units (1 page)

The expertise gained in the Laboratory of Prof. Dr Alain Goossens at the Department of Plant Systems Biology (PSB), UGent lead by Prof. Dr. Dirk Inzé during this BELSPO 2012 post-doctoral fellowship will be beneficial to the home institute and there will be prospects for future collaborations with the laboratory of Prof. Dr. Pablo Cerdán at Leloir Institute Foundation, Buenos Aires, Argentina. During this project, we studied the mode of action of E3-ubiquitin ligases and the regulation of their targets and gained new skills through technologies and new experimental approaches. After finishing my current BELSPO 2014-post-doctoral fellowship, I will return to Leloir Institute Foundation, where I was active as a PhD student and post-doctoral researcher, and where I will be able to apply the gained knowledge and experience with new experimental approaches in the research projects that are being carried out in the home Institute.

During the new BELSPO-2014 fellowship at PSB-UGent, I will continue with the characterization of ROX2, the Arabidopsis truncated haemoglobin with the aim to determine its role in the regulation of hormone signalling pathways. I will continue with the phenotypic characterization of the loss-of-function and gain-of-function Arabidopsis lines that we generated during the BELSPO-2012 fellowship to try to determine how ROX2 is involved in the regulation of hormone signalling pathways, taking into account the interesting ROX2-interacting proteins that were obtained during this project. We will also determine how ROX2 might be regulating the E3-ubiquitin ligases because we already know that ROX2 is not a direct target of RGLG3 and RGLG4 for proteasome degradation.

The experience gained by the study of plant response to biotic and abiotic stresses and to essential nutrients, together with the understanding of hormonal crosstalk could be definitely applied in the current running research lines in the home institute where they are studying how signalling is integrated at the transcriptional level. In that context the Arabidopsis Mediator complex is the subject of study and it is known that several subunits of this Mediator complex are involved in defence and SA and JA signalling pathways. Hence this and the future postdoctoral training set the foundation for future collaborations between the two research units.

5. Valorisation/Diffusion (including Publications, Conferences, Seminars, Missions abroad...

Participation in International Conferences:

1) Characterization of the role of the Arabidopsis truncated haemoglobin
Sabrina Iñigo, Astrid Nagels Durand, Andrés Ritter, Laurens Pauwels and Alain Goossens
Poster presentation on the 26th International Conference on Arabidopsis Research, Paris, France, 05-09/07/2015

2) Characterization of E3 ubiquitin ligases involved in jasmonate signalling
Sabrina Iñigo, Astrid Nagels Durand, Andres Ritter, Laurens Pauwels and Alain Goossens
Poster presentation on *the Plant Biology Europe FESPB/EPSO Congress 2014*, Dublin, Ireland, 22-26/06/2014

6. Skills/Added value transferred to home institution abroad (1/2 page)

The experience and expertise gained during both BELSPO post-doctoral fellowships in the laboratory of Prof. Dr. Alain Goossens at the Department of Plant Systems Biology, UGent, will definitely have an impact in my scientific career. This postdoctoral training will increase the chances of getting a permanent position in the Argentinean Council for Science and Technology (CONICET) which will allow me to start an independent career in the near future in any Research Institute or University in Argentina.

Hence, the expertise obtained during the post-doctoral training will be essential for my future research lines, where the integration of environmental signals at the transcriptional and posttranscriptional level through the Mediator complex and their importance in growth, development and defence are one of the main interests.