UNIVERSITEIT GENT

FACULTY OF SCIENCES

Hormone responses and RNA editing in plants

Jasper DUGARDEYN

Promoter: Prof. Dr. Dominique Van Der Straeten

Thesis submitted in partial fulfilment of the requirements
for the academic degree of
Doctor in Biology

Academic year 2012-2013

Department of Physiology
Laboratory of Functional Plant Biology

FPB FUNCTIONAL PLANT BIOLOGY

Acknowledgment

Some peoples are able to spread the enthousiasm which drives them, making other peoples to get the best out of themselves. Therefore I would like to thank my promoter, professor Van Der Straeten. Your way of teaching, letting us relive the experiments the founders of this branch of sciences performed, inspired me a lot. Since that course I knew I wanted to specialise in plant physiology.

Allready during my master thesis, I received a lot of help, tricks, good advice and further stimulation to get the best out of myself. These experiences made the choice whether to start as a PhD student in the HSB lab very easy.

Also during my time in the lab you continued to help me whenever the research was not going as we would have liked or when other peoples working on SLO left the lab. Not only were you my promoter, but during the years in the lab you became a true friend. I would especially like to thank you for your understanding and support when given the chance for a new job at the province of West-Flanders. During that time we never lost contact and you kept on persuading me to finish the PhD. As I am writing these words as one of the last steps to finish the thesis, I am glad I get the opportunity to think about these last years. Thanks for everything.

Of course I wouldn't have been able to complete this research without the help of the peoples who (have) work(ed) in the HSB lab. I would especially like to thank Zhu Qiang who continued the SLO research in a very profound way. It is a very nice feeling to see that "my project" is taking care in such determined and accurate way. I would also like to thank the colleagues who I worked with. The (in)formal talks were both useful for the research, but also helped a lot to keep positive and go to work in a good atmosphere.

Thirdly I musn't forget the researchers with who we collaborated to get some vital pieces of the SLO puzzle together. Espescially I would like to thank Jurgen Denecke who invited me to his lab to teach me the cloning and transformation in order to determine the subcellular localization of SLO2, which afterwards appeared to give a totally new insight in the function of this protein.

I would also like to thank the members of the reading and examination committee for their constructive suggestions and discussion to improve the quality of this PhD thesis.

Last but not least I would like to thank my friends and family. Without your help and support I would have given up already and this thesis would never have been. I would especially like to thank my wife and my parents. Unfortunately we already had to say goodbye. Therefore I would like to dedicate this work to Hilde and everyone else who has left us much to soon.

Jasper Dugardeyn

07 may 2013

List of abbreviations

ABA	abscissic acid
ABO	ABA overly-sensitive
ACC	1-amino-cyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
ABA	abscissic acid
ABF3	ABRE binding factor
ABRE	abscissic acid response element
ADP	adenosine diphosphate
AHG	ABA hypersensitive germination
AOX	alternative oxidase
AS	anthranilate synthase
ASA	anthranilate synthase □
ASB	anthranilate synthase □
ATCIB	B22 subunit of *Arabidopsis* complex I
ATP	adenosine triphosphate
AUX	auxin permease
AVG	aminoethoxyvinylglycine
AXR	auxin resistant
BA	6-benzylaminopurine
BLAST	basic local alignment search tool
BR	brassinosteroids
BRZ	brassinazole
CAF	CCR4 associated factor
CBB	cabbage
CBF	C-repeat-binding factor
CDP	ent-copalyl diphosphate
CHS	chalcone synthase
CIN	cytokinin insensitive
CLB	chloroplast biogenesis
CMS	cytoplasmic male sterile
COP	constitutive photomorphogenetic
COX	cytochrome oxidase
CPD	constitutive photomorphogenic dwarf
CPS	ent-copalyl diphosphate synthase
CRP	chloroplast RNA processing
CRR	chlororespiratory reduction
CRS	chloroplast splicing factor
CSS	changed sensitivity to cellulose synthesis inhibitors
CTR	constitutive triple response
DAB	diaminobenzidine
DET	de-etiolated
DG	delayed greening
DLE	degree of leaf emergence
DEX	dexamethasone
DREB	dehydration responsive element-binding factor
EBF	EIN3 binding F-box protein
EBR	epi-brassinolide

EFE	ethylene forming enzyme
EIL	ein3 like
EIN	ethylene insensitive
EIR	ethylene insensitive root
ER	endoplasmatic reticulum
EREBP	ethylene response element binding protein
ERF	ethylene response factor
ERS	ethylene response sensor
(m) ETC	(mitochondrial) electron transport chain
ETO	ethylene overproducer
ETR	ethylene resistant
FRO	frostbite
FRY	fiery
GA	gibberellin
GAI	gibberellic acid insensitive
GA2OX	GA_2 oxidase
GA3OX	GA_3 oxidase
GA20OX	GA_{20} oxidase
GFP	green fluorescent protein
GGP	geranylgeranyl diphosphate
GID	gibberellin insensitive dwarf
GL	glabra
GUN	genomes uncoupled
GUS	β-glucuronidase
HCF	high chlorophyll fluorescence
H_2DCF-DA	2,7-dichlorofluorescein diacetate
HLS	hookless
HRGP	hydroxyproline rich glycoproteins
HXK	hexokinase
IAA	indole-3-acetic acid
IP	immunoprecipitation
JA	jasmonic acid
JIN	jasmonate insensitive
KAO	ent-kaurenoic acid oxidase
KO	ent-kaurene oxidase
KS	kaurene synthase
LEH	length of the first epidermal cell with a visible root hair bulge
LNM	low nutrient medium
LOI	lovastatin insensitive
LPA	low *psiII* accumulation
1-MCP	1-methylcyclopropene
MeJA	methyl jasmonate
MEF	mitochondrial editing factor
mttB	membrane targeting and translocation
MV	methyl viologen
NAD^+	Nicotinamide adenine dinucleotide
NAD	NADH dehydrogenase
NBT	nitroblue tetrazolium
NCED	neoxanthine cis-epoxy-dioxygenase
NDA	alternative NAD(P)H dehydrogenase A
NDB	alternative NAD(P)H dehydrogenase B
NDC	alternative NAD(P)H dehydrogenase C
NDUFS	NADH dehydrogenase fragment S subunit

NF	norflurazon
NMAT	nuclear maturase
NPA	1-naphthylphthalamic acid
OPR	opague and growth reduction
OTP	organelle Transcript Processing
PAC	paclobutrazol
PC	plastocyanin
PÈRE(BP)	primary ethylene response element (binding protein)
PGN	pentatricopeptide repeat protein for germination on NaCl
PGR3	proton gradient regulation
PIN	pin formed
PMF	peptide mass fingerprinting
PNM	protein localized to the nucleus and mitochondria
PPR	pentatricopeptiderepeat
PPRL	PPR protein like
PTAC	plastid transcriptionally active chromosome
QC	quiescent center
RBCS	ribulose-1,5-bisphosphate carboxylase small subunit
REME	required for efficiency of mitochondrial editing
RF	restorer of fertility
RGA	repressor of ga1-3
RGL	RGA like
RHD	root hair defective
ROS	reactive oxygen species
RTY	rooty
SA	salicylic acid
SAM	S-adenosyl-methionine
SAX	hypersensitive to abscisic acid and auxin
SCF	skp1-cullin-F box
SERE(BP)	secondary ethylene response element (binding protein)
SLG	slow growth
SLO	slow growth
SLY	sleepy1
TAG	triacylglycerides
TIR	transport inhibitor response
Trp	tryptophan
TTG	transparent testa glabra
UQ	ubiquinone
WEI	weak ethylene insensitive
WT	wild type

Thesis outline

Chapter 1: General overview of the work

1.1 SLO2 is a PPR protein

SLO2 is a PPR protein. In *Arabidopsis*, this group of proteins is composed of about 450 members which contain repeats of a 35-amino-acid sequence (pentatricopeptide), leading to their name, and are typical plant specific (Lurin *et al.*, 2004; Andrés *et al.*, 2007; Schmitz-Linnewebber and Small, 2008). They were first described by Small and Peeters (2000). Next to this theme, the proteins may also contain variants of the PPR motif, called S-sequence (Short, 31 aa), or L-sequence (Long, 35 or 36 aa). Proteins which only carry the PPR motif are member of the P group. The others are part of the PLS group and may have a E, E+ or DYW sequences at their C-terminus. Based on the structure of these proteins and the variation in the sequences, they are predicted to interact with RNA (Lurin *et al.*, 2004). This was experimentally confirmed, showing that PPR proteins play a role in RNA stability, cleavage, splicing and editing (Andrés *et al.*, 2007; Schmitz-Linneweber and Small, 2008). RNA editing most often consists of a conversion of a cystidine in to a uridine nucleotide, a process occurring in plastids and mitochondria of land plants (Chateigner-Boutin and Small, 2010). Recently, the first mitochondrial RNA editing factors were identified (Zehrmann *et al.*, 2009). However, the majority of the characterised mutants in RNA editing factors do not show a visible phenotype (Takenaka *et al.*, 2010). An non-exhaustive overview of characterized PPR proteins from mitochondria, nucleus and chloroplasts can be found in table1, table2 and table3 respectively.

1.2 Isolation of SLO2

The central focus of this research is the characterization of the *SLO2 (SLOW GROWTH 2)* gene from *Arabidopsis thaliana*. The gene was isolated based on delayed leaf emergence of the *slo2* T-DNA insertion line on low nutrient medium (LNM) supplied with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid). Previously it was shown that ACC enhances the leaf emergence on this medium (Smalle and Van Der Straeten, 1997). The selected mutant showed a delayed emergence of leaves, suggesting a defect in ethylene signaling (Zhu *et al.*, 2012).

Characterized mitochondrial PPR proteins					
Name	**Locus**	**Subclass**	**Phenotype**	**Function**	**Reference**
ABO5	At1g51965	P	hypersensitive to ABA	splicing of *nad2*	Liu *et al.*, 2010
AHG11	At2g44880	E+	weak ABA hypersensitive	editing of *nad4*	Murayama *et al.*, 2012
MEF1	At5g52630	DYW	normal	editing of *rps4, nad7* and *nad2*	Zehrman *et al.*, 2009
MEF7	At5g09950	DYW	normal	editing of *cob, ccb206,nad2, nad4L*	Zehrman *et al.*, 2012
MEF 8	At2g25580	DYW	normal	editing of *nad5*	Takenaka *et al.*, 2010
MEF9	At1g62260	E	normal	editing of *nad7*	Takenaka, 2010
MEF11/ LOI1	At4g14850	E	enhanced tolerance to lovastatin	editing of *cox3, nad4* and *ccb203*	Tang *et al.*, 2010 / Verbitskiy *et al.*, 2010
MEF14	At3g26780	DYW	normal	editing of *matR*	Verbitskiy *et al.*, 2011
MEF 19	At3g05240	E	normal	editing of *ccb206*	Takenaka *et al.*, 2010
MEF 20	At3g18970	E	normal	editing of *rps4*	Takenaka *et al.*, 2010
MEF 21	At2g20540	E+	normal	editing of *cox3*	Takenaka *et al.*, 2010
MEF 22	At3g12770	DYW	normal	editing of *nad3*	Takenaka *et al.*, 2010
OTP43	At1g74900	P	dev. Delay / embryo lethal	splicing of *nad1* and *nad2*	De Longevialle *et al.*, 2007
OTP87*	At1g74600	E	dev. delay, small plants	editing of *nad7* and *atp1*	Hammani *et al.*, 2011a
PGN	At1g56570	E+	hypersensitive to B. *cinerea*, ABA, glucose, salinity	?	Laluk *et al.*, 2011
PNM1	At5g60960	P	embryo lethal	influence translation	Hammani *et al.*, 2011b
PPR40	At3g16890	P	semidwarf, hypersensitive to ABA, salt, ox. Stress	regulator of *cytC* reductase activity of complex III	Zsgimond *et al.*, 2008
PPR336	At1g61870	?	normal	associated with polysomes	Uytewaal *et al.*, 2008
PPR596	At1g80270	P	dev. delay, curled leaves	editing of *rps3*	Doniwa *et al.*, 2010
REME1	At2g03880	DYW	normal	editing of *orfX, nad2, matR, rpi5*	Bentolila *et al.*, 2010
SLG1	At5g08490	E+	slow growth, dev. Delay	editing of *nad3*	Yuan and Liu, 2012
SLO1	At2g22410	E	slow growth, dev. delay	editing of *nad4* and *nad9*	Sung *et al.*, 2010
SLO2	At2g13600	E+	slow growth, dev. Delay, ABA hypersensitive…	editing of *mttB, nad7, nad1*	Zhu *et al.*, 2012
RF1a	Petunia	P	no restoration of male sterility	RNA cleavage	Wang *et al.*, 2006
RF1b	Petunia	P	no restoration of male sterility	RNA degradation	Wang *et al.*, 2006
OGR1	Rice	DYW	Slow growth, dev. delay, dwarf, sterility	editing of *nad4, nad2, ccmC, cox2* and *cox3*	Kim *et al.*, 2009

Table 1: non-exhaustive list of charachterized mitochondrial PPR proteins (light grey = also chloroplasmatic, dark grey = also nuclear)

Characterized nuclear PPR proteins					
Name	**Locus**	**Subclass**	**Phenotype**	**Function**	**Reference**
GRP23	At1g10270	P	embryolethal	interaction with RNA polymerase II	Ding *et al.*, 2006
PNM1	At5g60960	P	embryo lethal	influence translation	Hammani *et al.*, 2011b

Table 2: non-exhaustive list of charachterized mitochondrial PPR proteins (grey = also mitochondrial)

Characterized chloroplasmatic PPR proteins					
Name	Locus	Subclass	Phenotype	Function	Reference
CAF1	At2g20020	?	pale green, ivory	splicing of *petD, rpl16, rps16, trnG-UCC, ndhB, ndhA, ycf3-1, clpP-1, rpoC1*	Asakura and Barkan, 2006
CAF2	At1g23400	?	embryo lethal, pale green, small	splicing of *ndhB, petB, rps12-1, ndhA, ycf3-1*	Asakura and Barkan, 2007
CLB19	At1g05750	E+	yellow leaves, seedling lethal	editing of *rpoA* and *clpP*	Chateigner-Boutin *et al.*, 2008
CRP1	Maize	P	pale green, seedling lethal	translation of *petA* and *psaC*	Schmitz-Linneweber *et al.*, 2005
CRR2	At3g46790	DYW	aberrant chlorophyl fluoresence	RNA cleavage of *ndhB*	Hashimoto *et al.*, 2003
CRR4	At2g45350	E+	aberrant chlorophyl fluoresence	editing of *ndhD*	Kotera *et al.*, 2005
CRS1	At5g16180	DYW	pale green, ivory, small	splicing of *atpF* intron	Asakura and Barkan, 2007
DG1	At5g67570	P	delayed greening of leaves	regulating plastid gene expression	Chi *et al.*, 2008
GUN1	At2g31400	P	lower anthocyanin content, ABA hypersensitive	editing of *accD* and *prs14* (in interaction with PPI)	Kakizaki *et al.*, 2012
HCF152	At3g09650	P	aberrant chlorophyl fluoresence	splicing of *petB*	Meierhoff *et al.*, 2003
LPA66	At5g48910	DYW	aberrant chlorophyl fluoresence	editing of *psbF*	Cai *et al.*, 2009
PDM1	At4g18520	PLS	albino leaves, seedling lethal	processing of *rpoA*	QianQian *et al.*,
PGR3	At4g31850	P	aberrant chlorophyl fluoresence	stabilization and translation of *petL* operon	Yamazaki *et al.*, 2004
PPR2	Maize	P	albino leaves, seedling lethal	ribosome acumulation	Williams and Barkan, 2003
PPR4	Maize	P	albino leaves, seedling lethal	splicing of *rps12*	Schmitz-Linneweber *et al.*, 2006
PPR5	Maize	P	albino leaves, seedling lethal	RNA stability of *trnG*	Beick *et al.*, 2008
PTAC2	At1g74850	P	albino leaves, seedling lethal	transcription of *psbA* and (see below),	Pfalz *et al.*, 2006
PTAC6	At1g21600	?	albino leaves, seedling lethal	RNA processing of *atpB, clpP, ndhB, ndhF, psaAB, rbcL, rps14* and *ycf3*	Pfalz *et al.*, 2006
PTAC12	At2g34640	?	albino leaves, seedling lethal	RNA processing of *atpB, clpP, ndhB, ndhF, psaAB, rbcL, rps14* and *ycf3*	Pfalz *et al.*, 2006
OsPPR1	Rice	E	albino leaves, seedling lethal	plastid biogenesis	Gothandam *et al.*, 2005
OTP51	At2g15820	P	aberrant chlorophyl fluoresence	splicing of *ycf3*	de Longevialle *et al.*, 2008
RARE1	At5g13270	DYW	normal phenotype	edting of *accD*	Robbins *et al.*, 2009

Table 3: non-exhaustive list of charachterized chloroplasmatic PPR proteins

1.3 The role of ethylene in plant growth

As slo2 was isolated as an ethylene mutant, we first wanted to confirm its altered sensitivity to ethylene. In *slo2* mutants, a defect in ethylene response was confirmed by investigating the triple response of the mutant. We showed that *slo2-1* mutants show a relatively smaller reduction of hypocotyl growth in the presence of ACC (Zhu *et al.*, 2013). The mutant roots also show an inhibition of growth as ACC is supplied, but this reduction of growth is less pronounced than the wild type (WT) response. As there is no difference in the exaggeration of the apical hook when ACC is added to the medium and the mutants show similar responses when ethylene is applied, it can be posted that the mutant is partially ethylene insensitive (Zhu *et al.*, 2013).

As ethylene is a hormone that has an effect in a myriad of plant processes, we first focused the research on ethylene (chapter 3). Therefore, based on an extensive literature search, we published a review of both ethylene biosynthesis and ethylene signaling. We also covered the role of ethylene in root growth, root hair formation, hypocotyl and stem growth and its role in the formation and exaggeration of the apical hook (Dugardeyn and Van Der Straeten, 2008).

The executed literature search showed an ambiguous role of ethylene in plant tissues, both stimulating and inhibiting growth. Pierik *et al.* (2006) presented a biphasic model to explain these differential responses to ethylene. They state that a dose-dependent response to ethylene differs between different plant(tissues) either leading to inhibition or stimulation of growth at certain ethylene levels. This differential response to different levels of ethylene has already been demonstrated at the gene level, by De Paepe *et al.* (2004a,b). These authors isolated a group of 30 genes which were more strongly upregulated by high ethylene levels than by low levels, but also detected a group of 7 genes which showed the opposite response. These findings further supported the idea that a change in ethylene signaling or synthesis may cause different effects in different tissues. A mutation in *SLO2* may provoke this change in ethylene signaling or synthesis. Given this possibility and the fact that the mutant was isolated based on a Degree of Leaf Emergence (DLE) screening on ACC we specifically focused our research on examining the role of SLO2 in ethylene signaling.

1.4 Interactions of ethylene with GA

Literature search clearly demonstrated a link between ethylene and GA signaling. Given the fact that GA plays an important role in growth processes (which clearly are disturbed in *slo2*) and the focus of the lab on these interactions, we first focused on further revealing this interaction mechanism. In hypocotyls, growth in the dark shows a clear crosstalk between ethylene and Gibberellic Acid (GA) by stabilizing the DELLA proteins, which are negative regulators of the GA response (Achard *et al.*, 2003 and Vriezen *et al.*, 2004). Besides this, the *ethylene overproducing 2-1* (*eto2-1*) and the *gibberellin-insensitive (gai*) mutant both show a shorter hypocotyl than WT when grown in light conditions and their double mutant shows an synergestic effect. This effect appears to be caused by an enhanced sensitivity of the double mutant to ethylene (De Grauwe *et al.*, 2008).

In roots ethylene has an inhibitory effect on growth rate, primarily caused by a reduction in cell length (Le *et al.*, 2001) but also by stimulating the number of cell divisions at the

quiescent centre of root tips (Ortega-Martinez *et al.*, 2007). Also in root tips, the regulation of cell elongation is at least partially caused by an interaction with GA since in WT seedlings, GA treatment can substantially overcome the ACC-induced inhibition of root growth (Achard *et al.*, 2003) by stabilizing the DELLA proteins.

As the *slo2* mutants show a reduced shortening of the root and the hypocotyls when treated with ACC and the literature search illustrated an obvious interaction between GA and ethylene to execute their effects, we wanted to examine the interaction between the GA and ethylene pathways into further detail. Therefore, we performed an in silico gene expression analysis of the root tips of *Arabidopsis* (chapter 4). Based on expression data of both ethylene and GA biosynthesis and signaling genes we suggested different working areas for both hormones.

1.5 Interaction of ethylene with other hormones

In our literature study, we also examined the interaction of ethylene with other hormones enticing different experiments to uncover the effect of a mutation in *SLO2* on the functionality of these hormones. GA interacts with ethylene to maintain the apical hook. In addition to GA, brassinosteroids and auxins are also important for this process (De Grauwe *et al.*, 2005).

Another hormone showing an obvious interaction with ethylene is abscissic acid (ABA). ABA is important during stress responses (Zhu, 2002) but is also important for normal plant growth. It plays a role in the closure of stomata (Schroeder *et al.*, 2001), germination (Finkelstein *et al.*, 2002) and root growth. Low concentrations of ABA stimulate root growth and high concentrations inhibit root growth (Ghassemian *et al.*, 2000; Beaudouin *et al.*, 2000). Also sugars regulate a vast amount of processes during plant growth (germination, embryogenesis, senescence, light response, pathogen responses (Sheen *et al.*, 1999; Xia *et al.*, 2000; Ohto *et al.*, 2001).

Together, ABA, sugar and ethylene form an intricate network (see Figure 1). Glucose enhances ABA concentrations and ABA biosynthesis mutants are glucose insensitive. The interaction of ABA and sugar is not only detected on a biosynthesis level, but also their signaling is correlated. Several sugar insensitive mutants are alleles of the *abi4* (ABA insensitive) ABA signaling mutant (Zhou *et al.*, 1998, Laby *et al.*, 2000, Arenas-Huertero *et al.*, 2000, Cheng *et al.*, 2002). Two sugar insensitive mutants *sis1* (sugar insensitive) and *gin4* (glucose insensitive) are alleles of *ctr1* (constitutive triple respons) (Gibson *et al.*, 2001and Cheng *et al.*, 2002) and the breakdown of EIN3 (ethylene insensitive) is stimulated by glucose (Yanagisawa *et al.*, 2003), linking the sugar and ethylene pathways. Ethylene interaction with ABA is illustrated by *etr1* (ethylene resistant) and *ein2*, both showing higher endogenous ABA levels (Beaudoin *et al.*, 2000). Ethylene also lowers the sensitivity for ABA, explaining the opposite effect of ABA (inhibiting) and ethylene (stimulating) on seed germination (Ghassemian *et al.*, 2000). The same authors suggest the same principle for root growth. To investigate these interactions, we examined the effect of sugar on growth of *slo2*-mutants which was highly stimulated in the presence of 1% of sucrose, but more strongly inhibited in the presence of 7% sucrose in mutants than in WT.

Since *slo2* was isolated as an ethylene mutant and given the above-mentioned interactions between ethylene and other hormones and sugar, our research was also focused on hormone interactions to discover the mechanism by which SLO2 is working.

Given the opposite effect of ethylene on sugar and ABA (and vice versa), we therefore examined the effect of a mutation in SLO2 on the germination rate and root growth in the absence and presence of ABA. The experiments showed an enhanced retardation of germination and root elongation of the mutants compared to WT in the presence of ABA, at least partially explained by a higher endogenous ABA level. We also wanted to check whether these physiological effects can be confirmed on a molecular gene level. Further evidence was gathered by a higher expression level of NCED3 (neoxanthine cis-epoxy-dioxygenase), shown to be crucial in the biosynthesis of ABA. This also resulted in higher expression level of different ABA signaling genes.

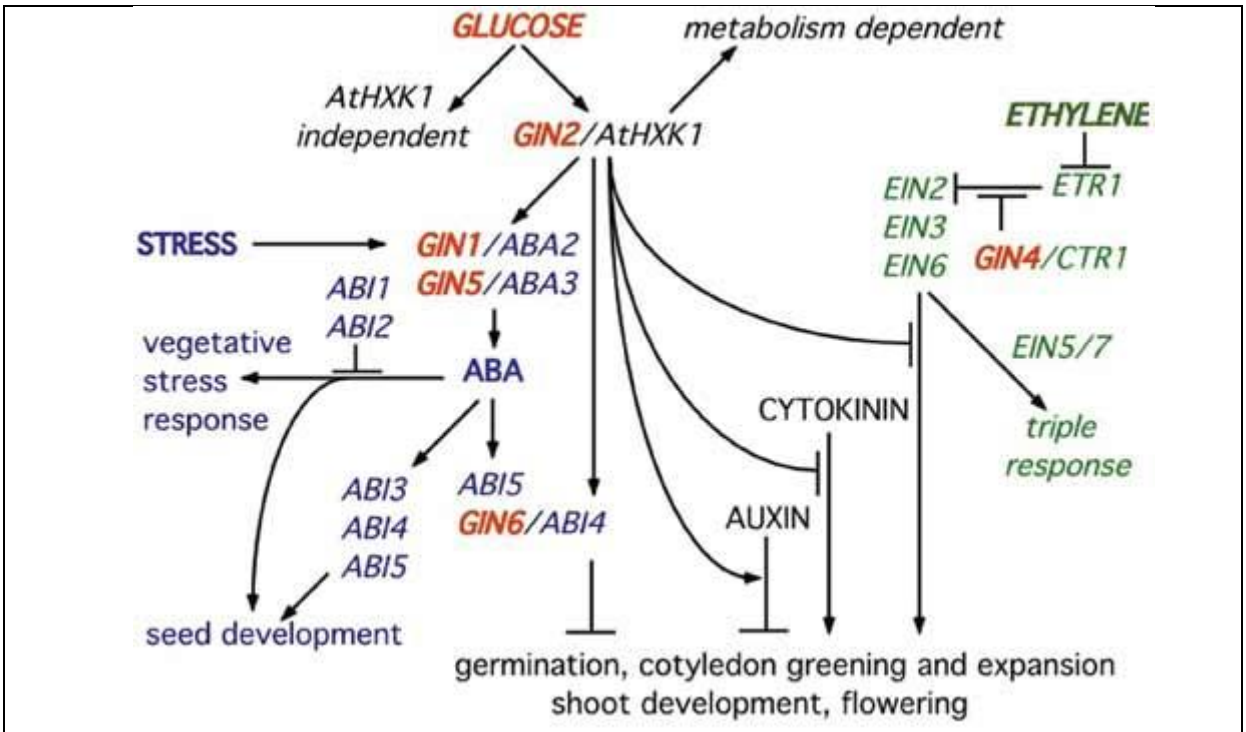

Figure 1: Overview of interactions between sugar, ABA and ethylene signalling (according to Jen Sheen Lab; http://molbio.mgh.harvard.edu/sheenweb/images/Sugar/sugar-reivew5.jpg) Glucose stimulates ABA signalling and biosynthesis and inhibits ethylene signalling.

As a lot of evidence was already published on the role of ABA in response to osmotic stress (Zhu, 2002), we also checked the phenotype of *slo2* mutants in the presence of salt or sorbitol. The mutants show a strong hypersensitivity to osmotic stress as shown by an impaired root and shoot growth and delayed germination. In order to determine the ABA dependence of this effect, we added NF (norflurazon, ABA synthesis inhibitor) to the medium, allowing the germination of *slo2* mutants to catch up with the germination of WT seeds.

Furthermore, we wanted to check whether the mutants are hypersensitive to biotic stress. This may be predicted given a higher infection rate of plants with *Botrytis cinerea* in the presence of ABA (Audenaert *et al.*, 2002) and the role of reactive oxygen in the mechanism of *Botrytis* infection (Heller and Tudzynski, 2011). When treated with *Botrytis*, *slo2* plants do

indeed show an increased infection rate as compared to the WT. This phenotype is reverted in complemented lines.

1.6 SLO2 is localized in the mitochondria

A crucial step in the research to the mechanism by which a gene functions, is to determine the localization of the protein it is encoding. Therefore, we created GFP-fusion proteins of SLO2. Although SLO2 contains no known mitochondrial or chloroplast localization sequence, its subcellular localization was shown to be mitochondrial. Since no direct effect of ethylene (or hormone) signaling has been shown in mitochondria, the focus of the research consequently shifted towards the carbon and energy metabolism of plants as mitochondria play a key role in the plant carbon and energy metabolism.

Within these organelles, the process of oxidative phosphorylation is executed making use of the mitochondrial electron transport chain. This chain consists of 4 large complexes, oxidates NADH resulting in an reduction of O_2 to H_2O. During this process, a flux of H^+ is generated, resulting in a H^+ gradient over the inner membrane of the mitochondria. H+ are transported back into the matrix by the ATPase complex, thereby converting ADP (adenosine diphosphate) into the energy rich ATP (adenosine triphosphate) molecule.

1.7 Change of perspective

As SLO2 is localized in the mitochondria, a role of SLO2 in ethylene hormone cascades is less probably. Therefore, the focus of the research shifted towards investigating the role of SLO2 in the mitochondria and the energy pathways of *Arabidopsis*. Moreover, this research can be directly correlated to the before executed sugar experiments, explaining the further focus of the research on sugar and ABA.

1.8 SLO2 functions in the energy and carbon metabolism of plants

Besides the previously mentioned results, showing the effects of sugar on the growth of *slo2*, we conducted experiments with CO_2 fertilization to check whether the effect seen with sugar treatments reflects a disturbance in the overall carbon status of the plants. The retarded growth and development phenotype of *slo2* was mostly reversed under high CO_2 concentrations compared to WT. When grown on MS/2 medium, *slo2* mutants can barely cope with the absence of sucrose. Also an increase in light dose reverses partially the *slo2* phenotype. We can therefore state that a mutation in *SLO2* interferes with the energy metabolism of plants.

1.9 Function of SLO2 in RNA editing

Given the fact that the mitochondrium transcriptome harbors over 500 editing sites and the obvious function of SLO2 in energy metabolism and the mitochondrial localization of SLO2 we investigated whether SLO2 has a function in editing the RNA for one (or more) of the mitochondrial complexes. SLO1 was shown to execute editing on different sites, located on 4

different mitochondrial transcripts. Four of these editing sites were located in transcripts of several subunits of complex I, being NAD4L, NAD7, NAD1 and mttB (membrane targeting and translocation or orfx).

1.10 Role of SLO2 in the respiratory pathway

Since it was already shown that the absence of NAD7 results in the absence of complex I (as for the CMSII mutant) we further investigated the molecular levels of the different complexes of the respiratory chain in *slo2* mutants. We were able to demonstrate lower complex I abundance in *slo2* mutants. We also saw a reduction in the levels of complex III and complex IV. Since these complexes play an important role in the energy metabolism of plants and the phenotype of *slo2* mutants, the levels of ATP and ADP were quantified, showing a severe reduction of the levels of ATP, ADP and the ATP/ADP ratio in the mutants. Next to this molecule, also NAD(H) plays an important role in the respiratory pathway. Our research showed that the $NADH/NAD^+$ ratio of the mutans is significantly higher. This balance is not only important for energy metabolism, but also for redox control and the response to oxidative stress (Shen *et al.*, 2006).

The results of changes in the respiratory pathway were further examined in the last part of our research (chapter 6). Since the respiratory pathway in *slo2* seems to be not working, mutants may switch to photorespiration in order to obtain the required energy levels. Therefore we measured the photorespiration rates of both mutants and WT. We were able to show an enhanced photorespiration in the mutant compared to the WT. We were also able to show higher levels of H_2O_2 in the mutants, probably caused by the enhanced photorespiration.In high light conditions however, ETR values were significantly lower. We were also able to demonstrate that plants try to compensate the lack of a functional respiratory pathway by a higher expression of complex I genes in the mutants. The same can be said for the genes coding for components of the retrograde respiratory pathway (Rasmusson *et al.*, 2004). The expression of these genes are already higher in mutants than in WT, but their expression are even more strongly enhanced by ABA than in WT seedlings.

1.11 Fundaments for further research

As SLO2 clearly has an effect on hormone responses and it is involved in a normal functioning of the mitochondrial respiration pathway, we wanted to check whether both processes could be linked with each other. Therefore, we checked the expression of NAD genes (coding for parts of the complex I of the electron transport chain) and showed that their expression levels are more strongly upregulated by ABA in the mutants than in WT, reflecting a role of the mitochondrial respiration in stress response.

As we were able to demonstrate this link, further research may investigate other processes requiring a fully functional SLO2. Therefore, we performed a micro-array to determine the overall gene expression in the mutant. In total, 778 genes showed a significant different expression level in the mutants. A vast majority of these genes are involved in stress responses, response to ABA, salicylic acid (SA), cytokinins and gibberellins. Interestingly, the micro-array also confirmed a change in expression of genes involved in plant development,

electron transport, light response and carbohydrate response, confirming the previously determined phenotypes and cross-talk mechanisms.

Summoned together, this research identified, characterized and partially explained the working mechanism of SLO2, a PPR protein involved in ethylene and other hormone responses, in energy and carbon metabolism and in both biotic and abiotic stress responses.

1.12 References

Achard, P., Vriezen, W.H., Van Der Straeten, D. and Harberd, N.P. (2003). Ethylene regulates *Arabidopsis* development via the modulation of DELLA protein growth repressor function. Plant Cell **15**, 2816-2825.

Alonso, J. M., Stepanova, A. N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F. M. and Ecker, J. R. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **100**, 2992-2997.

Andrés, C., Lurin, C. and Small, I. (2007). The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiologia Plantarum **129**, 14-22.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J. and León, P. (2000). Analysis of *Arabidopsis* glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes & Development **14**, 2085-2096.

Asakura Y, and Barkan A. (2006) *Arabidopsis* orthologs of maize chloroplast splicing factors promote splicing of orthologous and species-specific group II introns. Plant Physiology 142:1656-1663.

Asakura, Y., and Barkan, A. (2007) CRM Domain Protein Functions Dually in Group I and Group II Intron Splicing in Land Plant Chloroplasts[. The Plant Cell 19: 3864-3875.

Beaudoin, N., Serizet, C., Gosti, F. and Giraudat, J. (2000). Interactions between abscisic acid and ethylene signaling cascades. The Plant Cell **12**, 1103-1115.

Beick S, Schmitz-Linneweber C, Williams-Carrier R, Jensen B, Barkan A (2008) The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. Molecular and Cellular Biology **28**:5337-5347.

Bentolila, S., Knight, W. and Hanson, M. (2010) Natural variation in *Arabidopsis* leads to the identification of REME1, a pentatricopeptide repeat-DYW protein controlling the editing of mitochondrial transcripts. *Plant Physiol*. **154**: 1966-1982.

Cai, W., Ji, D., Guo, J., Ma, J., Zou, M., Lu, C., and Zhang, C. (2009) LPA66 is required for editing psbF chloroplast transcripts in *Arabidopsis*. Plant Physiology **150**:1260-1271.

Cai, W., Okuda, K. Peng, L., and Shikanai, T. (2011) PROTON GRADIENT REGULATION 3 recognizes multiple targets with limited similarity and mediates translation and RNA stabilization in plastids. Plant J. **67**:318-327.

Chateigner-Boutin *et al.* (2008) CLB19, a pentatricopeptide repeat protein required for editing of rpoA and clpP chloroplast transcripts. Plant J. **56**:590-602.

Chi, W., Mao, J., Li, Q., Ji, D., Zou, M., Lu, C., and Zhang, X. (2010) Interaction of the pentatricopeptide-repeat protein DELAYED GREENING 1 with sigma factor SIG6 in the regulation of chloroplast gene expression in *Arabidopsis* cotyledons. Plant J. **64**: 14-25.

De Grauwe, L., Vandenbussche, F., Tietz, O., Palme, K. and Van Der Straeten, D. (2005). Auxin, ethylene and brassinosteroids: Tripartite control of growth in the *Arabidopsis* hypocotyl. Plant and Cell Physiology **46**, 827-836.

De Grauwe, L., Chaerle, L., Dugardeyn, J., Decat, J., Rieu, I., Vriezen, W., Moritz, T., Beemster, G., Phillips, A., Harberd, N., Hedden, P. and Van Der Straeten, D. (2008). Reduced gibberellin response affects ethylene biosynthesis and responsiveness in the *Arabidopsis* gai eto2-1 double mutant. New Phytologist **177**,128-141.

de Longevialle, A.F., Meyer, E.H., Andres, C., Taylor, N.L., Lurin, C., Millar, A.H. and Small, I.D. (2007) The pentatricopeptide repeat gene OTP43 is required for trans-splicing of the mitochondrial nad1 Intron 1 in *Arabidopsis thaliana*. *Plant Cell*, **19**, 3256-3265.

De Paepe A. (2004a). Study of ethylene signalling in *Arabidopsis thaliana* by transcript profiling and mutant analysis. PhD thesis Ghent University.

De Paepe, A., Vuylsteke, M., Van Hummelen, P., Zabeau, M. and Van Der Straeten D. (2004b). Transcriptional profiling by cDNA-AFLP and microarray analysis reveals novel insights into the early response to ethylene in *Arabidopsis*. Plant Journal **39**, 537-559.

Ding, Y.H., Liu, N.Y., Tang, Z.S., Liu, J. and Yang, W.C. (2006) *Arabidopsis* GLUTAMINE-RICH PROTEIN23 is essential for early embryogenesis and encodes a novel nuclear PPR motif protein that interacts with RNA polymerase II subunit III. *Plant Cell*, **18**, 815-830.

Doniwa, Y., Ueda, M., Ueta, M., Wada, A., Kadowaki, K. and Tsutsumi, N. (2010) The involvement of a PPR protein of the P subfamily in partial RNA editing of an *Arabidopsis* mitochondrial transcript. *Gene*, **454**, 39-46.

Dugardeyn, J. and Van Der Straeten, D. (2008). Ethylene: Fine tuning plant growth by stimulation and inhibition of elongation. Plant Science **175**, 59-70.

Finkelstein, R.R., Gampala, S.S. and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14(Suppl)**, S15-S45.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y. and McCourt, P. (2000). Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. The Plant Cell **12**, 1117-1126.

Gibson, S.I., Laby, R.J. and Kim, D. (2001). The sugar-insensitive1 (sis1) mutant of *Arabidopsis* is allelic to ctr1. Biochemical and Biophysical Research Communications **280**, 196-203.

Gothandam, K.M., Kim, E.S., Cho, H., and Chung Y.Y OsPPR1, a pentatricopeptide repeat protein of rice is essential for the chloroplast biogenesis. Plant Molecular Biology **58**:421-433.

Hammani, K., Colas des Francs-Small, C., Takenaka, M., Tanz, S.K., Okuda, K., Shikanai, T., Brennicke, A. and Small, I. (2011a) The pentatricopeptide repeat protein OTP87 is essential for RNA editing of nad7 and atp1 transcripts in *Arabidopsis* mitochondria. *J. Biol. Chem*. **286**, 21361-21371.

Hammani, K., Gobert, A., Hleibieh, K., Choulier, L., Small, I. and Giegé, P. (2011b) An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation. Plant Cell, **23**, 730-740.

Hashimoto, M., Endo, T., Peltier, G., Tasaka, M., Shikanai, T. (2003) A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast ndhB in *Arabidopsis*. *Plant J.* **36**:*541-549*

Heller, J. and Tudzynski, P. (2011). Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annual review of phytopathology **49**, 369-390.

Laby, R.J., Kincaid, M.S., Kim, D. and Gibson, S.I. (2000). The *Arabidopsis* sugar-insensitive mutants sis4 and sis5 are defective in abscisic acid synthesis and response. The Plant Journal **23**, 587-596.

Kakizaki, T., Yazu, F., Nakayama, K., Ito-Inaba, Y., and Inaba T. (2012) Plastid signalling under multiple conditions is accompanied by a common defect in RNA editing in plastids. J. Exp. Bot. **63**:251-260

Kim, S.R., Yang, J.I., Moon, S., Ryu, C.H., An, K., Kim, K.M., and An, G. (2009). Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. Plant J. **59**, 738-749.

Koshiba, T. and Sheen, J. (2002). A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. The Plant Cell **14**, 2723-2743.

Kotera, E., Tasaka, M., and Shikanai, T. (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature **433**:326-330.

Laby, R.J., Kincaid, M.S., Kim, D. and Gibson, S.I. (2000). The *Arabidopsis* sugar-insensitive mutants sis4 and sis5 are defective in abscisic acid synthesis and response. The Plant Journal **23**, 587-596.

Laluk, K., Abuqamar, S., and Mengiste, T. (2011). The *Arabidopsis* Mitochondria-Localized Pentatricopeptide Repeat Protein PGN Functions in Defense against Necrotrophic Fungi and Abiotic Stress Tolerance. Plant Physiol **156**:2053-2068.

Le, J., Vandenbussche, F., Van Der Straeten, D. and Verbelen, J.P. (2001). In the early response of *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiology **125**, 519-522.

Liu, Y., He, J., Chen, Z., Ren, X., Hong, X. and Gong, Z. (2010) ABA overly-sensitive 5 (ABO5), encoding a pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3, is involved in the abscisic acid response in *Arabidopsis*. *Plant J*. **63**, 749-765.

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., Lecharny, A., Le Ret, M., Martin-Magniette, M.L., Mireau, H., Peeters, N., Renou, J.P., Szurek, B., Taconnat, L. and Small, I. (2004). Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16**, 2089-2103.

Meierhof , K., Felder, S., Nakamura, T., Bechtold, N., and Schuster, G. (2003) HCF152, an *Arabidopsis* RNA binding pentatricopeptide repeat protein involved in the processing of chloroplast psbB-psbT-psbH-petB-petD RNAs. The Plant Cell **15**:1480-1495

Murayama, M., Hayashi, S., Nishimura, N., Ishide, M., Kobayashi, K., Yagi, Y., Asami, T., Nakamura, T., Shinozaki, K., and Hirayama, T. (2012). Isolation of *Arabidopsis* ahg11, a weak ABA hypersensitive mutant defective in nad4 RNA editing. J Exp Bot **21:21.**

Ohto, M., Onai, K., Furukawa, Y., Aoki, E., Araki, T. and Nakamura, K. (2001). Effects of sugar on vegetative development and floral transition in *Arabidopsis*. Plant Physiology **127**, 252-261.

Ortega-Martinez, O., Pernas, M., Carol, R.J. and Dolan, L. (2007). Ethylene modulates stem cell division in the *Arabidopsis thaliana* root. Science **317**, 507-510.

Pfalz J, Liere K, Kandlbinder A, Dietz KJ, Oelmüller R. (2006) pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. Plant Cell **18**: 176–197.

Pierik, R., Tholen, D., Poorter, H., Visser, E. J. W. and Voesenek L. (2006). The Janus face of ethylene: growth inhibition and stimulation. Trends in Plant Science **11**, 176-183.

Rasmusson, A.G., Soole, K.L. and Elthon, T.E. (2004). Alternative NAD(P)H dehydrogenases of plant mitochondria. Annual Reviews Plant Biology **55**, 23-39.

Robbins, J.C., Wade, P.H., and Hanson, M. (2009) A comparative genomics approach identifies a PPR-DYW protein that is essential for C-to-U editing of the *Arabidopsis* chloroplastaccD transcript. RNA **15**:1142-1153

Schmitz-Linneweber C, Williams-Carrier R, Barkan A (2005) RNA immunoprecipitation and microarray analysis shows a chloroplast PPR protein to be associated with the 5'- region of mRNAs whose translation it activates. Plant Cell **17**: 2791-2804.

Schmitz-Linneweber, C. and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends in Plant Science **13**, 663-670.

Schroeder, J.I., Kwak, J.M. and Allen, G.J. (2001). Guard cell abscisic acid signaling and engineering drought hardiness in plants. Nature **410**, 327-330.

Sheen, J., Zhou, L. and Jang, J.C. (1999). Sugars as signaling molecules. Current Opinion in Plant Biology **2**, 410-418.

Small, I. and Peeters, N. (2000). The PPR motif – a TPR-related motif prevalent in plant organellar proteins. Trends Biochemical Science **25**, 46-47.

Smalle, J. and Van Der Straeten, D. (1997). Ethylene and vegetative development. Physiologia Plantarum **100**, 593-605.

Sung, T.Y., Tseng, C.C., and Hsieh, M.H. (2010). The SLO1 PPR protein is required for RNA editing at multiple sites with similar upstream sequences in *Arabidopsis* mitochondria. Plant J **63**:499-511.

Takenaka, M. (2010). MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the nad7 transcript in mitochondria of *Arabidopsis*. Plant Physiol **152**:939-947.

Takenaka, M., Verbitskiy, D., Zehrmann, A., and Brennicke, A. (2010). Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J Biol Chem **285**:27122-27129.

Tang, J., Kobayashi, K., Suzuki, M., Matsumoto, S., and Muranaka, T. (2010). The mitochondrial PPR protein LOVASTATIN INSENSITIVE 1 plays regulatory roles in cytosolic and plastidial isoprenoid biosynthesis through RNA editing. Plant J **61**:456-466.

Uytewaal, M., Mireau, H., Rurek, M., Hammani, K., Arnal, N., Quadrado, M., and Giegé, P. (2008) PPR336 is associated with polysomes in mitochondria. J. Mol. Biol. **375**: 626-636.

Verbitskiy, D., Hartel, B., Zehrmann, A., Brennicke, A., and Takenaka, M. (2011). The DYW-E-PPR protein MEF14 is required for RNA editing at site matR-1895 in mitochondria of *Arabidopsis thaliana*. FEBS Lett **585**:700-704.

Verbitskiy, D., Zehrmann, A., van der Merwe, J.A., Brennicke, A., and Takenaka, M. (2010). The PPR protein encoded by the LOVASTATIN INSENSITIVE 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J **61**:446-455.

Vriezen, W.H., Achard, P., Harberd, N.P. and Van Der Straeten, D. (2004). Ethylene-mediated enhancement of apical hook formation in etiolated *Arabidopsis thaliana* seedlings is gibberellin dependent. Plant Journal **37**, 505-516.

Wang. Z., *et al.* (2006) Cytoplasmic male sterility of rice with Boro II cytoplasm is caused by a cytoxic peptide and is restored by **two related PPR Motif Genes via Distinct Modes of mRNA silencing. The Plant Cell, 18:676-687.**

Williams PM and Barkan A. (2003) A chloroplast-localized PPR protein required for plastid ribosome accumulation. Plant J, **36**:675-686.

Xiao, W., Sheen, J. and Jang, J.C. (2000). The role of hexokinase in plant sugar signal transduction and growth and development. Plant Molecular Biology **44**, 451-461.

Yamazaki, H., Tasaka, M., and Shikanai, T. (2004) PPR motifs of the nucleus-encoded factor, PGR3, function in the selective and distinct steps of chloroplast gene expression in *Arabidopsis*. Plant J. **38**:152-163

Yanagisawa, S., Yoo, S.D. and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signaling in plants. Nature **425**, 521-525.

Yin, Q.Q., *et al.* The *Arabidopsis* pentatricopeptide repeat protein PDM1 is associated with the intergenic sequence of*S11-rpoA* for *rpoA* monocistronic RNA cleavage. (2012) Chinese Science Bulletin, **57**:3452-3459

Zehrmann, A., Verbitskiy, D., van der Merwe, J.A., Brennicke, A., and Takenaka, M. (2009). A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell **21**:558-567.

Zehrmann, A., van der Merwe, J., Verbitskiy, D., Hartel, B., Brennicke, A., and Takenaka, M. (2012). The DYW-class PPR protein MEF7 is required for RNA editing at four sites in mitochondria of *Arabidopsis thaliana*. RNA Biol **9**:156 - 162.

Zhou, L., Jang, J.C., Jones, T.L. and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. The Proceedings of the National Academy of Sciences U.S.A. **95**, 10294-10299.

Zhu, Q., Dugardeyn, J. Zhang, C., Mühlenbock, P., Valcke, R., De Coninck, B., Oden, S., Karampelias, M., Cammue, B., Prinsen, E., and Van Der Straeten, D. (2013). The *Arabidopsis* PPR RNA editing factor SLO2 participates in multiple stress and hormone responses. Molecular Plant, **accepted for publication**.

Zhu, Q., Dugardeyn, J., Zhang, C., Takenaka, M., Kuhn, K., Craddock, C., Smalle, J., Karampelias, M., Denecke, J., Peters, J., Gerats, T., Brennicke, A., Eastmond, P., Meyer, E.H. and Van Der Straeten, D. (2012). SLO2, a mitochondrial PPR protein affecting several RNA editing sites, is required for energy metabolism. The Plant journal **71**, 836-849.

Zhu, Q., E, H.M., and Van Der Straeten, D. (2013). Functional analysis of SLO2 provides new insight into the role of plant PPR proteins. Plant Signal Behav **7**:1209-1211.

Zhu, Q., Dugardeyn, J. Zhang, C., Mühlenbock, P., Valcke, R., De Coninck, B., Oden, S., Karampelias, M., Cammue, B., Prinsen, E., and Van Der Straeten, D. (2013). The *Arabidopsis* PPR RNA editing factor SLO2 participates in multiple stress and hormone responses. Molecular Plant, **accepted for publication**.

Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. Annual Reviews Plant Biology **53**, 247-273.

Zsigmond, L., Rigo, G., Szarka, A., Szekely, G., Otvos, K., Darula, Z., Medzihradszky, K.F., Koncz, C., Koncz, Z., and Szabados, L. (2008). *Arabidopsis* PPR40 connects abiotic stress responses to mitochondrial electron transport. Plant Physiol **146**:1721-1737.

Chapter 2: Objectives

The primary goal of this research is the isolation, identification and characterization of the *SLO2* gene of *Arabidopsis thaliana*. A mutant in this gene is isolated based on DLE on ACC supplied LNM medium.

The research consisted of the creation/identification of different mutant lines (T-DNA insertion lines, complemented lines, TILLING lines, GUS-fusions, GFP and YFP fusions and overexpressor lines driven by a 35S-promotor).

Since *SLO2* is isolated as a gene involved in ethylene response, the first aim of the research consisted of describing the different effects caused by a mutation in *SLO2* in response to ethylene and the different hormones with which ethylene interacts. Based on an extensive literature search and the experience of the lab in investigating the GA – ethylene crosstalk, the first focus is to investigate this interaction. As new data were produced, it became clear that SLO2 does not play a direct role in this process. Therefore, this part of the research remained restricted to an in-silico analysis of this interaction in root tips. Further physiological experiments are carried out to investigate the crosstalk between ethylene versus sugar/ABA. Furthermore, the response of the mutants in the presence of biotic and abiotic stress is investigated.

Another goal of the research was to determine the subcellular localization of SLO2. Based on the subcellular localization of SLO2, the focus of the research is shifted to the unraveling of the function of SLO2 in the mitochondrial electron transport chain and its role in the energy metabolism of plants.

As SLO2 is a PPR protein, further molecular research is performed in orde to identify the mitochondrial RNA targets of SLO2 and changes in gene expressions on a plant level.

Hence, it can be stated that the overall goal of this research is to lay the basis to explain the mechanism by which SLO2 executes its function.

Chapter 3:

Ethylene synthesis, signaling and cross-talk

Adapted from:

Ethylene: Fine tuning plant growth and development by stimulation and inhibition of elongation

Jasper Dugardeyn and Dominique Van Der Straeten

Plant architecture is determined by a subtle balance between growth stimulation and inhibition conferring optimal plasticity in response to environmental changes. Ethylene is a gaseous hormone which plays an essential role in a myriad of plant developmental processes, both by stimulation and inhibition of growth. Ethylene can promote root hair formation, flowering, fruit ripening and abscission, as well as leaf and petal senescence and abscission. Its role is dependent on the environmental conditions, and the plant developmental stage, besides also being species dependent. Within a plant and even within a given organ, the effect of ethylene treatment can differ, depending on endogenous and environmental cues. Moreover, ethylene signaling and response are a part of an intricate network in cross-talk with other hormones. In this review, current insights in the role of ethylene on growth processes and the influence of these different factors are discussed.

Plant Science, volume 175, Issues 1-2 July-August 2008, Pages 59-70
DOI : 10.1111/j.1365-313X.2012.05036.x

Jasper Dugardeyn wrote the first draft of this review.

3.1 Ethylene synthesis and signaling

3.1.1. Ethylene biosynthesis

Ethylene biosynthesis starts with methionine. This amino acid is converted to SAM (S-adenosyl-methionine). A reaction that is catalyzed by SAM (SAM synthetase) (Ravanel *et al.*, 1998). The next step is the conversion of SAM to ACC (1-aminocyclopropane-1-carboxylic-acid) and MTA (5'-methylthioadenosine). This step is catalyzed by ACS (ACC-SYNTHASE). It is a cytosolic enzyme that requires PLP (pyridoxal-5'-phosphate) as a cofactor (Adams *et al.*, 1979; Yang and Hoffman, 1984) and functions as homodimers (Yamagami *et al.*, 2003; Capitani *et al.*, 1999). In *Arabidopsis*, ACS is encoded by a gene family containing 12 members (*ACS1* and *ACS2* of Van Der Straeten and coworkers (1992) are named *ACS2* and *ACS4* respectively by Yamagami and coworkers (2003)). MTA is recycled to methionine in the Yang-cycle (Miyazaki and Yang, 1987). ACO (ACC-OXIDASE) catalyzes the conversion of ACC to ethylene. During this reaction ACC is oxidized and forms ethylene, CO_2 and HCN (Yang and Hoffman, 1984). In *Arabidopsis* ACO is part of a multi-gene family, as is ACS (Gomez-Lim *et al.*, 1993).

ACS is the major step regulating the rate of ethylene synthesis. One of the mechanisms used by plants to control ACS concentration is transcriptional regulation of *ACS* genes. The different functional members of the *ACS* gene family show unique and overlapping expression patterns. Furthermore, these patterns are different as environmental conditions change (Tsuchisaka and Theologis, 2004; 35; Rodrigues-Pousada *et al.*, 1993). In addition, different stress-promoting factors (cold, wounding, heat) have been shown to alter transcription of *ACS* genes, each in a specific manner for individual *ACS* genes (Tsuchisaka

and Theologis, 2004). Besides these abiotic conditions, hormones can also alter the expression of different *ACS* genes. Auxins are inducers of ethylene production (Tsuchisaka and Theologis, 2004; Yang and Hoffman, 1984; Abel *et al.*, 1995; Abeles and Morgan, 1992). The same holds true for cytokinins, which have been shown to up-regulate the expression of *ACS1/2*. In light-grown seedlings, *ACS4, 5*, and *7* are responsive to ABA (Wang *et al.*, 2005), while *ACS7* is also responsive to GA. Finally, also brassinosteroids play an inducing role by stimulating expression of *ACS2/4* in dark grown seedlings (Joo *et al.*, 2006). It was proposed that in particular conditions as upon wounding or during ripening and senescence, ACO also plays a role in regulating ethylene levels in plants (Kende, 1993).

For a more detailed review of ethylene synthesis and its regulation, see De Paepe and Van Der Straeten (2005).

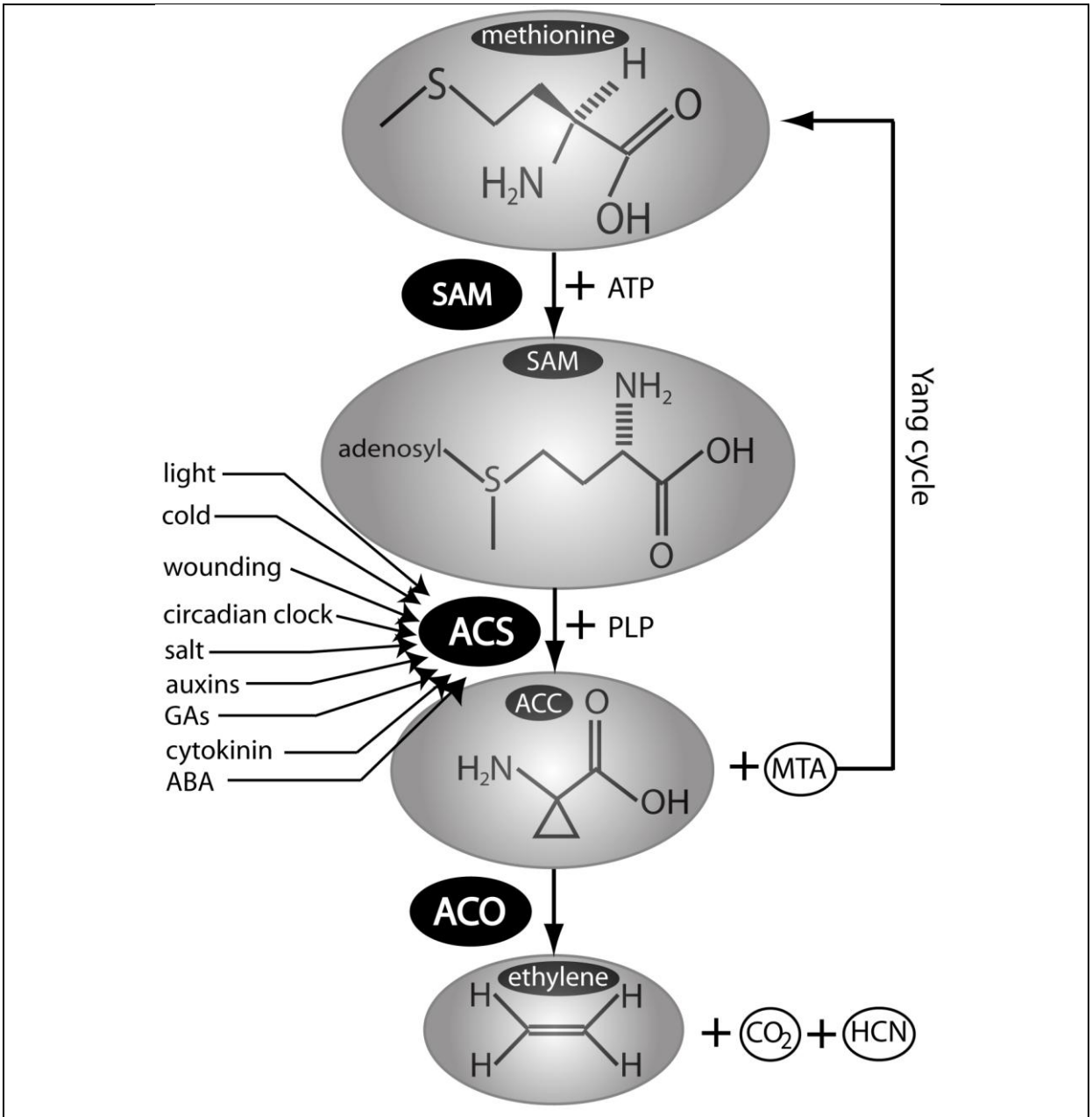

Figure 1: Ethylene biosynthesis: methionine is conversed to S-adenosyl-methionine by SAM-synthetase. SAM is further processed to ACC by ACS and subsequently to ethylene by ACO.

3.1.2. Ethylene signaling

In *Arabidopsis*, ethylene is perceived by 5 receptors, which are partially redundant in function and are localised on the endoplasmatic reticulum membrane (Chen *et al.*, 2002; Gao *et al.*, 2003). The receptors act as negative regulators of ethylene signaling by activating CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) in the absence of the hormone (De Paepe and Van Der Straeten, 2005; Hua and Meyerowitz, 1998).

CTR1 seems to be part of, and a negative regulator of a MAPK-cascade (Kieber *et al.*, 1993) which itself is a positive regulator of ethylene signaling. However, a direct biochemical connection between CTR1 and SIMKK (the next step in the MAPK-cascade) still has to be confirmed (Chang, 2003; Ouaked *et al.*, 2003). The MAPK-cascade activates EIN2 (ETHYLENE INSENSITIVE), an essential positive regulator of ethylene signaling (Hall and Bleecker, 2003). Subsequently, EIN3 and the EILs (EIN3-LIKE proteins) come into action. *eil1 ein3* double mutants are almost completely ethylene insensitive and almost indistinguishable from *ein2-5* (Alonso *et al.*, 2003). EIN3 is constitutively synthesized and degraded in a ubiquitin-dependent way. EBF1 (EIN3- BINDING F-BOX PROTEIN) and EBF2 mediate this breakdown (Gagne *et al.*, 2004; Potuschak *et al.*, 2003; Guo and Ecker, 2003). The expression of *EBF1/2* is regulated by EIN5, an exoribonuclease. *EIN5*-expression is up-regulated by ethylene and the corresponding protein is supposed to act in ethylene signaling by degrading EBF1/2 mRNA (Olmedo *et al.*, 2006; Potuschak *et al.*, 2006). EIN3 dimers are able to bind to the PERE (primary ethylene response element) in the promoter of *ERF1* (*ETHYLENE RESPONSE FACTOR1*) (Solano *et al.*, 1998). ERF1 is part of the family of EREBP (ETHYLENE RESPONSE ELEMENT BINDING PROTEINS) and is able to bind to the SERE (secondary ethylene-response element), a GCC box in the promoter region of ethylene regulated genes (Fujimoto *et al.*, 2000), regulating their expression.

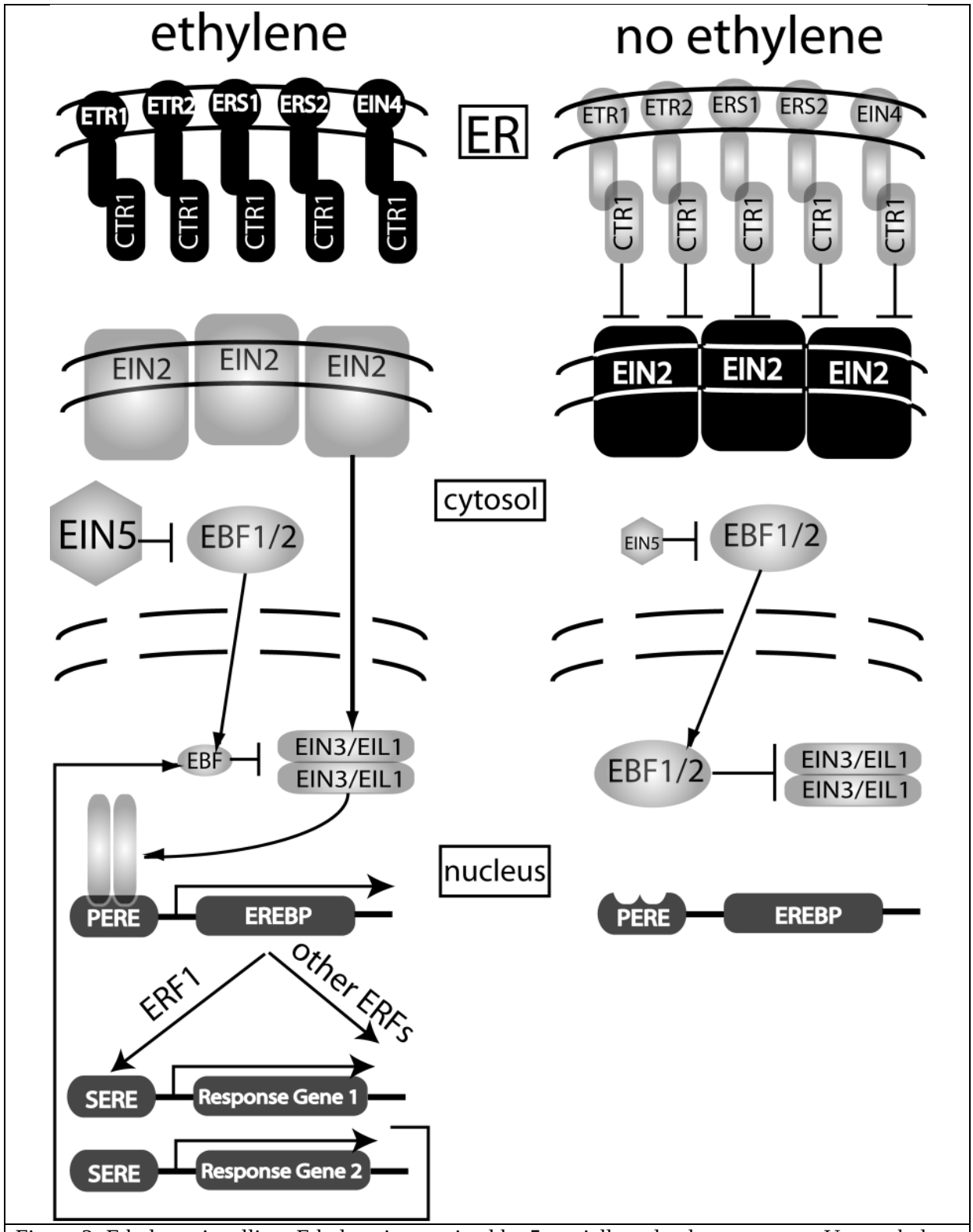

Figure 2: Ethylene signalling: Ethylene is perceived by 5 partially redundant receptors. Upon ethylene detection, interaction with the negative regulator CTR1 is interrupted. Because of this, EIN 2 is shifted to the nucleus, inhibiting the EBF mediated break down of EIN3/EIL1. EIN3 binds to the primary ethylene response element, leading to the expression of ethylene response element binding proteins. These EREBPs bind to the secondary ethylene response element, leading to the expression of ethylene response genes.

3.2 The role of ethylene in root growth

3.2.1. Ethylene represses root growth

The inhibitory effect of ethylene on root growth was described in 1901 by Neljubov. Like other ethylene responses, the response of roots also differs from species to species (Konings and Jackson, 1979). Plants growing on well-aerated soils (*Sinapsis alba L.*) show a high ethylene production and a strong inhibition of root growth upon exogenous ethylene treatment. In contrast, wetland species as *Oryza sativa L.*, have a lower rate of ethylene synthesis and are more resistant to exogenous ethylene treatment (Konings and Jackson, 1979). Three strategies may prevent the growth inhibitory effect of ethylene in wetland species (Visser and Pierik, 2007). Firstly, plants that grow in wetland conditions form aerenchyma, facilitating the diffusion of ethylene away from the root apex. Secondly, the root apices of these species also produce less ethylene compared to non-wetland species and thirdly, they are less susceptible to the inhibition of root growth by ethylene.

In land plants, ethylene strongly inhibits root growth. *Arabidopsis* (a non-wetland species) plants that have been grown in the presence of 0.1 µM ACC (1-amino-cyclopropane-1-carboxylic acid) for 7 days have a root that is half the length of plants that have been grown in the absence of the ethylene precursor (Achard *et al.*, 2003). For an overview of ethylene synthesis and signaling, see fig. 1 and fig. 2 respectively. This is correlated with a reduction of cell length. Le and coworkers (2001) demonstrated that the LEH (length of the first epidermal cell with a visible root hair bulge) is smaller upon ACC treatment. This inhibition of elongation occurs both in the elongation zone and closer to the root tip. Epidermis cells that are longer than or equal to the LEH at a given ethylene concentration immediately stop elongation. Cells closer to the root tip elongate until they reach the LEH (Le *et al.*, 2001). In cells shorter than the concentration-specified LEH, the transverse orientation of the microtubules is not changed upon ACC treatment, while in cells longer than the ACC-specified LEH the microtubules rearranged from a transverse to random (trichoblast) or longitudinal (atrichoblasts) orientation. This correlates well with the data of Swarup and coworkers (2007) who showed that ACC treatment has no effect on cell expansion rates in the apical 500 µm of the root but shortens the length of the elongation zone. Moreover, in the elongation zone, the maximum growth rate is reduced (Swarup *et al.*, 2007).

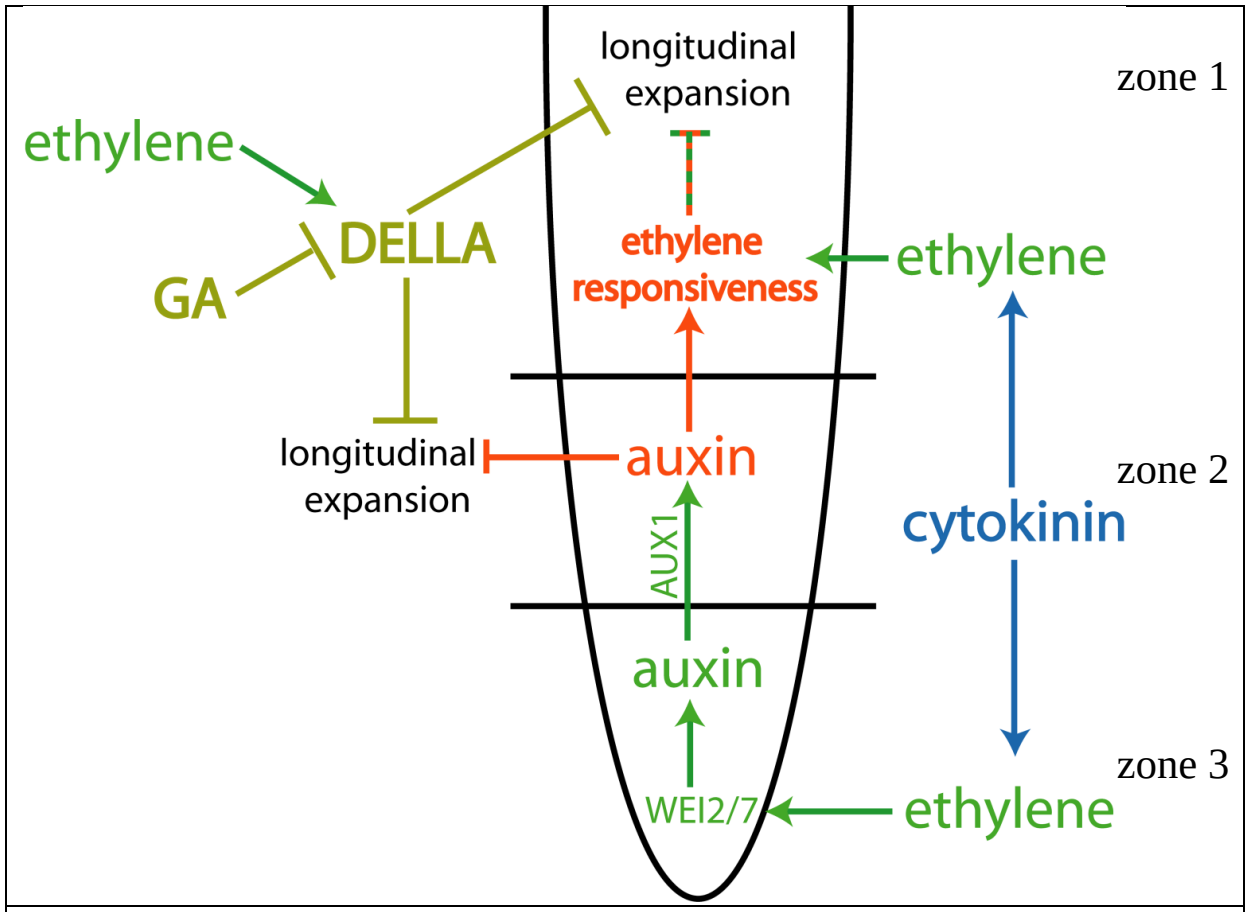

Figure 3: Exogenous application of ethylene to roots stimulates the expression of *WEI2* and *WEI7*, resulting in enhanced auxin synthesis. Auxin is subsequently transported to the transition zone. In this process, the auxin influx carrier AUX1 plays an important role. The higher auxin concentration in the transition zones will cause a change from longitudinal to radial cell expansion. Auxin further regulates ethylene sensitivity of cells moving from the transition to the elongation zone, such that they become sensitive for ethylene-induced growth repression. The growth inhibiting role of cytokinins is caused by an upregulation of ethylene synthesis. Finally, GAs induce proteasome-dependent degradation of DELLA proteins, which negatively regulate cell elongation and which are stabilized by ethylene.
This model is modified from (Swarup *et al.*, 2007; Ruzicka *et al.*, 2007 and Stepanova *et al.*, 2007). Zone 1 is the meristematic zone, zone 2 is the transition zone and zone 3 is the elongation zone (for more details, see figure 4B, paragraph 4.4)

It is however more likely that microtubule reorganisation is the consequence of an arrest in elongation rather than its cause, since in ACC treated plants, elongation stops within 10 minutes, while it still takes 1 h to establish a longitudinal microtubule orientation (Le *et al.*, 2004).

The concentration of ROS (reactive oxygen species), particularly H_2O_2, is higher in the cell walls but not in the cytosol of epidermal cells in the elongation zone of ACC-treated seedlings as compared to the control. For crosslinking of HRGPs (hydroxyproline-rich glycoproteins), the cross-linking sites must possess an amino acid that can be oxidized by peroxides (Schnabelrauch *et al.*, 1996). Upon combined treatment with ACC and tyrosine, the epidermal cells are larger than in seedlings treated with ACC only (De Cnodder *et al.*, 2005).

Thus it was proposed that ACC-treatment gives rise to an apoplastic oxidative burst, leading to crosslinking of HRGPs in the cell wall (De Cnodder *et al.*, 2005). ACC also stimulates callose deposition in epidermis and cortex cells of the elongation and differentiation zone. In *eto2* (*ethylene overproducer 2*), callose is also deposited in meristematic cells and in elongating cells (De Cnodder *et al.*, 2005). Callose is essential for plasmodesmata function (Roberts and Oparka, 2003). Therefore it is possible that cell-to-cell transport is also mediating inhibition of cell elongation under the influence of ACC (De Cnodder *et al.*, 2005).

Recently however it has been shown that ethylene does not only regulate root length by cell elongation, but also regulates root cell division. Alleles of the *eto1* mutant (*eto1-11* and *eto1-12*) show an extra division in the quiescent center (QC) that is not present in the wild type (WT) (Ortega-Martinez *et al.*, 2007). Using the ACC synthase inhibitor AVG (aminoethoxyvinylglycine), the authors also showed that this is an effect of ethylene overproduction, while treating WT-plants with ethylene stimulates supernumerary divisions of the QC. However, when seedlings are grown in the presence of 1 µM ACC (which already reduces the length of epidermal cells), neither the root meristem length, nor the differentiation of the columella initials change (Ruzicka *et al.* 2007).

In the past decade, it has become clear that all processes in plants are interconnected. Hence, the shortening of roots under the influence of ethylene is not an isolated process. DELLA proteins are repressors of the gibberellin pathway and their stability is negatively regulated by gibberellins (Dill and Sun, 2001; King *et al.*, 2001; Peng *et al.*, 1997; Silverstone *et al.*, 1998). Ethylene treatment delays the GA-induced disappearance of GFP-RGA (GREEN FLUORESCENT PROTEIN-RGA) from root cell nuclei by stabilizing the fusion protein (Achard et al., 2003). RGA (REPRESSOR OF *ga1-3*) is a DELLA protein, acting as a negative regulator of growth. Therefore, when grown in the presence of ACC, wild type seedlings have shorter roots than loss-of-function mutants of DELLA proteins. The roots of the double mutant *gai-t6 rga24* are longer than those of *rga24* which, in turn, are longer than those of *gai-t6* (*GA insensitive*) (Achard *et al.*, 2003). These differences become less apparent as the concentration of ACC rises. Furthermore, GA treatment can overcome the ACC-induced shortening of the root, but has only little effect on the growth of roots in the absence of ACC, suggesting that an optimal concentration of GA is naturally present.

In regulating root growth, ethylene does not only interact with GA but also with auxins. Although dark grown *wei2* (*weak ethylene insensitive 2*) (Alonso *et al.*, 2003) and *wei7* (Stepanova and Alonso, 2005) seedlings show a short hypocotyl and an exaggerated hook when treated with ethylene, root elongation is not inhibited. WEI2 has been previously identified as ASA1 (ANTHRANILATE SYNTHASE α) and WEI7 is also known as ASB1 (ANTHRANILATE SYNTHASE β) (Stepanova *et al.*, 2005). Both are subunits of the heteromeric AS1 (ANTHRANILATE SYNTHASE 1) complex which catalyzes the first step of tryptophan (Trp) biosynthesis, one of the precursors for auxin (Radwanski and Last, 1995). The ethylene insensitive phenotype can be rescued by addition of 10 µM anthranilate or 10 µM Trp and can be partially rescued by 10 µM IAA (indole-3-acetic acid) (Stepanova *et al.*, 2005). *wei1* shows a phenotype similar to that of *wei2*, with a short hypocotyl and exaggerated hook, but no shorter root when treated with ethylene. When treated with auxin, *wei1* seedlings have a longer root than the WT. *wei1* harbours a mutation in the *TIR1* gene

(*TRANSPORT INHIBITOR RESPONSE 1*) (Alonso *et al.*, 2003), which is responsible for ubiquitin labelling of AXR2/IAA7 (AUXIN RESISTANT2) and AXR3/IAA17 (Gray *et al.*, 2001) (both are repressors of the auxin signaling pathway). Therefore it can be stated that both auxin synthesis and auxin signaling are required for ethylene induced inhibition of root growth. Furthermore, in *aux1-7* (*auxin insensitive 1-7*) (which shows limited auxin influx in roots (Marchant *et al.*, 1999)) the repression of root growth induced by ACC is only 20 to 30 % of that of the WT (Stepanova *et al.*, 2007). The same can be seen in another *aux* allele, *aux1-22* (Swarup *et al.*, 2007). Also *eir1-1* (*ethylene insensitive root 1-1*, an allele of *pin2* (*pin formed 2*)) (Luschnig *et al*., 1998) and *35S:PIN1* exhibit ACC-resistant root and epidermal cell elongation (Ruzicka *et al.*, 2007). However, when ethylene mutants are supplied with auxins, they show an auxin-induced root shortening response similar to that of the WT (Stepanova *et al.*, 2007). These results suggest that a normal auxin synthesis, transport and signaling mechanism is required for ethylene to exert its effect on root growth (Stepanova *et al.*, 2005; Stepanova *et al.*, 2007).

DR5::GUS expression (*DR5* is a synthetic auxin reporter, coupled to β-glucuronidase) is enhanced by ethylene in the WT root tip (zone 1, according to the classification of Birnbaum and coworkers in 2003) (Stepanova *et al.*, 2005; Stepanova *et al.*, 2007). This is also confirmed by Swarup and coworkers (2007) who measured a higher de novo IAA synthesis in root tips after ACC or ethylene treatment. In the transition zone (zone 2 as defined by Birnbaum and coworkers (2003), containing meristematic cells and cells from the slow elongation zone), the *DR5::GUS* signal is not visible in the absence of ACC, but is present when ACC is added to the medium (Ruzicka *et al.*, 2007; Stepanova *et al.* 2007). This induction is however absent in *wei2*, *wei7* and *ein2-5* (*ethylene insensitive 2-5*) (Stepanova *et al.*, 2005). Likewise, the induction is not visible in *aux1* when grown in the presence of 5 µM or 10 µM ACC (Ruzicka *et al.*, 2007; Stepanova *et al.* 2007).. Furthermore, *eir1/pin2* and 35S:*PIN1* overexpressors show no signal in the presence of 1 µM ACC and a moderate ectopic expression in the presence of 5 µM ACC (Ruzicka *et al.*, 2007). These results suggest that ethylene induces a higher auxin concentration in the transition zone of the root (Stepanova *et al.*, 2007). The above-mentioned lines also all show an ethylene insensitive root phenotype. Conversely, exogenously applied ethylene induces the expression of *IAA2::GUS* (auxin responsive reporter, ((Luschnig *et al*., 1998; Swarup *et al.*, 2001)) in the lateral root cap, older meristematic cells and newly expanding cells (Swarup *et al.*, 2007; Ruzicka *et al.*, 2007). The *ctr1*-mutant (*constitutive triple response 1*) displays this strong expression in the absence of ethylene, while *ein2* and *aux1-22* do not show this upregulation (Swarup *et al.*, 2007). Furthermore, *eir1* has higher auxin levels in the root tip, but no *DR5::GUS* staining in the transition zone (Ruzicka *et al.*, 2007). Ethylene also regulates the expression of several PIN auxin efflux proteins and the AUX1 influx carrier (Ruzicka *et al.*, 2007). Together these data point towards an ethylene induced induction of auxin synthesis in the root apex, followed by transport to the transition zone (Swarup *et al.*, 2007; Ruzicka *et al.*, 2007). Although *rty1-1* (*rooty1-1*, an auxin over producer, (King *et al.*, 1995)) seedlings display a higher *DR5::GUS* expression in the transition zone, they have longer roots than WT plants when treated with ACC (Stepanova *et al.*, 2007; King *et al.*, 1995) indicating that a mere ethylene-induced auxin accumulation is not sufficient for complete ethylene-induced root shortening (Stepanova *et al.*, 2007).

The role of auxins in the regulation of root elongation can also in part be explained by induction of several *ACS* (*ACC SYNTHASE*) genes. By using GUS-fusions, Tsuchisaka and Theologis (2004) showed that in roots, *ACS2, 4, 5, 6, 7, 8* and *11* transcription is influenced by auxin. IAA enhances the expression of the aforementioned *ACS* genes and alters the pattern of expression of these in the root otherwise constitutively expressed *ACS*s. This confirms the data of Yamagami and coworkers (2003) who found higher levels of *ACS2, 4, 5, 6, 8* and *11* mRNA by RT-PCR upon auxin addition at the seedling level of 7 day-old seedlings. Stepanova and coworkers demonstrated that in the elongation zone (zone 3 by Birnbaum and coworkers (2003)) of *aux1-7 EBS::GUS* (synthetic EIN3-responsive promoter coupled to β–glucuronidase) expression is not visible after ACC treatment (10 µM), although this is the case in WT plants (Stepanova *et al.*, 2007). Therefore, a model can be proposed wherein an ethylene-mediated increase in auxin signal in the transition zone of the root is responsible for part, but not all, of the growth repressing effect of ethylene (Stepanova *et al.*, 2007). A microarray experiment performed by the same authors showed that 28% of the genes that were regulated by ethylene but not by auxin had an altered response to ethylene in *aux1*. In addition, 38 % of the auxin-regulated genes that are not regulated by ethylene show a different response in *ein2*. Together these data confirm the above-mentioned model (wherein auxin mediates ethylene responses), but also pinpoint that below certain threshold levels of ethylene signaling the response to auxin is altered. In this case, one hormone is sensitizing or conditioning cells for the other hormone. This kind of interactions however is not the only that occurs in the root. Other mechanisms that can be noticed are one hormone regulating the biosynthesis or signaling of the other (for example ethylene-mediated auxin responses) and both hormones acting independently on the same subset of genes (ethylene regulates a gene independent of auxin while auxin regulates the same gene independently of ethylene) (Stepanova *et al.*, 2007).

Other hormones, such as cytokinins and jasmonates (JA) also are interconnected to ethylene in root growth. At high levels of cytokinins (100 µM BA (6-benzylaminopurine)), seedling growth is strongly inhibited, but even at lower concentrations (5 nM) root growth inhibition is observed (Su and Howell, 1992). Cytokinins induce ethylene synthesis (Vogel *et al.*, 1998a). On the one hand, the induction of ethylene biosynthesis is regulated at the posttranslational level, by controlling the stability of ACS proteins. This is shown by *cin5* (*cytokinin insensitive 5*) seedlings which, at low cytokinin levels (<10 µM) produce much less ethylene than do wild-type seedlings (Vogel *et al.*, 1998a; Vogel *et al.*, 1998b). *cin5* harbours a mutation in *ACS5* (Vogel *et al.*, 1998a). The steady state level of a dexamethasone (DEX)-inducible form of myc-ACS5^{WT} is raised in seedlings grown in the presence of 9 nM DEX (Chae *et al.*, 2003). This upregulation is also reflected in an approximately two fold up-regulation of ethylene synthesis. Previously it was shown that the *eto2* mutation is caused by an insertion in the C-terminus of *ACS5*, resulting in enhanced accumulation of ACS5 (Vogel *et al.*, 1998a). ETO1 interacts with the C-terminus of ACS5. Because of this interaction, ACS5 interacts with a CUL3 ubiquitin E3 ligase, labelling ACS5 for proteasome dependent degradation (Wang *et al.*, 2004). The stabilisation of ACS5 by cytokinins is at least in part regulated by another mechanism since also in *eto2*, ethylene synthesis is raised in the presence of cytokinins. This correlates with the higher stability of myc-ACS5^{eto2} in the presence of cytokinins (Chae *et al.*, 2003). It can thus be stated that when WT seedlings are treated with

cytokinins, ACS5 steady state levels are increased, leading to higher ethylene concentrations (Chae *et al.*, 2003). Second, at least one *ACS* gene, *ACS1*, is induced by cytokinin treatment at the transcriptional level (up to 5-fold) (Rodrigues-Pousada *et al.*, 1999). A model combining these different responses is presented in figure 3. Finally, treating seedlings with jasmonates also inhibits root growth (Yamane *et al.*, 1981). However, this growth repression is at least in part independent of ethylene signaling, since both *jin1* (*jasmonate insensitive 1*) and *jin4* show reduced sensitivity to root growth inhibition by MeJA (methyljasmonate), but a WT response to ethylene. Furthermore, addition of silver nitrate did not alter MeJA inhibition of root growth (Berger *et al.*, 1996). It has also been shown that JA can form a conjugate with ACC in the form of JA-ACC, both in vitro and in vivo (Staswick and Tiryaki, 2004). It is however not likely that JA-ACC has a signaling function itself, but rather regulates the availability of ACC and JA for conversion to the respective active signals (Staswick and Tiryaki, 2004).

3.2.2. Ethylene induces root hair formation

In *Arabidopsis*, the root epidermis consists of trichoblasts and atrichoblasts. Trichoblasts are located in the cleft between underlying cortical cells and form root hairs. Atrichoblasts are located over a single cortical cell and do not form root hairs (Dolan *et al.*,1994; Galway *et al.*, 1994). The cell fate of root epidermal cells is regulated by a set of genes, either promoting trichoblast or atrichoblast fate. When seedlings are grown in the presence of 50 µM ACC or 5 µM AVG, ectopic root hair formation is induced (Bibikova and Gilroy, 2002) or repressed (Masucci and Schiefelbein, 1994; Masucci and Schiefelbein, 1996) respectively. In *ctr1* a modest increase in the proportion of root hair cells is noted (62 % in *ctr1*, 42 % in Colombia), with a corresponding frequency of ectopic root hair cells (32 % ectopic root hair cells) (Masucci and Schiefelbein, 1996). The initial patterning of the root epidermis however is identical to that of the wild-type.

RHD6 (ROOT HAIR DEFECTIVE 6) functions downstream of GL2 (GLABRA 2) and induces root hair formation (Masucci and Schiefelbein, 1994). GL2 is a homeodomain leucine zipper transcription factor that is thought to promote the hairless cell fate (Masucci *et al.*, 1996). TTG1 (transparent testa glabra 1) positively regulates GL2 expression, thereby promoting atrichoblast cell fate (Walker *et al.*, 1999). The *rhd6 gl2* and *rhd6 ttg1* double mutants have a higher frequency of root hair cells (17 and 23 % respectively) as compared to *rhd6* (4 %), but a lower proportion than WT (42 %) (Masucci and Schiefelbein, 1994; Masucci and Schiefelbein, 1996). Both double mutants also show some ectopic root hairs. When the double mutants are treated with ACC or IAA, both have the *ttg1* or *gl2* phenotype. This implies that ACC and IAA work downstream or independent of the *TTG/GL2* pathway (Masucci and Schiefelbein, 1996). Furthermore, AVG treatment of *ttg* and *gl2* mutants repressed the formation of root hairs. Taken together, it can be stated that ethylene acts downstream of TTG and GL2 (Masucci and Schiefelbein, 1996). This hypothesis is also confirmed with the *GL2::GUS* construct which is not differently expressed in *rhd6*, *axr2* or *ctr1* background when compared to untreated WT. These results confirm the hypothesis that *GL2* is not regulated by either one of these genes (Masucci and Schiefelbein, 1996), thus *rhd6*, *axr2* and *ctr1* do not abolish the early developmental events associated with the position-dependent differentiation of the epidermal cells. Moreover, since no effect on

GL2::GUS staining was noticed when WT seedlings are treated with ACC or AVG, ethylene is not likely to regulate these early stages (Masucci and Schiefelbein, 1996).

Not only ACC and IAA play a role in root hair formation, jasmonates also have been shown to stimulate root hair formation. 1 µM JA or MeJA stimulates root hair formation up to 2.5 and 4 times respectively (Zhu *et al.*, 2006). Both Ag^+ ions and AVG are able to antagonize the jasmonate induced root hair formation. Furthermore, MeJA has no effect in *etr1-1* (*ethylene resistant 1*) and a less pronounced effect in *etr1-3*. But also ethylene induced root hair formation is suppressed by JA biosynthesis inhibitors (Zhu *et al.*, 2006). The exact mechanism by which ethylene, auxin and jasmonates influence root hair formation is not elucidated yet.

3.3 The role of ethylene in hypocotyl growth

Ethylene plays a dual role in regulating hypocotyl length. In dark grown seedlings, one of the traits of the triple response is the repression of hypocotyl growth by ethylene (Guzman and Ecker, 1990). In the light however, low concentrations of ACC (1 µM) stimulate hypocotyl elongation of LNM (low nutrient medium) grown seedlings (Smalle *et al.*, 1997).

The Arabidopsis hypocotyl is composed of about 20-23 epidermal cells from base to top (Cheng *et al.*, 1995; Gendreau *et al.*, 1997), all of which are, in principle, of embryonic origin. This implies that hypocotyl growth mainly occurs through cell elongation (Gendreau *et al.*, 1997), even though both ethylene and gibberellins can stimulate cell division in the hypocotyl, giving rise to extra stomata (Saibo *et al.*, 2003). As in roots, elongation of hypocotyl cells is not uniform throughout the entire hypocotyl. Not only does elongation differ from cell to cell, it is dependent on whether plants are growing in the light or in darkness (Gendreau *et al.*, 1997). In white light, cell elongation occurs in all epidermal cells, but as the hypocotyl ages, the region with the highest elongation rate moves from the base of the hypocotyl to the middle (Le *et al.*, 2005). The elongating capacity of cells is correlated with the orientation of the cortical microtubules in the cytoskeleton. Three days old light grown seedlings show a transverse orientation of the microtubules in elongating cells at the base of the hypocotyl. In the upper part of the hypocotyl, the microtubules are randomly organized (Le *et al.*, 2005).

When seedlings are grown for three days in an 8 h/16 h dark/light cycle followed by three days without light, darkness strongly promotes cell elongation in the top two thirds of the hypocotyl, from cell eight upwards (Le *et al.*, 2005). In seedlings grown in continuous dark, the epidermal cells elongate along a steep acro-petal gradient, starting with growth in the basal cells after germination and ending with elongation in a small area below the apical hook at advanced growth stages (Gendreau *et al.*, 1997). The microtubules of three day-old dark grown seedlings are longitudinally organized at the base of the hypocotyl. In the middle and at the top of the hypocotyl, they are predominantly transversely oriented. As the seedlings grow older, the basal part of the hypocotyl shows fewer microtubules with a random orientation. Cells in the middle switch from a transverse to a longitudinal orientation. Only at the top the transverse orientation is maintained (Le *et al.*, 2005).

3.3.1. Ethylene represses hypocotyl growth

Application of ethylene to dark grown seedlings causes an inhibition of hypocotyl elongation (Guzman and Ecker, 1990). This response is already visible after 15 minutes and is

reversible (Binder *et al.*, 2004a). The growth inhibition is caused by a decrease in cell elongation (Le *et al.*, 2005), which is associated with a reorientation of the cytoskeleton. Four and five days-old seedlings grown in the presence of the ethylene precursor ACC have a relatively smaller zone with transverse microtubules, as compared to untreated seedlings (Le *et al.*, 2005). After 7 days, both treated and untreated seedlings show longitudinally oriented microtubules at the top (Le *et al.*, 2005).

The hypocotyl response to ethylene treatment is biphasic. Fifteen minutes after applying 10 µL/L ethylene there is a rapid decrease in growth rate. This rapid decrease is followed by a plateau stage where the growth rate stalls at 0.13 mm/h. This stage is reached 30 minutes after the start of the ethylene treatment and remains for 20 minutes. Subsequently, the growth rate decreases further during 20 minutes until a second plateau is reached during which the growth rate is only about 0.03 mm/h (Binder *et al.*, 2004a). After removal of ethylene, the growth rate is re-established to pre-treatment values after 90 minutes (Binder *et al.*, 2004a). The first phase of growth inhibition has a high sensitivity to ethylene. Moreover, the magnitude and time course of the growth rate in this phase is independent of the dose of ethylene, in contrast to the recovery to pre-treatment rates, which do show a dose dependence (Binder *et al.*, 2004b). The second phase shows a lower ethylene sensitivity. The dose-response characteristics of the second phase resemble that of long term inhibition of hypocotyl growth as determined by end-point analysis after 3 to 4 days by Bleecker and co-workers (Binder *et al.*, 2004b; Chen and Bleecker, 1995; Hall *et al.*, 1999).

The involvement of ethylene in this process is also demonstrated by a genetics approach. Constitutive ethylene mutants such as the ethylene overproducers *eto1*, *eto2* and *eto3* show shorter, thicker hypocotyls when grown in the dark in the absence of ethylene (Guzman and Ecker, 1990; Kieber *et al.*, 1993). Mutants in ethylene signaling genes confirm the function of ethylene in this process. The constitutive triple response mutant *ctr1*, which harbours a mutation in a repressor of ethylene response, shows the same phenotype as *eto1* mutants (Kieber *et al.*, 1993). A null mutation in *EIN2*, makes the mutants insensitive to hypocotyl shortening under the influence of ethylene (Alonso *et al.*, 1999). Mutations in *EIN3* and *EIL1* (*EIN3 LIKE 1*) demonstrate that both of these positive regulators of the ethylene signaling pathway have overlapping but also distinct functions in hypocotyl growth repression. This is illustrated by the work of Binder and coworkers (2007) who have shown that EIN3 plays a dominant role in the phaseII response to ethylene (since *ein3-1* mutants do not display the normal second stage response); while *eil1-3* mutants show a WT response in phase II. When the *ein3-1 eil1-2* double mutant is studied, the growth rate of the hypocotyl of the mutants is indistinguishable from WT during the first hour of ethylene treatment, (Binder *et al.*, 2004b). However, after the first plateau stage, the growth rate of the double mutant rises again, even in the presence of ethylene. The response is so brief (1-2 hours) that there is almost no effect on overall hypocotyl growth (Binder *et al.*, 2004b). Both EIN3 and EIL1 can be recognized by EBF1 (EIN3 BINDING F-BOX PROTEIN) and EBF2. EBF1 and EBF2 mediate the proteasome dependent breakdown of EIN3. Loss-of-function mutants in these genes, show higher levels of EIN3 (Gagne *et al.*, 2004; Potuschak *et al.*, 2003; Guo and Ecker, 2003). Both *ebf2-3* and *35S::EIN3* seedlings show a biphasic response to ethylene, but after removal of the ethylene signal, it takes them longer to recover (Binder *et al.*, 2007). *ebf1-3* seedlings have a similar onset and strength of phase I growth inhibition as compared to the wild type, but

proceed earlier to the second phase. In contrast to *ebf2-3* and 35S::*EIN3* seedlings, *ebf1-3* does not need a longer time to recuperate from the ethylene treatment (Binder *et al.*, 2007). Overexpressors (35S driven) of both *EBF1* and *EBF2* reach the phase I plateau in approximately the same time as do wild type seedlings, but they do never reach the second phase, since they resume fast elongation, even before the removal of the ethylene signal. After the ethylene signal is removed, they again reach normal growth levels. The triple mutant *ein3-1 ebf1-3 ebf2-3* initially responds to ethylene as wild type, but then immediately proceeds to phase II. During longer ethylene treatments, a partial restoration of growth rate is noted. Likewise, the quadruple mutant *ein3-1 eil1-3 ebf1-3 ebf2-3* shows a response that is initially indistinguishable from that of wild type. This mutant line however resumes its growth rate to that observed before ethylene exposure in the presence of saturating ethylene concentrations (Binder *et al.*, 2007). The response is so brief, that it has little effect on overall hypocotyl length (Binder *et al.*, 2007) as is seen in *ein3 eil1* mutants (Binder *et al.*, 2004b).These phenotypes lead to a model in which EIN2 inhibits interaction between EBF1/2 and EIN3/EIL1. As a result of this interaction, EIN3 and EIL1 levels rise, triggering an ethylene response. In the presence of ethylene, EBF1 levels remain constant, whereas levels of EBF2 rise, lowering EIN3 and EIL1 levels even when ethylene is still present (Binder *et al.*, 2007). It was concluded that EBF1 plays a role in air and in the initial phase of signaling, while EBF2 is implicated in the later stages and in the resumption of growth after ethylene removal.

Not only ethylene is known to inhibit hypocotyl elongation of dark grown plants, cytokinins induce the same effect. This is caused by an upregulation of ethylene synthesis (Vogel *et al.*, 1998b; Chae *et al.*, 2003) as described above. Recent research indicates that reduced gibberellin sensitivity suppresses ethylene overproduction in *eto2*, supporting the existence of GA control on ACS stability (De Grauwe *et al.*, 2008).

3.3.2. Ethylene stimulates hypocotyl growth

Under low nutrient conditions, low doses of ethylene enhance hypocotyl growth (Smalle *et al.*, 1997). This phenotype can also be noticed when plants are grown on a richer medium (MS/2), albeit less pronounced (up to 130 % on MS/2 against up to 200 % elongation on low nutrient medium, as compared to controls without ethylene). The ethylene dependence of this response is corroborated by experiments with ethylene signaling mutants, and with the ethylene action inhibitors 1-MCP (1-methylcyclopropene) and $AgNO_3$. Seedlings show either absence of elongation (as for *ein2-1, etr1-3* or for the wild type in the presence of 1-MCP or $AgNO_3$) or less pronounced elongation (as for *ein3* and *ein4*) (Smalle *et al.*, 1997). The overexpression of the C-terminus of EIN2 is sufficient to restore the ethylene induced hypocotyl elongation in *ein2-5* although it does not rescue the triple response (Alonso *et al.*, 1999).

Applying IAA also stimulates hypocotyl elongation (up to 125 % of the control value). Furthermore, application of ethylene to *pin1* and *pin3* mutants induces limited hypocotyl elongation. This is confirmed by treatments of the wild type with auxin transport inhibitors. Together, these data suggest a role for auxin transport in ethylene mediated hypocotyl elongation (Vandenbussche *et al.*, 2003a). The addition of 10 µM GA_3 to low nutrient medium of light grown seedlings enhances hypocotyl length (155 %) to the same extent as 50 µM ACC (160 %) When these treatments are combined, a synergestic effect (250 % as

compared to the control) is noted. The combination of IAA and GA_3 treatments also shows an at least additive effect (Saibo *et al.*, 2003). When 0.5 µM PAC (paclobutrazol, GA biosynthesis inhibitor) is added to low nutrient medium, the hypocotyls are shorter. This effect can almost completely be reversed by adding GA_3 to the medium.

When seedlings are grown on LNM, most of the hypocotyl growth occurred during three days after imbibition (Saibo *et al.*, 2003). Upon treatment with ACC, this period is prolonged by one day. In contrast, GA does not prolong the duration of elongation, but the elongation rate between day two and three is enhanced, resulting in a longer hypocotyl. Combination of both treatments results in a synergistic effect. Both GA and ACC do not only enhance hypocotyl length by stimulating cell elongation, but also generate additional cortex cells (Saibo *et al.*, 2003). As ACC, also IAA increases the duration of elongation. Furthermore, brassinosteroids (BR) also seem to play a role in ethylene induced hypocotyl elongation. De Grauwe and coworkers (2005) showed that wild type seedlings treated with Brz2001 (brassinazole, brassinosteroid synthesis inhibitor) and *cbb1* (*cabbage 1*) and *det2* (*de-etiolated 2*) (mutants in BR biosynthesis) do not show an increase in hypocotyl length in the light when treated with ACC. Moreover, the hypocotyl elongation of *hls1* (*hookless 1*, a mutation in an ethylene response gene that modulates the localization or sensitivity to auxin in hypocotyls (Lehman *et al.*, 1996)) responds synergistically to a treatment with both EBR (epi-brassinolide) and ACC (De Grauwe *et al.*, 2005). The hypocotyl of *sax1* (*hypersensitive to abscisic acid and auxin 1*, necessary for BR biosynthesis) (Ephritikhine *et al.*, 1999) is insensitive to ACC and GA. When EBR is applied, the hypocotyl regains its sensitivity to GA, but not to ACC (Ephritikhine *et al.*, 1999). These data indicate a role for brassinosteroids downstream or independent of the ethylene signal (De Grauwe *et al.*, 2005).

3.4 The role of ethylene in the formation and exaggeration of the apical hook

The exaggeration of the apical hook of dark-grown ethylene treated seedlings is part of the triple response. The apical hook protects the shoot apical meristem against mechanical stress during germination and early seedling growth (Vandenbussche and Van Der Straeten, 2004). In seedlings grown in darkness without exogenous ethylene, endogenous ethylene is required during hook maintenance (Vandenbussche and Van Der Straeten, 2004; Raz and Ecker, 1999). Addition of ethylene to etiolated seedlings causes an exaggeration of hook curvature, a trait that is also visible in air-grown ethylene overproducing mutants (*eto1, eto2* and *eto3*) and in constitutive ethylene signaling mutants (*ctr1*) (Guzman and Ecker, 1990; Kieber *et al.*, 1993; Roman *et al.*, 1995). Conversely, in ethylene insensitive mutants the apical hook is absent (Roman *et al.*, 1995). It must be mentioned however that ethylene treatment only has an effect when applied to seedlings 60-72 hours after germination. Formation of an exaggerated apical hook also requires continuous exposure to ethylene during this period (Raz and Ecker, 1999). Different features of the triple response can be dissected by a genetic approach, as mutants that are only ethylene insensitive regarding the formation of the apical hook have been identified. An example of such a mutant is *hls1* (Lehman *et al.*, 1996).

The formation of the apical hook is a perfect example of both the growth stimulating and growth inhibiting function of ethylene. As in tropic responses, bending of the apical hook

results from faster growth on one side of the hypocotyl (convex, outer side) as compared to the other side (concave, inner side). This is also reflected in cell length: apical cells from the outer side of the hook are longer than those at the inner side (Vriezen *et al.*, 2004). The exaggerated curvature formed by the apical hook upon ethylene treatment is accompanied by a longer arc (region between the beginning of the hook where the cells on the inner side become obviously smaller than those on the outer side of the arc and the bifurcation of the vascular bundle at the cotyledons of ethylene treated seedlings) resulting both from extra cell divisions and differential elongation of the convex versus the concave side of the hook (Vriezen *et al.*, 2004).

Li and coworkers (2004) showed that in *hls1-1* the cells at the inner side of the region where the hook normally arises, elongate up to 10 times and the cells at the outer side up to 2 times the level of WT plants, resulting in equally sized cells on both sides, explaining the absence of a hook (Lehman *et al.*, 1996). *HLS1* over-expression results in an exaggerated hook (Lehman *et al.*, 1996). *HLS1* is a downstream target of ethylene-dependent transcriptional regulators and is a negative regulator of auxin response. It is equally expressed throughout the apical hook and its expression is enhanced under the influence of ethylene.

When ethylene is applied, the abundance of *ACO2* (*ACC OXIDASE 2*) mRNA is enriched in cells at the outer side of the hook. In *hls1-1* and *cop2* (*constitutive photomorphogenic 2*) mutants, the expression of *ACO2* is also enhanced by ethylene, albeit to the same extent at both sides of the region where the hook normally arises to be formed (Raz and Ecker, 1999). Assuming that higher levels of *ACO2* mRNA lead to higher levels of ACO2 protein and consequently to higher ethylene levels, a role for ethylene in cell elongation can be proposed, given the fact that cell size in the apical hook of *hls1-1* mutants is equal (Lehman *et al.*, 1996), while in the wild type the length of the arc at the inner side is about one third of that at the outer side of the hook (Vriezen *et al.*, 2004).

Using a *DR5::GFP* construct, De Grauwe and coworkers (2005) showed that auxin concentrations are higher at the inner side of the hook. This effect was even enhanced and more localised when ACC was added to the medium. Li and coworkers (2004) have shown similar results. Furthermore, these authors showed that in *hls1* this differential expression of *DR5::GUS* was absent. Lehman and coworkers (1996) treated dark-grown seedlings with the auxin transport blocker NPA (1-naphtylphtalamic acid). Seedlings grown under these conditions do not form an apical hook. Consistent with these observations, known auxin transport mutants as *pin3* (Friml *et al.*, 2002) show a reduced hook maintenance. Both *pin3-3* and 35S::*PIN1* overexpressors do not show an exaggerated hook when treated with ACC (De Grauwe *et al.*, 2005). This is mainly resulting from a reduced elongation of the outer side of the hook (Friml *et al.*, 2002). Several other auxin signaling mutants lack the formation of a normal apical hook (reviewed by Vandenbussche and Van Der Straeten (2004)). The stronger auxin accumulation at the inner side of the hook, might make this region more sensitive to ethylene or locally enhance ethylene synthesis (De Grauwe *et al.*, 2005). Therefore it may be postulated that a differential distribution of auxin, controlled by ethylene, is required in order to maintain and form the (exaggerated) apical hook (possibly by regulating ethylene sensitivity or synthesis).

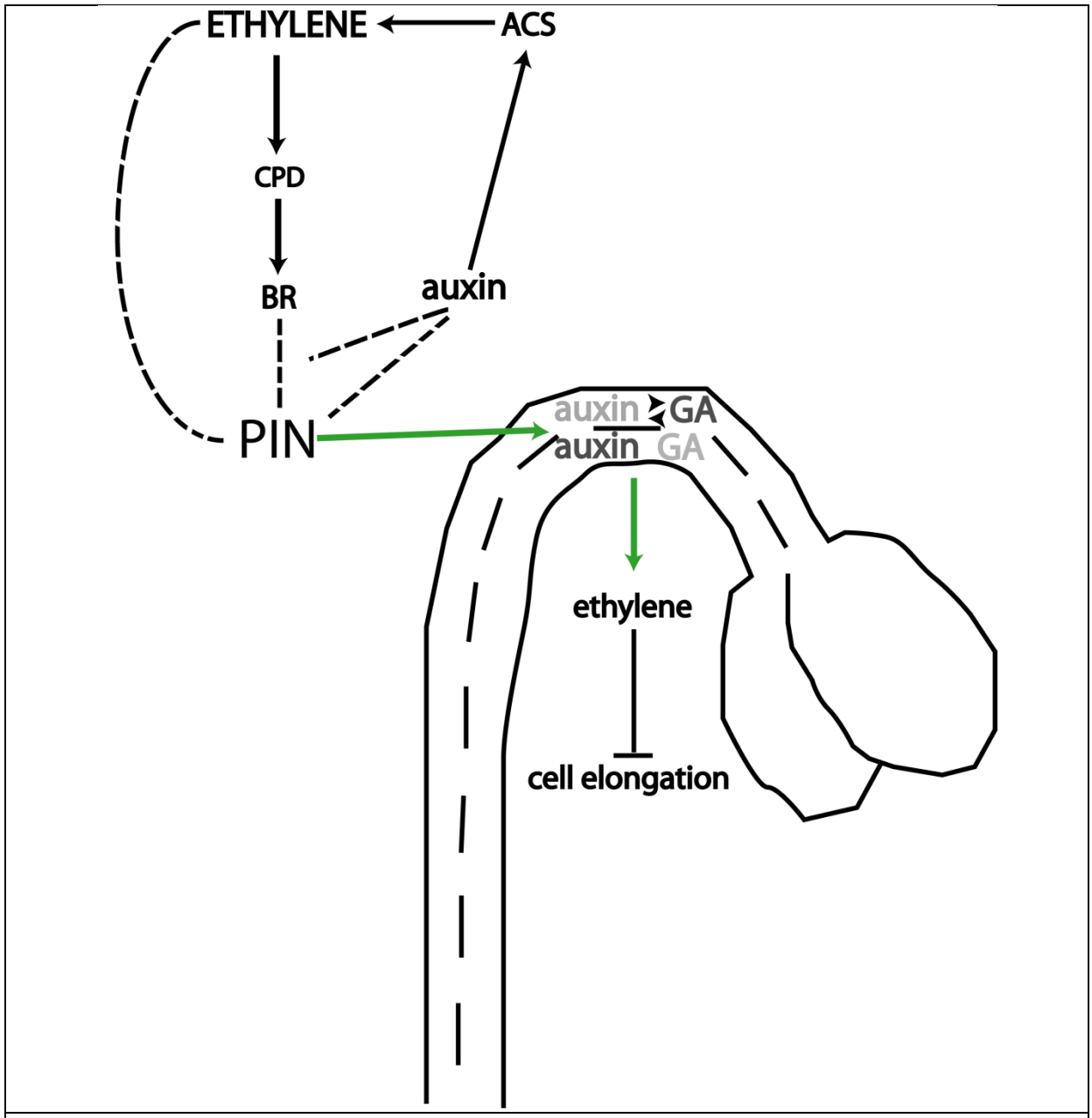

Figure 4: Formation and maintenance of the apical hook is an ethylene dependent process. Cell elongation at the outer side of the hook is related to enhanced GA sensitivity and signaling (Vriezen et al. 2004), possibly auxin-regulated. Bending is a natural consequence of the more pronounced elongation at the outer side as compared to the inner side of the hook. Shorter cells at the inner side may result from enhanced ethylene levels or enhanced ethylene sensitivity, caused by a higher auxin concentration at the inner side of the hook, a gradient which may be ethylene-regulated. The levels of auxin are differentialy regulated by the auxin efflux transporters PIN (PIN FORMED). The PINs are in turn regulated by ethylene, auxins and brassinosteroids. The regulation by ethylene can be a direct effect or caused by modulating brassinsosteroid synthesis over CPD (CONSTITUTIVE PHOTOMORPHOGENIC DWARF). Auxin could also directly regulate the PINs or may interfere with the brassinosteroid-induced regulation of PIN. Model based on De Grauwe *et al.* (2005) and Vriezen *et al.* (2004) , dotted lines represent regulations, T-bars represent repression and lines with an arrowhead represent induction.

Besides auxins, brassinosteroids are required to form the apical hook. *CPD*::*GUS* (*CONSTITUTIVE PHOTOMORPHOGENIC DWARF*; brassinosteroid marker) shows a stronger expression in the outer side of the hook. When ACC is applied, *CPD*::*GUS* expression is up-regulated on both the outer and inner side of the apical hook, but the expression is stronger on the outer side (De Grauwe *et al.*, 2005). When both ACC and NPA are added, staining is only visible in the stele. Seedlings that are grown on IAA stain uniformly in the region where the hook is supposed to arise. Phenotypical proof for a role for brassinosteroids in apical hook formation is given by the BR biosynthesis mutants *cbb1* (Kauschmann *et al.*, 1996) and *det2* (Chory *et al.*, 1991). These mutants lack a normal hook when grown in the dark, although the other traits of the triple response are still visible (De Grauwe *et al.*, 2005). Treatment of auxin inducible GUS reporter lines with the combination of ACC and EBR, shows a reduction of the exaggerated hook and a more homogenous distribution of auxin reporter gene expression. These data suggest that BR work downstream or independent of ethylene to establish a differential distribution of auxin (De Grauwe *et al.*, 2005). Seedlings that are grown in the presence of PAC lack the apical hook (Achard *et al.*, 2003; Vriezen *et al.*, 2004; Alabadi *et al.*, 2004), as do GA biosynthesis mutants, indicative for an essential role for GA in apical hook maintenance. Inactivation of the DELLA proteins RGA and GAI (negative regulators of GA signaling) fully restored apical hook formation in a *ga1-3* background (Achard *et al.*, 2003; Alabadi *et al.*, 2004). Together these data strongly support a function of gibberellins in hook formation. ACC enhances the stimulating effect of GA on cell division in the formation of an exaggerated apical hook of etiolated seedlings, and stabilizes RGA, resulting in smaller cells (Vriezen *et al.*, 2004). A stronger up-regulation of *GASA1::GUS* (a GA reporter line (Raventos *et al.*, 2000)) is noticed at the outer side of the hook. This differential expression seems to be independent of RGA, since no significant difference in stabilization of RGA between the outer or inner side of the hook can be noted. A possible explanation of this result may lay in tissue specific differences in ethylene or gibberellin sensitivity (Vriezen *et al.*, 2004). A simplified model of the interactions in the formation of the apical hook is shown in fig. 4.

3.5 The role of ethylene in stem growth

3.5.1. Ethylene represses stem growth

Ethylene represses stem growth. One of the most conspicuous examples of this shoot growth repressing function is seen in *ctr1* which displays a stunted and thick inflorescence stem (Kieber *et al.*, 1993). Likewise, a tobacco ethylene overproducing mutant shows a shorter stem, resulting from shorter internodes (Knoester *et al.*, 1997).

Ethylene does not only repress stem growth, but also leaf growth. In wild type plants, *ACS1* mRNA levels are lower during leaf surface expansion than during leaf emergence or senescence. ACC has been shown to stimulate leaf emergence (Smalle and Van Der Straeten, 1997) and *ACS* mRNA levels can be associated with ethylene levels (as suggested by ethylene measurements at the time of the emergence of the cotyledons and the first leaf pair) (Smalle *et al.*, 1999). Together, these data point to a growth repressing role of ethylene in leaf growth (Smalle *et al.*, 1999). The leaf surface of constitutive ethylene mutants (*ctr1*) is smaller than that of WT (Kieber *et al.*, 1993). This reduction in leaf size is primarily caused by a

repression of cell growth since both *ctr1* or ethylene treated WT plants show smaller epidermal leaf cells (Kieber *et al.*, 1993; Rodrigues-Pousada *et al.*, 1993). Ethylene insensitive mutants on the other hand show larger leaves. It can be assumed that this is correlated with occurrence of larger leaf cells, but no direct measurements are available (Bleecker *et al.*, 1988; Hua *et al.*, 1995). In conclusion, ethylene seems to be required for leaf emergence, but later on, ethylene levels are lowered, leading to a relief of ethylene-induced repression of cell expansion.

3.5.2. Ethylene stimulates stem growth

As seen in other organs, ethylene also plays a dual role in stem growth. Besides its role in repressing leaf expansion and stem growth, ethylene is required for optimal shade avoidance by stimulating growth (Pierik *et al.*, 2004a; Pierik *et al.*, 2004b; Vandenbussche *et al.*, 2003b). It is shown that ethylene induces an upward movement of *Arabidopsis* leaves, caused by a differential growth at the end of both petiole and leaf blade (albeit the response varies depending on the conditions) (Millenaar *et al.*, 2005; Vandenbussche *et al.*, 2005).

When shaded, plants will try to catch as much light as possible in order to maximise photosynthesis (Vandenbussche *et al.*, 2005). Since plants are non-mobile and *Arabidopsis* is a rosette plant, shaded plants can only reach out of the shade by changing the angle of their leaves and by petiole growth. Plants grown under a dense canopy, are not only confronted with a quantitative change in light (the light doses lower), but also the spectrum alters, with a decrease in the ratio of red / far red light (shifts towards far red when shaded) (Vandenbussche *et al.*, 2005). In tobacco, this shift of spectrum is sufficient to induce hyponasty independent of ethylene. Ethylene however determines the rate of stem and petiole elongation under these conditions (Pierik *et al.*, 2004b). Furthermore it is shown that under aberrant R/FR conditions ethylene production is enhanced (Pierik *et al.*, 2004a; Pierik *et al.*, 2004b; Vandenbussche *et al.*, 2003b). Lowering the light dose cannot enhance stem elongation or induce a hyponastic response in ethylene resistant seedlings (Pierik *et al.*, 2004b). This dose dependent response is a blue light response (Pierik *et al.*, 2004b).

As seen in other processes, ethylene interacts with other hormones in this process. When GA-synthesis is blocked in tobacco, ethylene-induced hyponastic movements and stem and petiole elongation are prevented, indicating an essential role of GA in these responses. However, when the ratio of R/FR light is altered in the presence of PAC, stem and petiole elongation is inhibited, but hyponastic movements still occur. In the wild type and in ethylene resistant tobacco mutants, petiole elongation seems to be equally sensitive to GA. Stem elongation however is more sensitive for GA in wild type than in ethylene resistant tobacco mutants. Both processes are more pronounced in low R:FR ratios (Pierik *et al.*, 2004a). These data support a model in which ethylene stimulates hyponasty in a GA dependent manner. A changing R/FR ratio however can induce hyponasty both in an ethylene dependent and independent way. Nevertheless, the elongation response is strictly dependent of GA.

3.6 Conclusions

In this review, several examples of differential responses of tissues to ethylene have been illustrated. There is clearly no simple way to predict how a tissue will react to ethylene. Ethylene exerts its function based on multiple inputs (internal, environmental and species-specific) that all together determine the reaction of tissues to ethylene. Ethylene responses should therefore not be seen as growth stimulating or growth repressing, but as growth modulating. In addition, it is important to keep in mind that hormones act in a tight network, wherein ethylene cannot exert its effect without the functionality of other hormonal pathways. Subsets of response genes with similar expression patterns can in part explain the different ethylene responses. Nonetheless, further research is needed in order to identify the factors that modulate these tissue specific ethylene responses.

Besides the factors mentioned above, the ethylene concentration may also be of great importance. Several genes have already been shown to respond differentially to different ethylene concentrations. De Paepe (De Paepe, 2004; De Paepe *et al.*, 2004) isolated a group of 30 genes that show a stronger up-regulation when treated with 10 µl/l ethylene than when treated with 0.1 µl/l ethylene. Seven other genes showed a stronger up-regulation with the lower ethylene concentration. Thus, dose dependent ethylene responses deserve further exploration in the future.

3.7 Addendum

Since August 2012 the exact mechanism by which EIN exerts its function has been elucidated by Qia *et al.* (2012). A brief update can be found in figure 5.

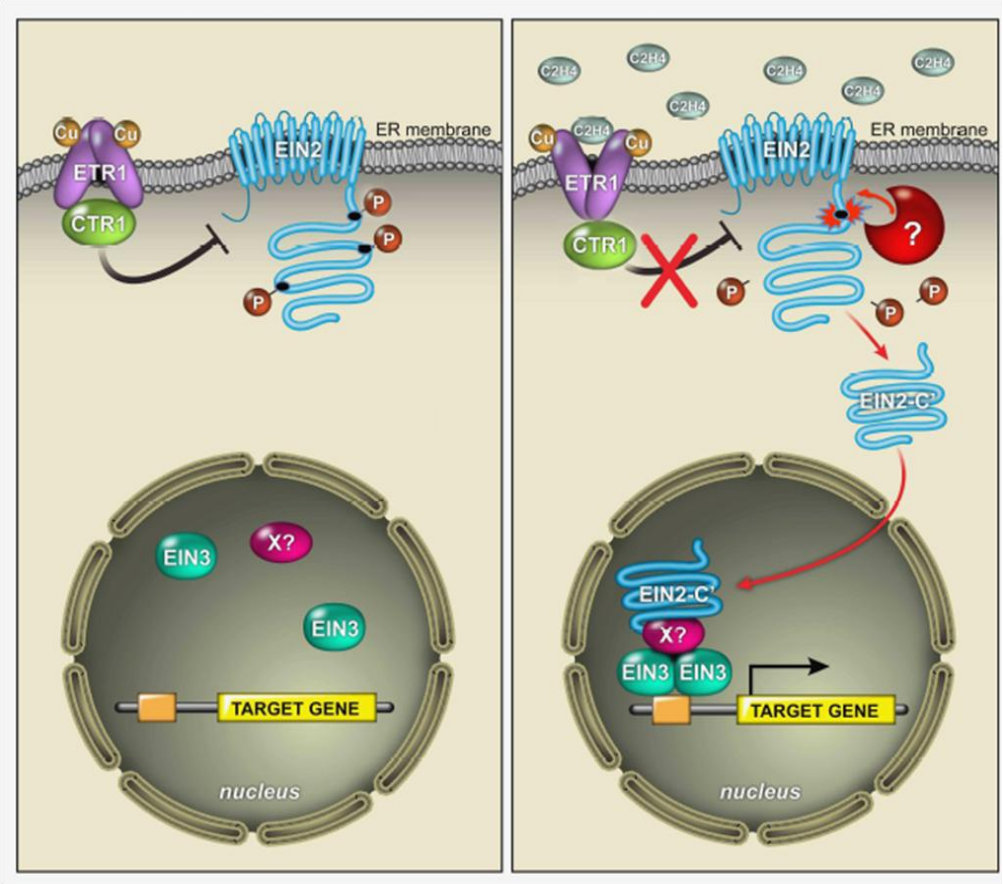

Figure 5: functiononal mechanism of EIN2
(Left panel) Model for the phosphorylation-dependent proteolysis and ER to nucleus translocation of EIN2 C' polypeptide in response to ethylene. In the absence of hormone, EIN2 is localized in the ER and shows CTR1-dependent phosphorylation, resulting in suppression of ethylene responses.
(Right panel) Upon the perception of ethylene gas in the ER by the ethylene receptor ETR1(*27*), dephosphorylation of EIN2 leads to proteolytic cleavage and release of a large carboxyl-terminal fragment (EIN2-C'), which rapidly translocates to the nucleus and activates EIN3 /EIL1-dependent transcription through direct or indirect interaction with EIN3/EIL1. Adapted from Qia *et al.* (2012)

3.8 References

Abel, S., Nguyen, M.D., Chow, W. and Theologis, A. (1995) *ASC4*, a Primary Indoleacetic Acid-Responsive Gene Encoding 1-Aminocyclopropane-1-Carboxylate Synthase in *Arabidopsis-thaliana*. J. Biol. Chem. **270**, 26020-26020.

Abeles, F.B., Morgan, P.W. and Saltveit, M.E. (1992) Ethylene in Plant Biology, Academic Press, San Diego.

Achard, P., Vriezen, W.H., Van Der Straeten, D. and Harberd, N.P. (2003) Ethylene regulates *Arabidopsis* development via the modulation of DELLA protein growth repressor function. Plant Cell **15**: 2816-2825.

Adams, D.O. and Yang, S.F. (1979) Ethylene Biosynthesis - Identification of 1-Aminocyclopropane-1-Carboxylic Acid as an Intermediate in the Conversion of Methionine to Ethylene. Proceedings of the National Academy of Sciences of the United States of America **76**, 170-174.

Alabadi, D., Gil, J., Blazquez, M.A. and Garcia-Martinez, J.L. (2004) Gibberellins repress photomorphogenesis in darkness Plant Physiology **134**, 1050-1057.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker JR. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284**, 2148-2152.

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M. and Ecker, J. R. (2003) Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **100**, 2992-2997.

Berger, S., Bell, E. and Mullet, J.E. (1996) Two methyl jasmonate-insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. Plant Physiology **111**, 525-531.

Binder, B.M., O'Malley, R.C., Wang, W.Y., Moore, J.M., Parks, B.M., Spalding, E.P. and Bleecker, A.B. (2004a) *Arabidopsis* seedling growth response and recovery to ethylene. A kinetic analysis. Plant Physiology **136,** 2913-2920.

Binder, B.M., Mortimore, L.A., Stepanova, A.N., Ecker, J.R. and Bleecker, A.B. (2004b) Short-term growth responses to ethylene in *Arabidopsis* seedlings are EIN3/EIL1 independent. Plant Physiology **136**, 2921-2927.

Binder, B.M.,, Walker, J.M., Gagne, J.M., Emborg, T.J., Hemmann, G., Bleecker, A.B. and Vierstra, R.D. (2007) The *Arabidopsis* EIN3 binding F-box proteins EBF1 and EBF2 have distinct but overlapping roles in ethylene signaling. Plant Cell **19** 509-523.

Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W. and Benfey, P.N. (2003) A gene expression map of the *Arabidopsis* root. Science **302**, 1956-1960.

Bleecker, A.B., Estelle, M.A., Somerville, C. and Kende, H. (1988) Insensitivity to Ethylene Conferred by a Dominant Mutation in *Arabidopsis-thaliana.* Science **241**, 1086-1089.

Capitani, G., Hohenester, E., Feng, L., Storici, P., Kirsch, J.F. and Jansonius, J.N. (1999) Structure of 1-aminocyclopropane-1-carboxylate synthase, a key enzyme in the biosynthesis of the plant hormone ethylene. Journal of Molecular Biology **294**, 745-756.

Chae, H.S., Faure, F. and Kieber, J.J. (2003) The eto1, eto2, and eto3 mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. Plant Cell **15**, 545-559.

Chang, C.R. (2003) Ethylene signaling: the MAPK module has finally landed. Trends in Plant Science **8**, 365-368.

Chen, Q.H.G. and Bleecker, A.B. (1995) Analysis of Ethylene Signal-Transduction Kinetics Associated with Seedling-Growth Response and Chitinase Induction in Wild-Type and Mutant *Arabidopsis.* Plant Physiology **108**, 597-607.

Chen, Y.F., Randlett, M.D., Findell, J.L. and Schaller, G.E. (2002) Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of *Arabidopsis*. Journal of Biological Chemistry **277**, 19861-19866.

Cheng, J.C., Seeley, K.A. and Sung, Z.R. (1995) Rml1 and Rml2, *Arabidopsis* Genes Required for Cell-Proliferation at the Root-Tip. Plant Physiology **107**, 365-376.

Chory, J., Nagpal, P. and Peto, C.A. (1991) Phenotypic and Genetic-Analysis of *Det2*, a New Mutant That Affects Light-Regulated Seedling Development in *Arabidopsis.* Plant Cell **3**, 445-459.

De Cnodder, T., Vissenberg, K., Van Der Straeten, D. and Verbelen, J.P. (2005) Regulation of cell length in the *Arabidopsis thaliana* root by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid: a matter of apoplastic reactions. New Phytologist **168**, 541-550.

De Grauwe, L., Vandenbussche, F., Tietz, O., Palme, K. and Van Der Straeten, D. (2005) Auxin, ethylene and brassinosteroids: Tripartite control of growth in the *Arabidopsis* hypocotyl. Plant and Cell Physiology **46**, 827-836.

De Grauwe, L., Chaerle, L., Dugardeyn, J., Decat, J., Rieu, I., Vriezen, W., Moritz, T., Beemster, G., Phillips, A., Harberd, N.P., Hedden, P. and Van Der Straeten, D. (2008) Reduced gibberellin response affects ethylene biosynthesis and responsiveness in the *Arabidopsis* gai eto2-1 double mutant. New Phytologist **177**,128-141.

De Paepe A. (2004) Study of ethylene signaling in *Arabidopsis thaliana* by transcript profiling and mutant analysis. PhD thesis Ghent University.

De Paepe, A., Vuylsteke, M., Van Hummelen, P., Zabeau, M. and Van Der Straeten D. (2004) Transcriptional profiling by cDNA-AFLP and microarray analysis reveals novel insights into the early response to ethylene in *Arabidopsis*. Plant Journal **39**, 537-559.

De Paepe, A. and Van Der Straeten D. (2005) Ethylene Biosynthesis and Signaling: An Overview. Vitamins and Hormones **72** 399-430.

Dill, A. and Sun, T.P. (2001) Synergistic derepression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana.* Genetics **159**, 777-785.

Dolan, L, Duckett, C.M., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, S. and Roberts, K. (1994) Clonal Relationships and Cell Patterning in the Root Epidermis of *Arabidopsis.* Development **120**, 2465-2474.

Ephritikhine, G., Fellner, M., Vannini, C., Lapous, D. and Barbier-Brygoo, H. (1999) The *sax1* dwarf mutant of *Arabidopsis thaliana* shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid. Plant Journal **18**, 303-314.

Friml J., Wisniewska, J., Benkova, E., Mendgen, K. and Palme, K. (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis.* Nature **415,** 806-809.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12**, 393-404.

Gagne, J.M., Smalle, J., Gingerich, D.J., Walker, J.M., Yoo, S.D., Yanagisawa, S. and Vierstra, R.D. (2004) *Arabidopsis* EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proceedings of the National Academy of Sciences of the United States of America **101**, 6803-6808.

Galway, M.E., Masucci, J.D., Lloyd, A.M., Walbot, V., Davis, R.W. and Schiefelbein, J.W. (1994) The Ttg Gene Is Required to Specify Epidermal-Cell Fate and Cell Patterning in the *Arabidopsis* Root. Dev. Biol. **166**, 740-754.

Gao, Z.Y., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.L., Kieber, J.J. and Schaller, G.E. (2003) Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of *Arabidopsis* through participation in ethylene receptor signaling complexes. Journal of Biological Chemistry **278**, 34725-34732.

Gendreau, E, Traas, J., Desnos, T., Grandjean, O., Caboche, M. and Hofte, H. (1997) Cellular basis of hypocotyl growth in *Arabidopsis thaliana.* Plant Physiology **114**, 295-305.

Gomez-Lim, M.A., Valdeslopez, V., Cruzhernandez, A. and Saucedoarias, L.J. (1993) Isolation and Characterization of a Gene Involved in Ethylene Biosynthesis from *Arabidopsis-Thaliana*. Gene **134**, 217-221.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001) Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. Nature **414**, 271-276.

Guo, H.W. and Ecker, J.R. (2003) Plant responses to ethylene gas are mediated by SCF (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115**, 667-677.

Guzman, P. and Ecker, J.R. (1990) Exploiting the Triple Response of *Arabidopsis* to Identify Ethylene-Related Mutants. Plant Cell **2**, 513-523.

Hall, A.E., Chen, Q.H.G., Findell, J.L., Schaller, G.E. and Bleecker, A.B. (1999) The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiology **121**, 291-299.

Hall, A.E. and Bleecker, A.B. (2003) Analysis of combinatorial loss-of-function mutants in the *Arabidopsis* ethylene receptors reveals that the ers1etr2 double mutant has severe developmental defects that are EIN2 dependent. Plant Cell **15**, 2032-2041.

Hua, J., Chang, C., Sun, Q. and Meyerowitz, E.M. (1995) Ethylene Insensitivity Conferred by *Arabidopsis* Ers Gene. Science **269**, 1712-1714.

Hua, J. and Meyerowitz, E.M. (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana.* Cell **94**, 261-271.

Joo, S., Seo, Y.S., Kim, S.M., Hong, D.K., Park, K.Y. and Kim, W.T. (2006)Brassinosteroid induction of *AtACS4* encoding an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase 4 in *Arabidopsis* seedlings Physiol. Plant. **126**, 592-604.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L. and Altmann, T. (1996) Genetic evidence for an essential role of brassinosteroids in plant development. Plant Journal **9**, 701-713.

Kende, H. (1993) Ethylene Biosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **44**, 283-307.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker J.R. (1993) Ctr1, a Negative Regulator of the Ethylene Response Pathway in *Arabidopsis*, Encodes a Member of the Raf Family of Protein-Kinases. Cell **72**, 427-441.

King, J.J., Stimart, D.P., Fisher, R.H. and Bleecker, A.B. (1995) A mutation altering auxin homeostasis and plant morphology in *Arabidopsis.* Plant Cell **7**, 2023-2037.

King, K.E., Moritz, T. and Harberd, N. P. (2001) Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. Genetics **159**, 767-776.

Knoester, M., Linthorst, H.J.M., Bol, J.F. and vanLoon, L.C. (1997) Modulation of stress-inducible ethylene biosynthesis by sense and antisense gene expression in tobacco. Plant Science **126**, 173-183.

Konings, H. and Jackson, M.B. (1979) Relationship between Rates of Ethylene Production by Roots and the Promoting or Inhibiting Effects of Exogenous Ethylene and Water on Root Elongation. Zeitschrift Fur Pflanzenphysiologie **92**, 385-397.

Le, J., Vandenbussche, F., De Cnodder, T., Van Der Straeten, D. and Verbelen, J.P. (2005) Cell elongation and microtubule behavior in the *Arabidopsis* hypocotyl: Responses to ethylene and auxin. Journal of Plant Growth Regul. **24**, 166-178.

Le, J., Vandenbussche, F., Van Der Straeten, D. and Verbelen, J.P. (2001) In the early response of *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiology **125**, 519-522.

Le, J., Vandenbussche, F., Van Der Straeten, D. and Verbelen, J.P. (2004) Position and cell type-dependent microtubule reorientation characterizes the early response of the *Arabidopsis* root epidermis to ethylene. Physiologia Plantarum **121**, 513-519.

Li, H., Johnson, P., Stepanova, A.N., Alonso, M. and Ecker J.R (2004) Convergence of signaling of differential cell growth pathways in the control in *Arabidopsis.* Dev. Cell **7**, 193-204.

Lehman, A., Black, R. and Ecker J.R (1996) *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. Cell **85**, 183-194.

Luschnig, C., Gaxiola, R.A., Grisafi, P. and Fink, G.R. (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana.* Genes & Development **12**, 2175-2187.

Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C. and Bennett, M. J. (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. Embo Journal **18**, 2066-2073.

Masucci, J.D. and Schiefelbein, J.W. (1994) The Rhd6 Mutation of *Arabidopsis-thaliana* Alters Root-Hair Initiation through an Auxin-Associated and Ethylene-Associated Process. Plant Physiology **106**, 1335-1346.

Masucci, J.D. and Schiefelbein, J.W. (1996) Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. Plant Cell **8**, 1505-1517.

Masucci, J.D., Rerie, W.G., Foreman, D.R., Zhang, M., Galway, M.E., Marks, M.D. and Schiefelbein, J.W. (1996) The homeobox gene *GLABRA 2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana.* Development **122**, 1253-1260.

Millenaar, F.F., Cox, M.C.H., van Berkel, Y., Welschen, R.A.M., Pierik, R., Voesenek, L.A. and Peeters, A.J.M. (2005) Ethylene-induced differential growth of petioles in *Arabidopsis*. Analyzing natural variation, response kinetics, and regulation. Plant Physiology **137**, 998-1008.

Miyazaki, . J.H. and Yang, S. F. (1987) Inhibition of the Methionine Cycle Enzymes. Phytochemistry **26,** 2655-2660.

Neljubov, D. (1901) Uber die horizontale Nutation der Stengel von Pisum sativum und einiger Anderer Pflanzen. Beih. Bot. Zentralb. **10**, 128-139.

Olmedo, G., Guo, H.W., Gregory, B.D., Nourizadeh, S.D., Aguilar-Henonin, L., Li, H.J., An, F.Y., Guzman, P. and Ecker J.R (2006) *ETHYLENE-INSENSITIVE5* encodes a 5 '-> 3 ' exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2. Proceedings of the National Academy of Sciences of the United States of America **103**, 13286-13293.

Ortega-Martinez, O., Pernas, M., Carol, R.J. and Dolan, L. (2007). Ethylene modulates stem cell division in the *Arabidopsis thaliana* root. Science **317**, 507-510.

Ouaked, F., Rozhon, W., Lecourieux, D. and Hirt, H. (2003) A MAPK pathway mediates ethylene signaling in plants. Embo Journal **22**, 1282-1288.

Peng, J.R., Carol, P. Richards, D. E. King, K. E. Cowling, R. J. Murphy, G. P. and Harberd, N. P. (1997) The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes & Development **11**, 3194-3205.

Pierik, R., Cuppens, M.L.C., Voesenek, L. and Visser, E.J.W. (2004a) Interactions between ethylene and gibberellins in phytochrome-mediated shade avoidance responses in tobacco. Plant Physiology **136**, 2928-2936.

Pierik R, Whitelam GC, Voesenek L, de Kroon H, Visser EJW. 2004b. Canopy studies on ethylene-insensitive tobacco identify ethylene as a novel element in blue light and plant-plant signaling. Plant Journal **38**, 310-319.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, S., Genschik, P. (2003) EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F-box proteins: EBF1 and EBF2. Cell **115**, 679-689.

Potuschak, T., Vansiri, A., Binder, A.B.M., Lechner, E., Vierstra, R.D. and Genschik, P. (2006) The Exoribonuclease XRN4 is a Component of the Ethylene Response Pathway in *Arabidopsis.* Plant Cell **18**, 3047-3057.

Qia, H., Shen, Z., Huang, S.C., Schmitz, R.J., Urich, M.A., Briggs, S.P. and Ecker, J.R. (2012) Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. Science **338**, 390-393.

Radwanski, E.R. and Last, R.L. (1995) Tryptophan Biosynthesis and Metabolism - Biochemical and Molecular-Genetics. Plant Cell **7**, 921-934.

Ravanel, S., Gakiere, B., Job, D. and Douce, R. (1998) The specific features of methionine biosynthesis and metabolism in plants. Proceedings of the National Academy of Sciences of the United States of America **95**, 7805-7812.

Raventos, D., Meier, C. Mattsson, O., Jensen, A.B., and Mundy, J. (2000) Fusion genetic analysis of gibberellin signaling mutants. Plant Journal **22**, 427-438.

Raz, V. and Ecker, J.R. (1999) Regulation of differential growth in the apical hook of *Arabidopsis*. Development **126**, 3661-3668.

Roberts, K.J. and Oparka (2003) Plasmodesmata and the control of symplastic transport. Plant Cell Environ. **26**, 103-124.

Rodrigues-Pousada, R., Derycke, R., Dedonder, A., Vancaeneghem, W., Engler, G., Vanmontagu, M. and Van Der Straeten, D. (1993) The *Arabidopsis* 1-Aminocyclopropane-1-Carboxylate Synthase Gene-1 Is Expressed During Early Development. Plant Cell **5**, 897-911.

Rodrigues-Pousada, R., Vancaeneghem, W., Chauvaux, N., Van Onekelen, H., Vanmontagu, M. and Van Der Straeten, D. (1999) Hormonal cross-talk regulates the *Arabidopsis thaliana* 1-aminocyclopropane-1-carboxylate synthase gene 1 in a developmental and tissue-dependent manner. Physiologia Plantarum **105**, 312-320.

Roman, G, Lubarsky, B., Kieber, J.J., Rothenberg, M. and Ecker J.R. (1995) Genetic-Analysis of Ethylene Signal-Transduction in *Arabidopsis-thaliana* - 5 Novel Mutant Loci Integrated into a Stress-Response Pathway Genetics **139**, 1393-1409. **Ruzicka, K., Ljung, K., Vanneste, S., Podhorska, R., Beeckman, T., Friml, J. and Benkova, E.** (2007) Ethylene

regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell **19**, 2197-2212.

Saibo, N.J.M., Vriezen, W.H., Beemster, G.T.S. and Van Der Straeten D. (2003) Growth and stomata development of *Arabidopsis* hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. Plant Journal **33**, 989-1000.

Schnabelrauch, L.S., Kieliszewski, M., Upham, B.L., Alizedeh, H. and Lamport, D.T.A. (1996) Isolation of pl 4.6 extensin peroxidase from tomato cell suspension cultures and identification of Val-Tyr-Lys as putative intermolecular cross-link site. Plant Journal **9**, 477-489.

Silverstone, A.L., Ciampaglio, C.N. and Sun, T.P. (1998) The *Arabidopsis RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway Plant Cell **10**, 155-169.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M. and Van Der Straeten, D. (1997) Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. Proceedings of the National Academy of Sciences of the United States of America **94**, 2756-2761.

Smalle, J. and Van Der Straeten, D. (1997). Ethylene and vegetative development. Physiologia Plantarum **100**, 593-605.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M. and Van Der Straeten, D. (1997) Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. Proceedings of the National Academy of Sciences of the United States of America **94**, 2756-2761.

Smalle, J., Haegman, M., Mertens, J., Vangronsveld, J., Van Montagu, M. and Van Der Straeten, D. (1999) The expression pattern of the *Arabidopsis* ACC synthase gene 1 during rosette leaf development. Journal of Experimental Botany **50**, 1561-1566.

Solano, R., Stepanova A.N., Chao, Q.M., Ecker, J.R. (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes & Development **12**, 3703-3714.

Staswick, P.E. and Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis.* Plant Cell **16**, 2117-2127.

Stepanova, A.N. and Alonso, J.M. (2005) Ethylene signaling and response pathway: a unique signaling cascade with a multitude of inputs and outputs. Physiologia Plantarum **123**, 195-206.

Stepanova, A.N., Hoyt, J.M., Hamilton A.A. and Alonso, J.M. (2005) A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in *Arabidopsis.* Plant Cell **17**, 2230-2242.

Stepanova, A.N., Yun, J., Likhacheva, A.V. and Alonso, J.M. (2007) Multilevel interactions between ethylene and auxin in *Arabidopsis* roots. Plant Cell **19**, 2169-2185.

Su, W.P. and Howell, **S.H.** (1992) A Single Genetic-Locus, Ckr1, Defines *Arabidopsis* Mutants in Which Root-Growth Is Resistant to Low Concentrations of Cytokinin. Plant Physiology **99**, 1569-1574.

Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K. and Bennett, M. (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. Genes & Development **15**, 2648-2653.

Swarup, R., Kramer, E.M., Perry, P., Knox, K., Leyser, H.M.O., Haseloff, J., Beemster, G.T.S., Bhalerao, R. and Bennett, M.J. (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nature Cell Biology **7**, 1057-1065.

Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G.T.S., Sandberg, G., Bhalerao, R., Ljung, K. and Bennett, M.J. (2007) Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. Plant Cell **19**, 2186-2196.

Tsuchisaka, A. and Theologis, A. (2004) Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiology **136**, 2982-3000.

Vandenbussche, F., Smalle, J., Le, J., Saibo, N.J.M., De Paepe, A., Chaerle, L., Tietz, O., Smets, R., Laarhoven, L.J.J., Harren, F.J.M., Van Onckelen, H., Palme, K., Verbelen, J.P. and Van Der Straeten, D. (2003a) The *Arabidopsis* mutant *alh1* illustrates a cross talk between ethylene and auxin. Plant Physiology **131**, 1228-1238.

Vandenbussche, F., Vriezen, W.H., Smalle, J., Laarhoven, L.J.J., Harren, F.J.M. and Van Der Straeten, D. (2003b) Ethylene and auxin control decreased light intensity. Plant Physiology **133**, 517-527.

Vandenbussche, F. and Van Der Straeten, D. (2004) Shaping the shoot: a circuitry that integrates multiple signals. Trends in Plant Science **9**, 499-506.

Vandenbussche, F., Pierik, R., **Millenaar, F.F., Voesenek, L.A. and Van Der Straeten, D**. (2005) Reaching out of the shade Current Opinions in Plant Biology **8**, 462-468.

Van Der Straeten, D., Rodrigues-Pousada, R.A., Villarroel, R., Hanley, S., Goodman, H.M. and Van Montagu, M. (1992) Cloning, genetic mapping, and expression analysis of an *Arabidopsis thaliana* gene that encodes 1 aminocyclopropane 1 carboxylate synthase. Proceedings of the National Academy of Sciences of the United States of America **89**, 9969-9973.

Visser, E.J.W. and Pierik, R. (2007) Inhibition of root elongation by ethylene in wetland and non-wetland plant species and the impact of longitudinal ventilation. Plant Cell Environ. **30**, 31-38.

Vogel, J.P., Woeste, K.E., Theologis, A. and Kieber, J.J. (1998a) Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. Proceedings of the National Academy of Sciences of the United States of America **95**, 4766-4771.

Vogel, J.P., Schuerman, P., Woeste, K.E., Brandstatter, I. and Kieber, J.J. (1998b) Isolation and characterization of *Arabidopsis* mutants defective in the induction of ethylene biosynthesis by cytokinin. Genetics **149**, 417-427.

Vriezen, W.H., Achard, P., Harberd, N.P. and Van Der Straeten, D. (2004). Ethylene-mediated enhancement of apical hook formation in etiolated *Arabidopsis thaliana* seedlings is gibberellin dependent. Plant Journal **37**, 505-516.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D. and Gray J.C. (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. Plant Cell **11**, 1337-1349.

Wang, K.L.C., Yoshida, H., Lurin, C. and Ecker J.R (2004) Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. Nature **428**, 945-950.

Wang, N.N., Shih, M.C. and Li, N. (2005) The GUS reporter-aided analysis of the promoter activities of *Arabidopsis* ACC synthase genes *AtACS4*, *AtACS5*, and *AtACS7* induced by hormones and stresses, Journal of Experimental Botany **56**, 909-920.

Yamagami, T., Tsuchisaka, A., Yamada, K., Haddon, W. F., Harden, L.A. and Theologis, A. (2003) Biochemical diversity among the 1-amino-cyclopropane-1-carboxylate synthase isozymes encoded by the *Arabidopsis* gene family J. Biol. Chem. **278**, 49102-49112.

Yamane, H., Takagi, H., Abe, H., Yokota, T. and Takahashi, N. (1981) Identification of Jasmonic Acid in 3 Species of Higher-Plants and Its Biological-Activities. Plant Cell Physiology **22**, 689-697.

Yang, S.F. and Hoffman, N.E. (1984) Ethylene Biosynthesis and Its Regulation in Higher-Plants. Annual Review of Plant Physiology and Plant Molecular Biology **35**, 155-189.

Zhu, C.H., Gan, L. J. Shen, Z. G. and Xia, K. (2006) Interactions between jasmonates and ethylene in the regulation of root hair development in *Arabidopsis.* Journal of Experimental Botany 57, 1299-1308..

Chapter 4:

Ethylene-gibberellin cross-talk

Adapted from

To grow or not to grow: what can we learn on ethylene–gibberellin cross-talk by in silico gene expression analysis?

Jasper Dugardeyn, Filip Vandenbussche and Dominique Van Der Straeten*

Ethylene and gibberellins (GAs) are known to influence plant growth by mutual cross-talk and by interaction with other hormones. Transcript meta-analysis shows that GA and ethylene metabolism genes are expressed in the majority of plant organs. Both GAs and the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) may thus be synthesized ubiquitously. Transport of both hormones has been described and might hence lead to a controlled distribution. Transcript meta-analysis also suggests that applying exogenous ethylene to plants represses the expression of GA metabolism genes. Conversely, upon treatment with GAs, the expression of some ethylene synthesis genes is up-regulated. The analysis further shows that the genes coding for signaling components of these hormones are expressed throughout the entire plant. However, a tissue-specific transcript meta-analysis of ethylene synthesis and signaling genes in *Arabidopsis* roots suggests a more localized function of ethylene in the fast elongation and specialization zone, while GA seems to act in the (pro)meristematic zone and in the transition zone. Recent research has shown that brassinosteroids and auxins exert their function at the epidermis, consequently driving organ growth. From transcript meta-analysis data of *Arabidopsis* roots, it appears that GAs might also act in a cell type-specific manner.

Journal of Experimental Botany , 2008 ;59(1) : 1-16
DOI:10.1093/jxb/erm349

Jasper Dugardeyn wrote the first draft with the exception of the paragraph 4.5.2

4.1 Introduction

During development, plants are highly dependent on hormonal interplay for determination of their architecture and plasticity in response to environmental changes (reviewed by Vandenbussche and Van Der Straeten, 2004). Auxins are primary regulators of plant form (reviewed by Friml, 2003). Gibberellins (GAs) and brassinosteroids (BRs) stimulate elongation while ethylene most often leads to an inhibition of longitudinal expansion and promotes radial outgrowth (reviewed by Vandenbussche and Van Der Straeten, 2007). Insight into the mechanisms of hormonal interactions has expanded in recent years, often indicating cross-regulation of the stability of key factors in either biosynthesis or signal transduction (Achard *et al.*, 2003, 2006; Chae *et al.*, 2003; Fu and Harberd, 2003; Vriezen *et al.*, 2004).

Hormones were long thought to exert their effects on a whole-organ basis. However, recent evidence points towards a cell type-specific action of both auxins and BRs exerting their effects at the level of the epidermis, consequently driving whole-organ growth (Swarup *et al.*, 2005; Savaldi-Goldstein *et al.*, 2007), while it is believed that their synthesis also occurs in other cell types. It is not known whether this is a general mechanism that applies for all hormones.

An overview of interactions between the ethylene and GA pathways is presented here and in silico experimental evidence has been compiled supporting long-distance action of ethylene and GAs, thus complying with the original definition of hormones (Starling, 1905).

4.1.1. Effects of ethylene and GA on extension growth: state of the art

Ethylene is mostly known as a growth-inhibiting hormone (reviewed by Smalle and Van Der Straeten, 1997). The clearest example of this growth-inhibiting response is the triple response (Guzman and Ecker, 1990). The role of ethylene, however, is not only restricted to growth inhibition. In lower concentrations, ethylene can also stimulate growth of the hypocotyl and is necessary for shade avoidance and elongation after submergence (reviewed by Smalle *et al.*, 1997; Vriezen *et al.*, 2003; Vandenbussche and Van Der Straeten, 2004). The stimulatory role of GAs on elongation has been widely documented (Tanimoto, 1987; Hedden and Phillips, 2000; Fu and Harberd, 2003; reviewed by Alvey and Harberd, 2005). The central components for this response are the DELLA proteins, acting as growth repressors in the absence of GAs. In roots, the growth inhibitory effect of ethylene is primarily caused by a reduction in cell length (Le *et al.*, 2001). The LEH (length of the first epidermal cell with a visible root hair bulge) was defined as a parameter to assess the effect of ethylene on root cell elongation. Epidermal cells that are longer than or equal to the LEH at a given ethylene concentration immediately stop elongating, while cells closer to the root tip elongate until they reach the LEH value. Recently, it has also been shown that ethylene regulates cell division as well, leading to supernumerary divisions of the quiescent centre (Ortega- Martinez *et al.*, 2007).

Ethylene regulates cell elongation at least in part by cross-talk to the GA pathway (Achard *et al.*, 2003). At reduced ethylene levels, the growth of *gai-t6 rga-24* (*GA insensitive t6-repressor of ga1-3 24*, double loss-of-function DELLA mutant, leading to constituous GA response) is more resistant to the effects of 1 amino-cyclopropane 1 carboxylic acid (ACC) than that of the wild type. Furthermore, in wild-type seedlings, GA treatment can substantially overcome the ACC-induced inhibition of root growth (Achard *et al.*, 2003). These effects can be explained by a stabilization of REPRESSOR of *ga1-3* (RGA) and GA INSENSITIVE (GAI) by ethylene as shown for GFP fusion proteins in the root (Achard *et al.*, 2003).

4.2 Biosynthesis and signaling pathways

4.2.1. Ethylene synthesis

A schematic overview of the ethylene synthesis pathway is shown in Fig. 1. The first step in the synthesis of ethylene is the conversion of methionine into S-adenosylmethionine (SAM). The reaction is ATP dependent and catalysed by SAM synthetase (Ravanel *et al.*, 1998). In *Arabidopsis*, SAM synthetase is encoded by two genes (SAM1, SAM2). The next (and the rate-limiting) step in ethylene biosynthesis is the conversion of SAM to ACC. The reaction is catalysed by ACC synthase (ACS) (Adams and Yang, 1979; Capitani *et al.*, 1999), and the first genes of the multigene family have been characterized in tomato (Olson *et al.*, 1991; Rottmann *et al.*, 1991; Van Der Straeten *et al.*, 1992). In *Arabidopsis*, ACS is encoded by a gene family of 12 members, two of which, however, are not biologically active and two others do not function as ACSs but as aminotransferases (Tsuchisaka and Theologis, 2004b).

ACSs have been demonstrated to function as homodimers; however, based on the formation of 17 heterodimers in Escherichia coli, it is also possible that heterodimers are formed in planta (Tsuchisaka and Theologis, 2004b). Because of possible heterodimerizations and thus interference of inactive with active ACS isoforms, the expression of all 12 members of this family is analysed in silico. The last step in the ethylene biosynthesis is an oxidation of ACC to ethylene catalysed by ACC oxidase (ACO) (reviewed by Yang and Hoffman, 1984). In *Arabidopsis*, ACO is also encoded by a multigene family, for which thus far only ACO1, ACO2, and the ethylene-forming enzyme (EFE) have been described as ethylene related (Gomez-Lim *et al.*, 1993; Bovy *et al.*, 1999; Raz and Ecker, 1999).

4.2.2. Ethylene signaling

The ethylene signal transduction pathway has been largely elucidated (Fig. 1) [for comprehensive reviews see Alonso and Stepanova (2004), De Paepe and Van Der Straeten (2005), and Vandenbussche *et al.* (2006)]. In *Arabidopsis*, ethylene is perceived by five endoplasmic reticulum (ER) membrane-associated receptors which are partially redundant and show homology with bacterial two-component histidine kinases (Chen *et al.*, 2002; Gao *et al.*, 2003). CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) is a mitogen- activated protein kinase kinase kinase (MAPKKK) acting as a negative regulator of a MAPK cascade (Kieber *et al.*, 1993) which itself is a positive regulator of ethylene response (Chang, 2003; Ouaked *et al.*, 2003). It is stimulated by the receptors in the absence of ethylene (reviewed by De Paepe and Van Der Straeten, 2005). The MAPK cascade in its turn stimulates ETHYLENE INSENSITIVE 2 (EIN2) (Ouaked *et al.*, 2003), an N-RAMP protein and positive regulator of the ethylene signaling pathway (Hall and Bleecker, 2003). The next stage in the ethylene signaling pathway is composed of EIN3 and the EIN3 like (EIL) transcription factors. EIN3 dimers are able to bind the primary ethylene response element (PERE) in the promoter of *ETHYLENE RESPONSE FACTOR 1* (*ERF1*) and other ethylene response genes. Also EIL1 and EIL2 are able to bind this region (Solano *et al.*, 1998). EIN3 is degraded in a proteasome-dependent manner after ubiquitination mediated by EIN3 BINDING F-BOX PROTEIN (EBF1) and EBF2 (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004). ERF1 and other ethylene response element-binding proteins (EREBPs) can in turn bind the secondary ethylene response element (SERE) in the promoter region of ethylene-regulated genes (Fujimoto *et al.*, 2000).

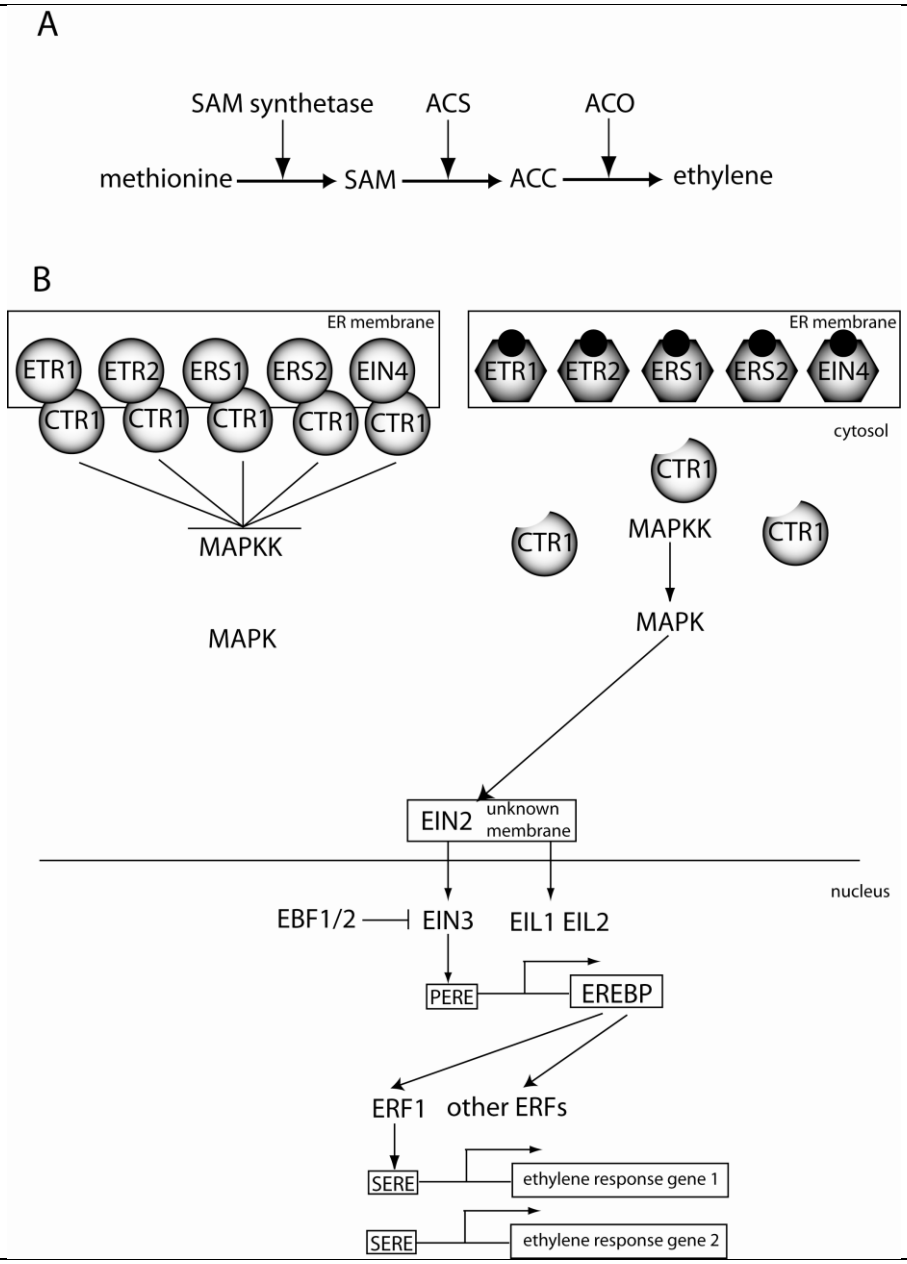

Figure 1 (A) Ethylene synthesis:
methionine is converted to S-adenosyl-methionine (SAM) by SAM synthetase. ACC synthase converts SAM to 1-amino-cyclopropane-1- carboxylic acid (ACC) which is oxidized to ethylene by ACC oxidase (ACO).
Figure 1 (B) Ethylene is perceived by a family of five ethylene receptors. They are called ETR1, ETR2, ERS1, ERS2, and EIN4. All of these receptors are localized in the membrane of the endoplasmatic reticulum
and are (in the absence of ethylene) associated with CTR1. CTR1 is a negative regulator of a MAPKKK pathway which in turns stimulates the ethylene response by stimulating EIN2. When ethylene is present (represented by black circles), the conformation of the receptors changes. As a result of this change, CTR1 is no longer repressing the MAPK pathway, thus EIN2 is active. When EIN2 is active, this leads to a stimulation of EIN3 and the EILs. They are able to regulate expression of ERF1 (ethylene response factor) and probably of other ethylene response element-binding proteins (EREBPs) by binding to their primary ethylene response element (PERE). EREBPS can interact with the secondary element response element (SERE) of ethylene-regulated genes, thus regulating their expression.

4.2.3. Gibberellin synthesis

All steps involved in GA biosynthesis have been characterized (Zhao *et al.*, 2007). The synthesis of GA can be divided into three stages (Fig. 2) corresponding to different subcellular compartments. The first stage is localized in proplastids (Sun and Kamiya, 1994; Aach *et al.*, 1997), where geranylgeranyl diphosphate (GGPP) is converted to ent-copalyl diphosphate (CDP) by entcopalyl diphosphate synthase (CPS). In *Arabidopsis*, this enzyme is encoded by GA1 (Sun *et al.*, 1992). From CDP, ent-kaurene is synthesized by kaurene synthase (KS), encoded by *GA2* in *Arabidopsis* (Yamaguchi *et al.*, 1998). In the second stage of this pathway, ent-kaurene is transformed to GA12 and GA53. Three oxidations are carried out by ENT-KAURENE OXIDASE (KO) leading to the formation of ent-kaurenoic acid (Helliwell *et al.*, 1999). The next three steps in the formation of GA12 are catalysed by ENT-KAURENOIC ACID OXIDASE 1 and 2 (KAO) (Helliwell *et al.*, 2001a). By using green fluorescent protein (GFP) fusions, Helliwell *et al.* (2001b) were able to show that KO is located on the outer surface of the chloroplast envelope and that KAO1 and KAO2 are associated with the ER. In total, >135 GA derivatives have been discovered, but in plants only a few are physiologically active. The main active form in *Arabidopsis* at the vegetative stage is GA4 (reviewed by Olszewski *et al.*, 2002). GA4 is formed by the subsequent oxidation of GA53 and GA12 by 2-oxoglutarate-dependent dioxygenases, GA20 oxidase (GA20OX) and by GA3 oxidase (GA3OX) (reviewed by Zhao *et al.*, 2007). Both are encoded by a multigene family of at least four members for *GA3OX* and at least four for *GA20OX* (reviewed by Hedden and Phillips, 2000). A search in the TAIR database showed the presence of four *GA3OX* genes and five *GA20OX* genes.

Plants modulate their levels of bioactive GAs, in part, by conversion to inactive forms through 2b-hydroxylation. This reaction is catalysed by GA2 oxidases (GA2OX) (reviewed by Zhao *et al.*, 2007). Also GA2OX is encoded by a multigene family (reviewed by Hedden and Philips, 2000). Finally, it must be mentioned that concentrations of active GA species are also determined by the levels of methylation (Varbanova *et al.*, 2007) and epoxidation (as shown in rice; Zhu *et al.*, 2006).

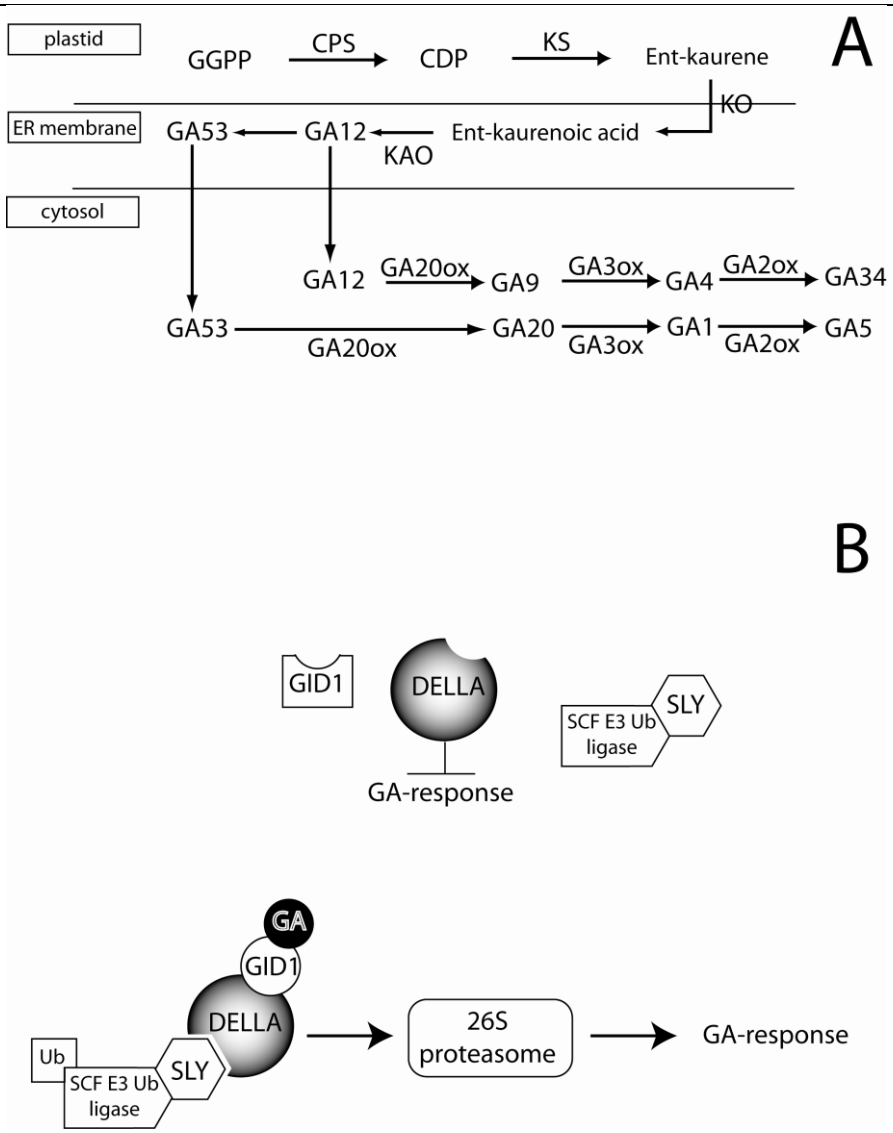

Figure 2 (A) The synthesis of GA can be divided into three stages which correspond to different subcellular localizations. In the first stage, GGPP (geranylgeranyl diphosphate) is converted to ent-copalyl diphosphate (CDP) by ent-copalyl diphosphate synthase (CPS). CDP is in turn converted to ent-kaurene by kaurene synthase (KS). These reactions occur in the proplastids. KO (ent-kaurene oxidase) is localized on the outer surface of the chloroplast envelope and oxidizes ent-kaurene to ent-kaurenoic acid which in turn is oxidized to GA12 by the ER-localized KAO (ent-kaurenoic acid oxidase). In the ER, ent-kaurene is transformed to GA12 and GA53. In the cytoplasm, GA20OX converts GA12 and GA53 to GA9 and GA20, respectively. GA3OX further oxidizes GA9 and GA20 to GA4 and GA1, respectively. The levels of GA9 and GA20 can be regulated by GA2OX which converts GA4 to GA34 and GA1 to GA5.

Figure 2 (B) The signal transduction of gibberellins starts with the perception of GA by the GID1 receptors. Because of binding of GA, they undergo a conformational shift which makes it possible for them to interact with the DELLA proteins. DELLA proteins are repressors of the GA response. Interaction of GA receptors with DELLA causes SLY1 to interact with the DELLA proteins, leading to their ubiquitination and subsequent proteasome-dependent degradation. The lower concentrations of DELLA proteins lead to a lower repression, thus, GA response can occur.

4.2.4. Gibberellin signaling

In *Arabidopsis*, GAs are perceived by three receptors, GIBBERELLIN-INSENSITIVE DWARF 1A (GID1A), GID1B, and GID1C (Fig. 2) (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006). The findings of Griffiths *et al.* (2006) support a model in which the perception of GA leads to interaction of GID1 with a member of the DELLA protein family (Fig. 2). This family consists of RGA1, GAI, RGL1 (RGA LIKE), RGL2, and RGL3. They act as growth repressors in the absence of GA.Upon GA treatment or endogenous GA accumulation, DELLA proteins are marked for degradation, via an SCF (Skp1–Cullin–F-box) E3 ubiquitin ligase complex (Silverstone *et al.*, 2001). The interaction of GID1 and DELLA proteins results in binding of the DELLA partner to SLY1 (SLEEPY1) via its GRAS domain (Griffiths *et al.*, 2006). SLY1 is the F-box component of the SCFSLY1 E3 ubiquitin ligase, which labels DELLA proteins for proteasome degradation (Dill *et al.*, 2004). Thus, growth results from derepression of the GA pathway.

4.2.5. In silico analysis of expression patterns of genes in ethylene and GA synthesis and signaling

As evidence for cell type specificity of hormone action is growing, it is conceivable that hormonal interplay may be regulated by co-ordinated expression of genes crucial for multiple hormone responses. This possibility was investigated using transcript meta-analysis to visualize overlaps in the expression pattern of genes belonging to the biosynthesis and signaling pathways of GAs and ethylene. Literature data, together with Genevestigator data (using GeneAtlas and ResponseViewer on https://www.genevestigator.ethz.ch) (Zimmermann *et al.*, 2004), were compiled, to map GA and ethylene synthesis gene expression. By doing the same for genes in their signaling pathways, overlaps in expression patterns were mapped, which may uncover potential interaction sites in the control of elongation growth. The GeneAtlas tool provides the average signal intensity of values of a gene of interest in all organs or tissues annotated in the database. The anatomy annotation was based on standard anatomy terms as defined by the Plant Ontology Consortium (www.plantontology.org). The ResponseViewer tool provides the same functionalities as GeneAtlas based on stress response annotations. For each condition, one or several representative experiments were chosen. Each factor is given with the corresponding control from these experiments, allowing direct comparison (Zimmerman *et al.*, 2004).

Likewise, cell type-specific expression patterns of ethylene and GA synthesis and signaling genes in roots were compared using the digital in situ database AREXdb (www.arexdb.org). This website compiles *Arabidopsis* gene expression data from various sources (microarrays, in situ hybridizations, promoter::reporter constructs, etc.) into a single database. The different colours in the pictures reflect hybridization intensity differences based on normalized microarray data, therefore allowing comparison between different genes. Many of the experiments used to identify cell type-specific expression patterns used protoplasts derived from intact roots and fluorescence-activated cell sorting (FACS). Although a very powerful tool, some care should be taken with the interpretation of the generated data, as the preparation of protoplasts could influence the expression of certain genes. Furthermore, because of the experimental approach used, some of the expression zones are not as clear-cut as illustrated. Protoplasts from cell types were isolated from whole roots, not regions of the

root. The expression zones were afterwards reconstructed based on comparison of protoplast microarray data with data from excised root sections.

4.2.6. Organ-specific expression of ethylene biosynthesis genes

Data on different genes of the ethylene synthesis pathway were retrieved from GeneAtlas. It can be seen that both SAM synthetase genes are expressed at high levels in every plant organ.

With respect to *ACS* genes, *ACS6* and *ACS10* are the most common transcripts found in *Arabidopsis* plants (Fig. 3A). They are highly expressed in inflorescence stems and in roots. In contrast, *ACS1*, *3*, *5*, *9*, and *11* show a uniform expression, albeit at low levels. In leaves, *ACS2*, *4*, *7*, and *8* are moderately expressed. In roots, the expression of *ACS2*, *ACS7*, and *ACS12* is also modest. Whenever possible, the GeneAtlas data were compared with those generated by Rodrigues-Pousada *et al.* (1993) and Tsuchisaka and Theologis (2004b) who performed a detailed analysis of the expression of *ACS* genes using promoter–GUS (β-glucuronidase) fusions (for *ACS1/2*, *4*, *5*, *6*, *7*, *8*, *9*, and *11*) [the *ACS1* gene, described by Rodrigues-Pousada *et al.* (1993), was renamed *ACS2* by Tsuchisaka and Theologis (2004a)]. Overall, these results are in accordance with the GeneAtlas data. Contrasting results between GeneAtlas data and previous histochemical analyses may indicate low expression levels in a particular organ, as compared with other *ACS* genes (nevertheless visible in histochemical assays) or differences in mRNA stability.

Based on the data generated by GeneAtlas (Fig. 3), *ACO2* is highly expressed in all plant organs, with a very strong expression in the stem (Fig. 3B). *ACO1* is expressed at low levels in most organs when compared with *ACO2* or *EFE*, but shows a strong expression in roots and in the hypocotyl and radicle of seedlings (albeit not as strong as that of *ACO2*). In leaves, *EFE* is the most abundant *ACO*; in most other organs its expression is intermediary between that of *ACO1* and *ACO2*.

Taken together and supposing that gene expression generally reflects sites of enzyme activity, the data suggest that every organ is capable of ethylene synthesis, but perhaps not all to the same extent. Previously it had been shown that the major rate-limiting step in ethylene synthesis is ACS (reviewed by Yang and Hoffman, 1984). It is therefore not surprising to find extensive diversity in expression patterns of *ACS* genes, since this is a primary way to modulate ethylene levels both temporally and spatially. The unique (and overlapping) expression patterns of *ACS* genes can lead to the formation of heterodimers, possibly with different biochemical properties and, consequently, variable ethylene synthesis capacities (Tsuchisaka and Theologis, 2004a, b).

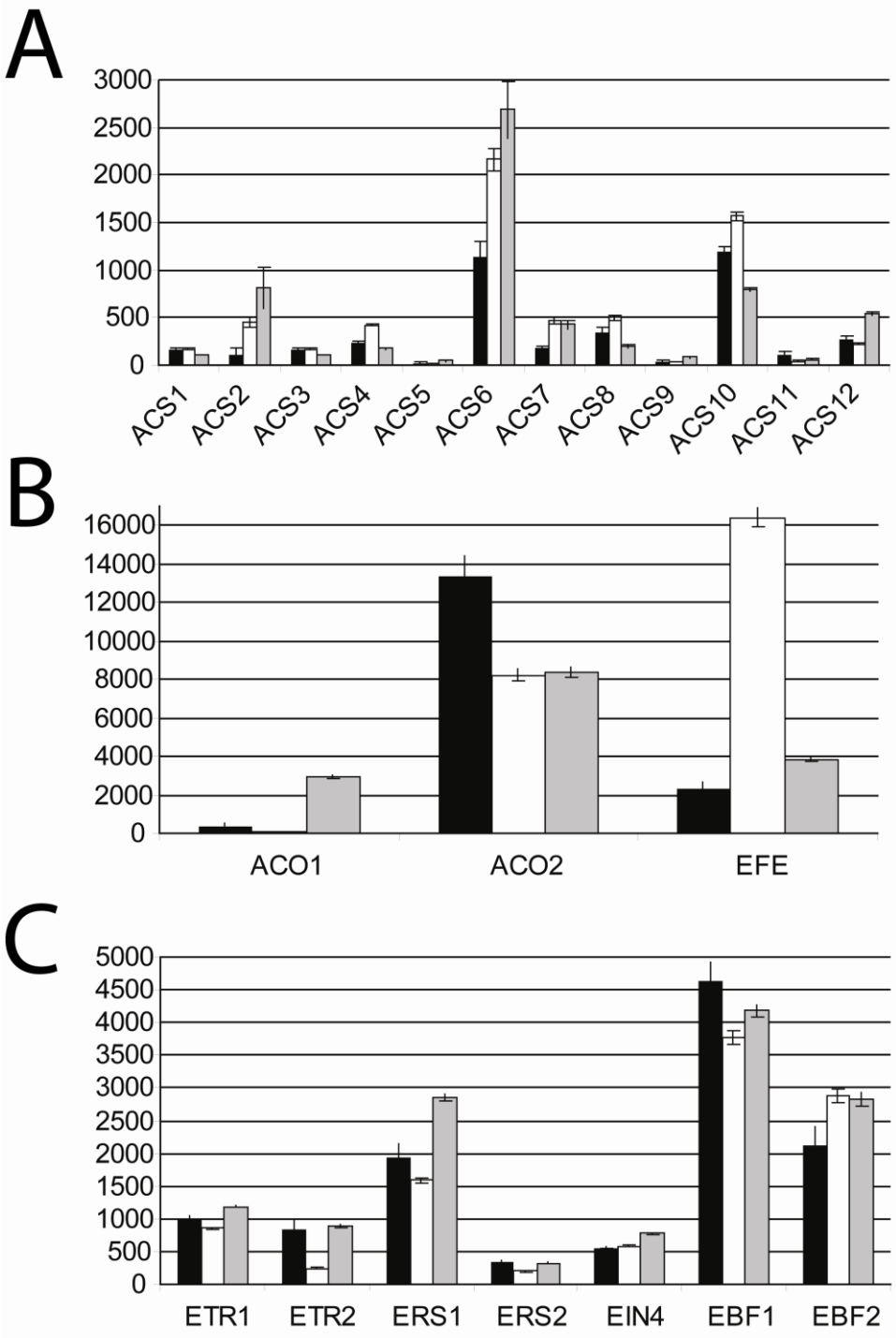

Figure 3 Expression of *ACS* genes (A), *ACO* genes (B), and ethylene signaling genes (C) in inflorescence stem (black), adult leaves (white), and roots (grey). Data were derived from GeneAtlas. Error bars represent the standard error (inflorescence stem: n=33, adult leaf: n=274, root: n=260) .

4.2.7. Cell type-specific expression of ethylene biosynthesis genes

Cell type-specific expression of genes involved in ethylene synthesis in root tips of *Arabidopsis* was examined using AREXdb. The nomenclature of the different regions discussed is presented in Fig. 4. The expression pattern of different ethylene biosynthesis genes (*SAM SYNTHETASE, ACS*, and *ACO*) is shown in Fig. 5. It is remarkable that the ethylene synthesis genes can be divided into three groups. Only *ACS8*, *AC11*, *ACO1*, and *EFE* show little if any expression in zone 3 (fast elongation zone and specialization zone) of the root when compared with other genes.

The second group encompasses members (*SAM1*, *ACS2, 5, 6, 7, 9*, and *ACO2*) which show the strongest expression in zone 3 as compared with the other zones. The third group (*SAM2, ACS3, ACS4, ACS10*, and *ACS12*) is formed by genes that are highly expressed in every tissue examined. Remarkably, ACS8 is mostly expressed in the lateral root cap. Previously ACS8 expression was proven to be strongly up-regulated by auxin (Tsuchisaka and Theologis, 2004b), which may indicate a role for this gene in root growth and tropic responses.

A more detailed investigation reveals further cell type specialization of ethylene synthesis genes. In zone 3, *SAM1* is strongly expressed, while *SAM2* is moderately expressed. In the root transition zone (zone 2), *SAM1* is expressed at moderate levels whereas *SAM2* is highly expressed. The expression patterns in vascular tissue, endodermis, and cortex are essentially the same for both genes.

Based on AREXdb data, *ACS7* shows expression in all cells examined (except in the columella); *ACS9* is expressed in zone 3, the root cap, and in the cortex and endodermis of the transition zone and in the root cap. *ACS2, 5, 6, 7*, and *9* are more expressed in zone 3 of the root than in the apical zones. The expression of *ACS2, 3, 7*, and *9* is the strongest in the endodermis. Only *ACS5* shows a stronger expression in the vascular tissue of zone 3 as compared with the endodermis. Except for *ACS2* and *ACS9*, all *ACSs* show clear expression in the vascular tissue. *ACS3, 4, 10*, and *12* are expressed relatively strongly over the entire root tip. *ACS8* is strongly expressed in the root cap zone and in the endodermis of zone 3. In contrast to the results of Tsuchisaka and Theologis (2004b), the AREXdb site does show expression in the root cap for *ACS4, 7, 9*, and *10. ACS11* shows almost no expression in the root tip. There are no digital in situ data available for *ACS1*.

ACO1 and *ACO2* are both very strongly expressed in the columella. *ACO1* is also highly expressed in the lateral root cap, whereas the strongest expression of *ACO2* is found in the vascular tissue of zone 3. *ACO1* is not expressed in that region. The expression of *EFE* shows the same pattern as that of *ACO1*.

The stronger expression of *ACO2* in the roots compared with *ACO1*, as revealed by GeneAtlas, is confirmed by AREXdb data. Furthermore, also on a root tissue typespecific basis (AREXdb), unique and overlapping expression patterns of *ACS* genes are observed, as seen on an organ basis (GeneAtlas, Fig. 3A).

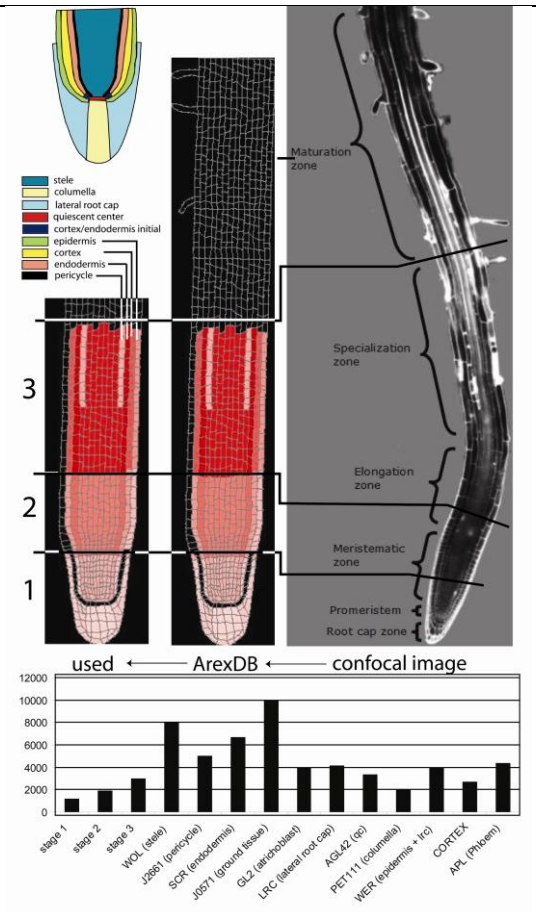

Figure 4 Nomenclature of the studied regions by AREXdb. The nomenclature of different root zones is based on a confocal image by Noritaka Matsumoto (Benfey Laboratory, source: http://www.arexdb.org/database.jsp; panel on the right hand). The picture in the upper left corner shows the different cell types in the root tip (based on Taiz and Zeiger (2006), Chapter 19). The middle panel shows an approximate, apportioned expression pattern as downloaded from the AREXdb site for the gene encoding SAM synthetase 1 (SAM1) (Birnbaum *et al.*, 2003; darker red represents higher expression, while white reflects no expression and black indicates absence of data); the lower left panel shows the part covered in Figs 5 and 7. The bar graph reflects raw expression values for the SAM1 gene in each of the developmental zones and cell types (qc: quiescent centre; lrc: lateral root cap). The horizontal lines that cross the confocal picture and the AREXdb profiles in the middle and lower left panels represent the section limits of different root zones (based on developmental stages) used to build the gene expression map as described by Birnbaum *et al.* (2003). In stage 1, the root tip reaches its full diameter (about 0.15 mm from the root tip). The corresponding zone 1 therefore contains the lateral root cap, columella, promeristem, and part of the meristematic zone. Stage 2 represents the cells that transition from being optically dense to a more transparent appearance as they begin longitudinal expansion (about 0.30 mm from the root tip). Zone 2 is referred to by Stepanova *et al.* (2007) as the transition zone and is composed of meristematic cells and cells from the slow elongation zone. Stage 3 occurs where the root hairs were fully elongated (about 0.45–2 mm from the root tip). Zone 3 is therefore composed of cells from the fast elongation zone and the specialization zone where root hairs start to appear (onset of differentiation: Le *et al.*, 2001). In the AREXdb pictures, in certain cases, different signal intensities are observed within a single layer in a zone (as for instance in the pericycle in this figure being less expressed in zone 3 as compared to the surrounding tissue in this zone). This is due to the use of several markers for a single cell type.

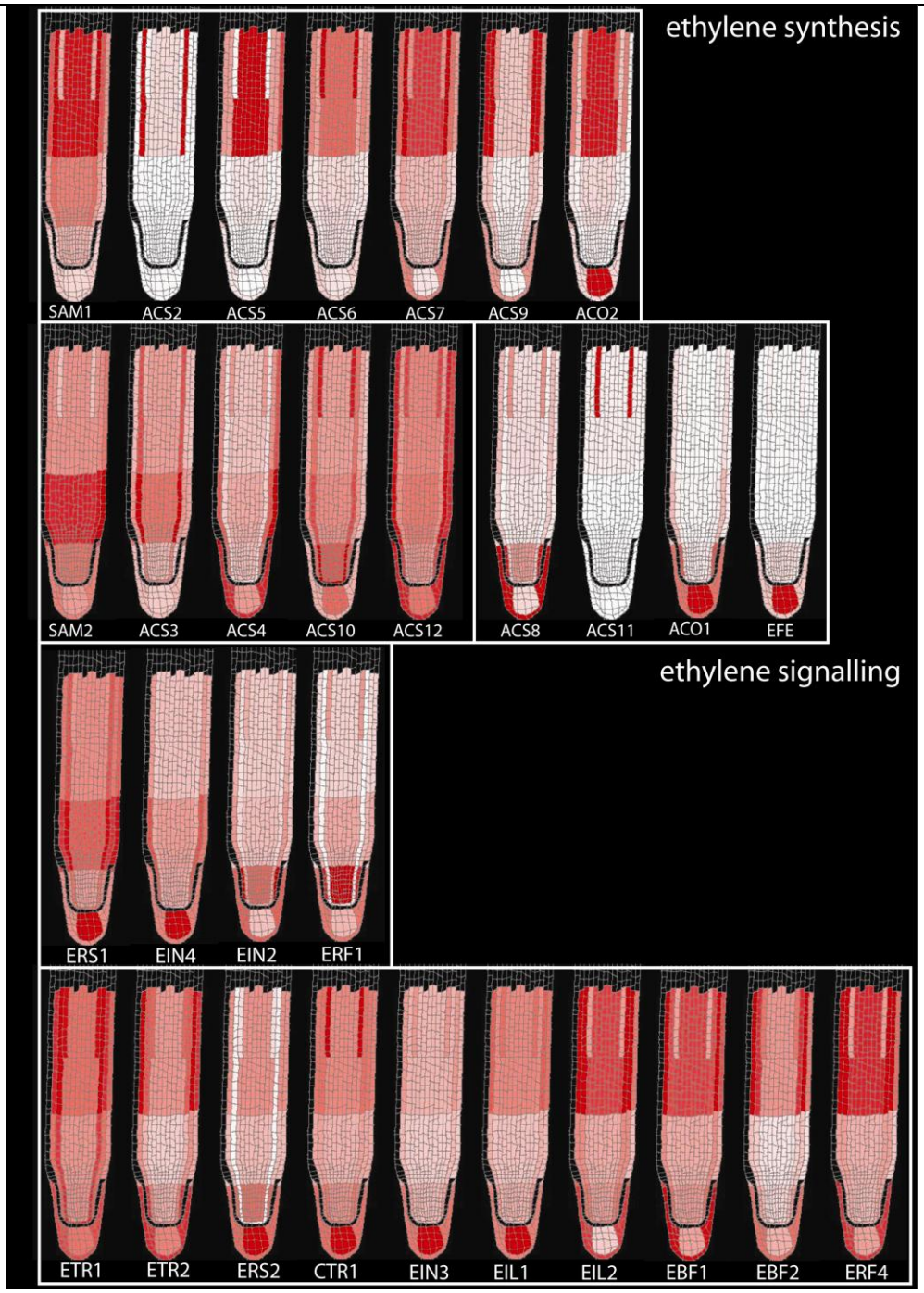

Figure 5 The upper part of the figures shows the expression patterns of the ethylene synthesis genes studied. Three groups can be identified. One group (*SAM1*, *ACS2*, *ACS5*, *ACS6*, *ACS7*, *ACS9*, and *ACO2*) shows a stronger expression in zone 3 (fast elongation zone and specialization zone) than in the other regions investigated. Members of the second group (*SAM2*, *ACS3*, *ACS4*, *ACS10*, and *ACS12*) are expressed strongly over the whole root, whereas members of the third group (*ACS8*, *ACS11*, *ACO1*, and *EFE*) show an overall low expression (except in some very specific regions). The lower part of the picture shows the expression patterns of the ethylene signaling genes that were discussed. The ethylene signaling genes were divided into two groups. One group contains members that are more strongly expressed in the elongation zones than in other zones of the root investigated (*ETR1*, *ETR2*, *ERS2*, *CTR1*, *EIN3*, *EIL1*, *EIL2*, *EBF1*, *EBF2*, and *ERF4*). The other group contains the other ethylene signaling genes that were investigated. Expression patterns were obtained from www.arexdb.org.

These results suggest that the highest level of ethylene synthesis is reached in zone 3 (covering the fast elongation zone and the specialization zone). This correlates well with the current knowledge on the function of ethylene in root growth. In the absence of ethylene, cells that are already differentiating elongate more slowly than cells in the elongation zone (Le *et al.*, 2001). Application of exogenous ethylene to roots inhibits growth by decreasing cell elongation in the elongation zone, where ethylene responses are also controlled by auxin (Le *et al.*, 2001; Ruzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007). It is therefore conceivable that endogenous ethylene, needed in order to fine-tune root length, is synthesized at higher levels in zone 3. Stronger ethylene response in this zone was demonstrated by using an EBS::GUS reporter line (Stepanova *et al.*, 2007). Higher ethylene concentrations could induce an elongation repressing signal at the end of the fast elongation zone and in the specialization zone. Ethylene may thus control a switch from elongation to differentiation.

4.2.8. Organ-specific expression of ethylene signaling components

Hormones can only exercise their function when they are perceived by specific receptors and if the signal arising from receptor recognition is transduced. Therefore, the expression of the genes encoding enzymes that are required for effective signal transduction was also studied.

As shown in Fig 3C, *ETHYLENE RESISTANT1* (*ETR1*) and *ETR2* are expressed in all organs, but the levels of *ETR1* expression are higher (Hua *et al.*, 1998; Sakai *et al.*, 1998; GeneAtlas). *ETHYLENE RESPONSE SENSOR1 (ERS1)* and *ERS2* are also present in every organ. The expression level of ERS1 is even higher than that of *ETR1* and is particularly high in roots (Hua *et al.*, 1998;GeneAtlas; Fig. 3C). *ERS2* is the least abundantly expressed receptor in the root (Hua *et al.*, 1998; GeneAtlas). The GeneAtlas data reveal a uniform expression for *ETHYLENE INSENSITIVE4* (*EIN4*), which is comparable with that of *ETR2*. The same conclusion can be drawn from the results of Hua *et al.* (1998), at least for those tissues included in the GeneAtlas.

CTR1 is expressed in every organ according to GeneAtlas (Fig. 3C). Likewise, genes encoding the N-RAMP protein EIN2 (Alonso *et al.*, 1999) and the transcription factors EIN3 and EIL1 show high and ubiquitous expression (GeneAtlas). The most prominent expression of *EIN2* is seen in senescent leaves. *EBF1* shows an expression that is 30% higher than that of *EBF2*, but both genes are expressed relatively uniformly in the whole plant (Fig. 3C). *ERF1* itself is moderately expressed in all organs, but shows a peak in senescent leaves. *ERF4* is highly expressed in all tissues (GeneAtlas). Yang *et al.* (2005) showed that *ERF4* is indeed constitutively expressed in roots, leaves, and stems of *Arabidopsis*. According to ResponseViewer, *ERF1* is up-regulated by ethylene (2.6-fold), while *ERF4* is not ethylene regulated. This result correlates with the previously shown up-regulation of the expression of *ERF1* by ethylene (Solano *et al.*, 1998; Lorenzo *et al.*, 2003). *ERF4*, however, has also been shown to be up-regulated by ethylene (Yang *et al.*, 2005).

4.2.9. Cell type-specific expression of ethylene signaling genes

Exploring the tissue-specific expression of ethylene signaling genes in the root using AREXdb (Fig. 5) allowed the genes to be divided into two groups based on their expression in zone 3 (fast elongation zone and specialization zone). The expression of *ERS1*, *EIN4*,

EIN2, and *ERF1* is lower in zone 3 as compared to zone 2 (transition zone). *ETR1, 2* and *ERS2* are more strongly expressed in zone 3 than in the other investigated root zones. Their expression is the strongest in the cortex and endodermis. *ERS1* is expressed highly in the transition zone as compared with the other receptors. In the columella, *EIN4* and *ERS1, 2* show very strong expression. It is furthermore remarkable that *ERS2* expression is absent in the endodermis. *CTR1* shows a strong expression in zone 3 and in the columella. *EIN2* is strongly expressed in the promeristem and the root cap. The components downstream of EIN2 (i.e. *EIN3, EIL1, EIL2, EBF1*, and *EBF2*) are expressed at high levels in zone 3. This correlates once again with the function of ethylene in root growth as discussed before (Le *et al.*, 2001; Ruzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007). *EIN1* and *EIL1* are also strongly expressed in the columella. Furthermore, *ERF4* is also expressed at high levels in zone 3 and in the root cap, while *ERF1* is highly expressed in the promeristem and the root cap. Higher expression of ethylene synthesis (*ACS4,8; ACO1, 2* and *EFE*) and response genes in root tips might be linked to induction of auxin biosynthesis (Ruzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007).

Both *ERF1* (Lorenzo *et al.*, 2003) and *ERF4* (Yang *et al.*, 2005) are up-regulated by ethylene. The difference in response between *ERF1* and *ERF4* can be caused by tissue-specific regions in their respective promoters.

4.2.10. GA biosynthesis: organ-specific expression

p*GA1*::GUS fusions showed that *CPS* has a weak but specific promoter. *CPS* is mainly expressed in rapidly growing tissues. The expression is high in shoot apices and root tips. The promoter is also active in vascular tissue of some non-growing organs (e.g. mature leaves). These organs may therefore function as sites for synthesis of GA that is transported to other organs (Silverstone *et al.*, 1997).

The expression pattern of *KS* is comparable with that of *CPS* (being expressed in the same tissues (data not shown), although *KS* is expressed at higher levels than *GA1* according to GeneAtlas (Fig. 6A).

KO is present in high levels in all tissues (Fig. 6B). As further shown by GeneAtlas, both *KAO* genes are expressed in every tissue examined, with a higher expression in inflorescence stems than in leaves and roots. For *KAO*, these data are confirmed by Helliwell *et al.* (2001a). Based on the data represented in GeneAtlas, *GA20OX2, 3, 4*, and *5* have a relatively low expression in vegetative tissues and show little organ specificity in those tissues (Fig. 6C). The expression of *GA20OX2*, however, is high in flowers and siliques, as is the expression of *GA20OX3*. When compared with other *GA20OX* genes, *GA20OX1* shows the highest expression in elongating vegetative tissues such as stems and juvenile leaves. These data correlate well with the results of Phillips *et al.* (1995) who found high expression of *GA20OX1* in stems, of *GA20OX2* in flowers and siliques, and of *GA20OX3* in siliques. *GA3OX1* expression is relatively high in all plant organs (Fig. 6C). *GA3OX1* is the most prominently expressed *GA3OX* family member in the stem, and is also expressed in developing cotyledons, the vegetative shoot apical meristem, and in parts of the roots (see below) (Mitchum *et al.*, 2006). Based on the GeneAtlas data, especially *GA3OX1* is strongly expressed in roots. Also *GA20OX2* and *GA3OX2* show a stronger expression than the other *GAxOXy* biosynthesis genes, although not as pronounced as *GA3OX1* (data not shown). This

explains the semi-dwarfed phenotype of the ga3ox1 mutant (Chiang *et al.*, 1995). *GA3OX2* is also expressed in all organs, but at extremely high levels in the early stages of development (until 5-d-old) and at very low levels during later stages of development (in the stem, flowers, and siliques) (Mitchum *et al.*, 2006). *GA3OX4* is expressed at lower levels than *GA3OX1* and *GA3OX2* except at later stages, where it reaches levels higher than *GA3OX2*, but still much lower than those of *GA3OX1*. In later stages (especially in flowers), *GA3OX3* and 4 reach levels higher than *GA3OX2*, but lower than *GA3OX1* (Mitchum *et al.*, 2006; GeneAtlas; Fig. 6C). The *ga3ox2* loss-of-function mutant does not show a phenotype in aerial parts of the plant, whereas *ga3ox1* does. The double mutant *ga3ox1 ga3ox2* does not show a phenotype as severe as *ga1-3*, but has a 30% smaller rosette diameter and is 37% shorter than *ga3ox1*. This intermediate phenotype of the double mutant may be explained by GA production driven by GA3OX4 which accumulated during early stages (until 5-d-old) and by GA transport from flowers and siliques produced by GA3OX3 and GA3OX4 (Mitchum *et al.*, 2006). Furthermore, it is possible that there are more than four GA3OXs. In the *ga3ox1* single mutant, the rosette phenotype probably results from GA production in the early stages (until 2 d) driven by GA3OX2 or again by transport from generative structures. In roots, the double mutant shows a more severe phenotype than *ga1-3*, although *ga3ox2* shows no phenotype and *ga3ox1* roots are only slightly shorter than the wild type. The double mutant has a lower level of GA4 than *ga3ox1*, but a higher level when compared with *ga1-3* (Mitchum *et al.*, 2006).

Northern blotting revealed *GA2OX2* expression in the lower stem and in leaves. *GA2OX3* was not detected in any of these organs (Thomas *et al.*, 1999). These observations are only in part confirmed by GeneAtlas data which show that *GA2OX2*, 3, and 6 are the most abundant genes in the stem and in the roots (Fig. 6D). In roots, *GA2OX2* and *GA2OX6* are strongly expressed, while the expression of *GA2OX3* is 2-fold lower than that of *GA2OX2* and 4-fold lower than *GA2OX6* expression. *GA2OX2* and *GA2OX6* are further strongly expressed in adult leaves. In these tissues, the other *GA2OX* genes reach maximum levels that are at least six times lower than that of *GA2OX6* and three times lower than that of *GA2OX2*. Based on the GeneAtlas data, in roots, a stronger expression of *GA2OX6*, *GA2OX2* and *GA2OX3* can be noticed, with *GA2OX6* showing the highest expression levels (data not shown).

An attempt has been made to link published experimental data (obtained by GUS histochemical assays, Q-PCR, or RNA blotting) with those retrieved from GeneAtlas (which are based on microarray studies). Discrepancies are possible since the conditions between these experiments may vary and mRNA stability can cause differences between GUS expression patterns and microarray data. Nevertheless, the results generated by GeneAtlas consistently show clear trends that are confirmed by other experimental data. Taken together, it is most likely that GA is synthesized in every elongating plant tissue. Different isozymes with their unique and overlapping expression patterns enable GA synthesis where and when needed.

Since one biosynthetic (*GA3OX1*) and two catabolic genes (*GA2OX2* and *6*) are highly expressed in mature grown leaves, it is possible that GA concentrations are also regulated precisely here and transported to organs that still need to elongate. Distribution and transport of GA are discussed further below.

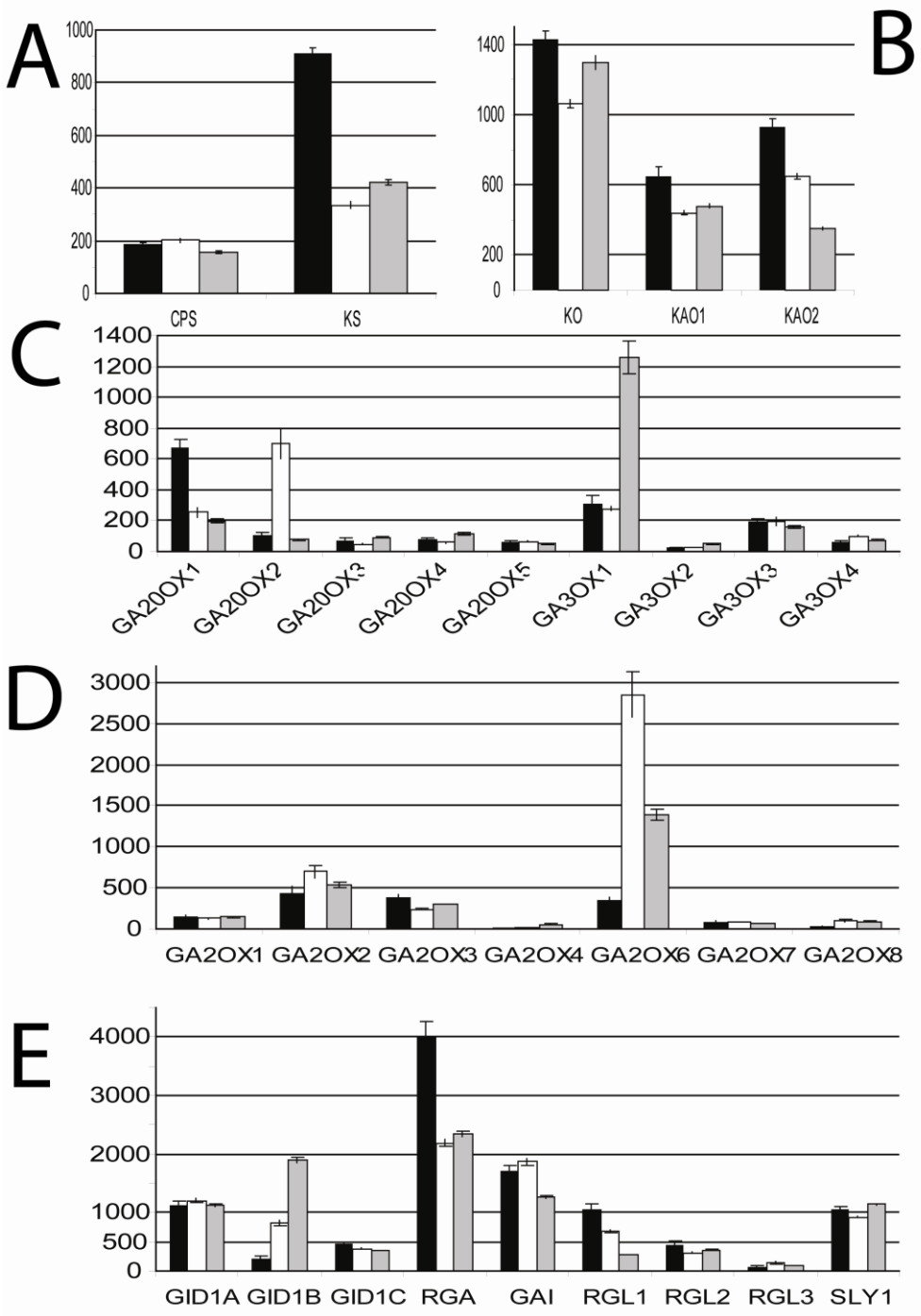

Figure 6 (A) Expression of *CPS* and *KS* in shoot apex (black), adult leaves (white), and roots (grey). (B) Expression of *KO* and *KAO1, 2* in inflorescence stems (black), adult leaves (white), and roots (grey). (C) Expression of GA synthesis genes in inflorescence stems (black), flowers (white), and juvenile leaves (grey). (D) Expression of GA catabolism genes in inflorescence stems (black), flowers (white), and juvenile leaves (grey). (E) Expression of GA signaling genes in inflorescence stems (black), adult leaves (white), and roots (grey). Data were derived from GeneAtlas. Error bars represent the standard error (shoot apex: n=116, adult leaf, n=274, root: n=260, inflorescence stem: n=33, flower: n=125, and juvenile leaf: n=91).

4.2.11. Cell type-specific expression of GA biosynthesis genes

Experimental evidence challenging the concept that the synthesis and perception of hormonal signals are organ specific, and proposing a rather cell- and tissue-specific action, is accumulating (Swarup *et al.*, 2005; Savaldi- Goldstein *et al.*, 2007). Therefore, root data in AREXdb were investigated in order to check whether this also applies to GA biosynthesis and signaling genes (Fig. 7). Based on their expression pattern, the GA metabolism genes could be divided into two groups. One group shows a stronger expression in the endodermis and cortex, while the second group of genes does not.

CPS and *KS* are strongly expressed in the root tip. The expression of *CPS* is the strongest in the endodermis, whereas *KS* is expressed very strongly in the columella and does not show higher expression in the endodermis as compared with surrounding tissues.

GA20OX1, *2*, and *3* are primarily expressed in the cortex and the endodermis. *GA20OX1* is primarily expressed in the cortex. This expression is the strongest in the (pro)meristem zone (zone 1) and the transition zone (zone 2). Based on the AREXdb data, the only *GA20OX* genes that are expressed in the columella are *GA20OX4* and *5*, with a stronger expression of *GA20OX4* in this region. Both genes are expressed strongly in the entire part of the root investigated by AREXdb. *GA20OX4* shows no endodermal expression.

Mitchum *et al.* (2006) investigated the expression of *GA3OX1* in different plant tissues. In roots, *GA3OX1* expression was only seen in the regions above the elongation zone. By contrast, *GA3OX2* is expressed in the elongation zone, meristem, and the columella of the primary root tip and lateral roots (Mitchum *et al.*, 2006); the expression in the quiescent centre and in the columella as shown by these authors is, however, not confirmed by AREXdb. The pattern of *GA3OX3* mirrors that of *CPS*, showing a strong expression in all root zones, with the strongest expression in the endodermis. Except for the endodermis (where the expression is very high), *GA3OX4* shows a lower expression in these regions as compared with *GA3OX3*, but a higher level than *GA3OX1* and *GA3OX2*. Mitchum *et al.* (2006) reported that in 5-d-old plants, only *GA3OX1* and *GA3OX2* were expressed, which is in contrast to the data retrieved from AREXdb (which do show expression of *GA3OX3* and *GA3OX4*).

All *GA2OX* genes are expressed most strongly in the root endodermis, except *GA2OX1* and *GA2OX2*. *GA2OX1* shows a strong expression in the vascular tissue of zone 3, while *GA2OX2* is mostly expressed in the cortex of this zone. *GA2OX3* is expressed strongly in every part of the root tip, *GA2OX1*, *2*, *6*, and *8* are more expressed in zone 3, while *GA2OX4*, and *7* are stronger in the transition zone (*GA2OX4*) and in the (pro)meristem (*GA2OX7*).

From these data, it is clear that most of the genes belonging to the *GA20OX*, *GA3OX*, or GA2OX gene families are differentially expressed in different root tip tissues. It is remarkable that a majority of the genes implicated in regulating GA levels in roots are expressed more strongly in the endodermis than the surrounding tissues. Obvious examples are *CPS*, *GA20OX2*, *GA20OX3*, *GA3OX3*, *GA3OX4*, *GA2OX3*, *GA2OX4*, *GA2OX7*, and *GA2OX8*. Often there is also strong expression in the cortex as seen for *GA20OX1*, *GA20OX2*, *GA3OX4*, *GA2OX2*, *GA2OX7*, and *GA2OX8*. For the other genes, expression in the cortex and/or endodermis is concomitant with expression in the vascular tissue. Hence, it is possible that a GA gradient is formed in the root, with inner tissues accumulating more GA than the epidermal layer. GA concentrations may be modulated by tissue-specific signals to which genes in GA metabolism respond differentially.

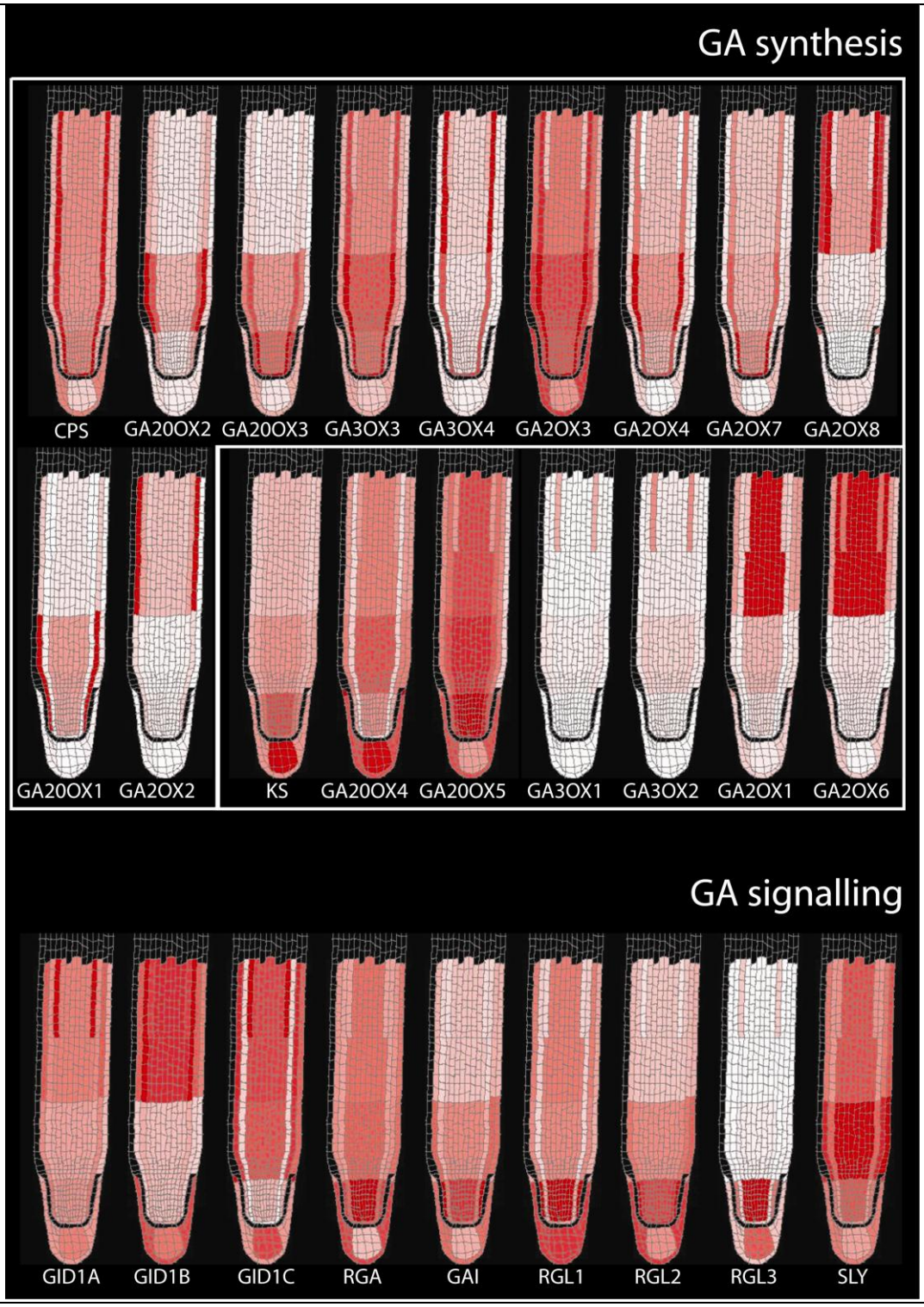

Figure 7 The first two rows of the figure show the GA synthesis or catabolism genes that were studied. They are divided into two groups. The first group contains all of the genes which show a stronger expression in the endodermis (first row) or in the cortex (second row) than in the surrounding tissues. This group consists of *CPS*, *GA20OX1*, *GA20OX2*, *GA20OX3*, *GA3OX3*, *GA3OX4*, *GA2OX2*, *GA2OX3*, *GA2OX4*, *GA2OX7*, and *GA2OX8*. In the other group (*KS*, *GA3OX1*, *GA3OX2*, *GA20OX4*, *GA20OX5*, *GA2OX1*, and *GA2OX6*), this is not the case. The GA signaling genes (lower row) were not placed in different groups. Expression patterns were obtained from www.arexdb.org.

To confirm the existence of such a gradient in GA concentration, further research will be needed. Furthermore, analysis of the AREXdb data reveals that in the (pro)meristematic zone, seven out of 11 biosynthetic genes are expressed (*CPS*, *KS*, *GA3OX3*, and *GA20OX1*, *3*, *4*, *5*) and three out of seven *GA2OXs* (*GA2OX3*, *4* and *7*). Also, more GA biosynthesis genes are strongly expressed in the transition zone of the root (*CPS*, *KS*, *GA20OX1*, *2*, *3*, *4*, *5*, and *GA3OX3*, giving a ratio of eight out of 11) as compared to zone 3 (*CPS*, *GA3OX3*, and *GA20OX4*, *5*, giving a ratio of four out of 11). Moreover, in zone 3 (fast elongation zone and specialization zone), all seven *GA2OX* genes are clearly expressed (*GA2OX1*, *2*, *3*, *4*, *6*, *7*, and *8*) whereas in the transition zone only two out of seven are strongly expressed (*GA2OX4* and *7*). Based on these expression patterns, it can be speculated that in zone 3 GA breakdown is more important than synthesis, while in the transition and (although less pronounced) in the (pro)meristematic zone, synthesis is more prevalent. It has been shown that in the differentiation zone, cells stop elongating (Le *et al.*, 2001). This is in accordance with the promoting role of GA in cell elongation and cell division as shown in hypocotyls (Vriezen *et al.*, 2004).

4.2.12. Organ-specific expression of gibberellin signaling components

Nakajima *et al.* (2006) showed that GID1A, *GID1B*, and *GID1C* are expressed at similar levels in stems, leaves, and roots. Griffiths *et al.* (2006) confirmed that all three receptor genes are expressed in every tissue examined, but that *GID1A* is present at the highest levels. In contrast, *GID1C* is expressed at the lowest level in most organs, with the exception of the 24-d-old stem where its expression is 2-fold higher than that of *GID1B*, albeit much lower than that of *GID1A*. *GID1B* is expressed at levels intermediate between those of *GID1A* and *GID1C* in most organs, but is the most important receptor in the roots (Griffiths *et al.*, 2006). These data correlate well with those in the GeneAtlas database (Fig. 6E). According to GeneAtlas, the DELLA gene *RGA* shows strong expression in most tissues examined. It is the most abundant DELLA protein in the roots of mature plants (Fig. 6D). The expression pattern of *GAI* is strongly similar, although its mRNA appears less abundant than that of *RGA*. In stems, the expression of *RGL1* is high but not as high as that of *RGA* and *GAI*. *RGL2* and *3* are expressed at low levels in the organs considered in GeneAtlas, with *RGL3* being the weakest compared with all other DELLA genes. *SLY1* is expressed highly and uniformly through all plant organs investigated by GeneAtlas.

In conclusion, both the GA receptors and the DELLA proteins show unique and overlapping expression patterns. The receptors have redundant functions in developmental processes that are known to be controlled by individual DELLA proteins (reviewed by Alvey and Harberd, 2005; Griffiths *et al.*, 2006). Mutations in *GID1a* cause more severe phenotypes than mutations in other receptors. The *gid1b-1 gid1c-1* double mutant has a phenotype similar to that of the wild type with respect to stem length, silique length, and fertility, whereas *gid1a* double mutants display aberrant phenotypes. This correlates with the higher expression of *GID1a* in the corresponding tissues. However, such a phenotype is not seen in every organ where *GID1a* expression is more abundant. For example, the rosette diameter of *gid1a* double mutants does not differ significantly from that of the wild type. It is therefore possible that the localized higher expression of some signaling genes has an effect on the response of a certain organ to GA. Obviously this is not the only form of regulation. Post-translational effects have

been shown to be very important in GA signaling. For instance, ethylene stabilizes RGA in *Arabidopsis* roots and in darkgrown seedlings (Achard *et al.*, 2003; Vriezen *et al.*, 2004), while auxins have the opposite effect (Fu and Harberd, 2003).

4.2.13. Cell type-specific expression of gibberellin signaling components

Arabidopsis GA receptors are all strongly expressed in zone 3 (fast elongation zone and specialization zone) (Fig. 7). *GID1b* shows a strong expression in the endodermis, whereas *GID1c* does not show any expression in the endodermis. Furthermore, *GID1c* is also strongly expressed in the transition zone. In addition, every receptor is expressed in the columella and the root cap, with *GID1C* showing the strongest expression in the columella. It is remarkable that *RGA*, *GAI*, *RGL1*, *RGL2*, *RGL3*, and *SLY1* are all strongly expressed in the promeristematic region of the root. These genes (except *RGL3*) are also expressed in the transition zone. The expression of *RGA*, *RGL1*, and *SLY1* in the roots is present in the entire root tip, but stronger in the promeristem for *RGA* and *RGL1* and in the transition zone for *SLY*. *RGL1* does not show expression in the endodermis. While the GA biosynthesis genes seem to be quite strictly regulated with regard to cell type, this is clearly not the case for the signaling genes, which have largely overlapping patterns and a wide field of expression. These data are confirmed by the distribution of a p*RGA::GFP-RGA* fusion in 5-, 7-, and 8- d-old seedlings (Fu and Harberd, 2003; Achard *et al.*, 2003; Silverstone *et al.*, 2001, respectively). In our analysis, a relative high level of *RGA* and *GAI* expression is noticed in both the (pro)meristem and in the transition zone. Post-translational regulation of these proteins appears to play a pivotal role in the process of root growth (Achard *et al.*, 2003; Fu and Harberd, 2003). The tissue-specific GA response may therefore primarily be regulated by the specificity of GA biosynthesis, as discussed above.

4.3 Ethylene and gibberellin cross-talk

In contrast to GAs, ethylene generally inhibits root and shoot cell expansion (Kieber *et al.*, 1993; Rodrigues-Pousada *et al.*, 1993; Smalle *et al.*, 1997). In *Arabidopsis*, the DELLA proteins GAI and RGA mediate reduction of root growth caused by ethylene. Ethylene slows down the GA-induced breakdown of GFP–RGA fusion proteins (Achard *et al.*, 2003). Since DELLA protein degradation is necessary for full root elongation (Fu and Harberd, 2003), this may at least in part explain the ethylene induced root growth inhibition. Besides the interaction at the DELLA level, ethylene and GAs also appear to influence response gene expression reciprocally (De Grauwe *et al.*, 2007).

Also in other plant parts, a close interaction between ethylene- and GA-regulated DELLA proteins was found. After hypogeic germination, seedlings force their way up through the soil. Because of darkness, the cotyledons remain folded and the apical hook is maintained, in order to protect the apical meristem. Ethylene is a key factor in the maintenance of the apical hook, determining the degree of curvature (Raz and Ecker, 1999), while its exogenous application results in an exaggeration of the hook. However, it was recently demonstrated that the maintenance of the apical hook is equally dependent on endogenous GA and on the absence of the DELLA proteins GAI and RGA (Achard *et al.*, 2003; Vriezen *et al.*, 2004).

Depending on the species and on the environmental signals, ethylene can stimulate or inhibit elongation (reviewed by Smalle and Van Der Straeten, 1997; Vriezen *et al.*, 2003).

Induction of elongation, mediated by ethylene, has long been mainly associated with semiaquatic plants such as Rumex palustris and rice (reviewed by Vriezen *et al.*, 2003). It has become clear that some terrestrial plants also use ethylene as a signal for elongation growth. Ethylene induces elongation of wheat (Suge *et al.*, 1997), of the hypocotyl of *Arabidopsis* (Smalle *et al.*, 1997), and of internodes of tobacco (Pierik *et al.*, 2004a). The latter phenotype has been linked to the shade avoidance response. Moreover, a role for GA signaling has been suggested therein (Pierik *et al.*, 2004a). Entrapment of ethylene in the canopy, albeit to a lower concentration than in roots of flooded plants, causes internode elongation (Pierik *et al.*, 2004b). For pea, there are indications that phytochromes negatively control ethylene production, which in turn lowers GA synthesis (Foo *et al.*, 2006). In the *Arabidopsis* hypocotyl, ethylene and GA have a synergistic effect on elongation in the light (De Grauwe *et al.*, 2007). The exact mechanism of interplay of both hormones in light-grown seedlings remains to be elucidated.

4.4 Reciprocal effect on transcript levels between ethylene and GA pathways

Apart from the earlier published effect of ethylene on DELLA stabilization, ethylene and GA pathways may affect each other's pathways by other means. An overview of transcript patterns in plants treated with either hormone could provide more information. Data derived from microarray experiments and data kept in the Genevestigator databases were therefore used. By analysing data produced by the ResponseViewer (Fig. 8A), both GA synthesis genes (*GA20OX1*, *3*, *GA3OX1*, and *KS*) and GA catabolism genes (*GA2OX2*, *4*, *and 7*) of Col0 petioles (plants were in stage 3.9 as described by Boyes *et al.*, 2001) that were treated with 5 ppm ethylene for 3 h were down-regulated by ethylene. Other members of the same families (*GA20OX5* and *GA2OX3*,*6*, and *8*) are upregulated. Up- and down-regulation of different GA biosynthesis and catabolism genes by ethylene has also been found in *Arabidopsis* seedlings (Vandenbussche *et al.*, 2007). However, overall it appears from these data that applying ethylene to plants lowers the expression of GA synthesis and catabolic genes in the petiole. As indicated above, *GA2OX4* and *GA2OX7* are expressed at very low levels. A further down-regulation of their expression might not have a clear effect on GA levels.

Analysis of the expression of the *ACS* genes using ResponseViewer indicates that there is an up-regulation of *ACS2*, *5*, *6*, *7*, *8*, and *11* when 1 lM GA is applied to 7-d-old Col0 seedlings (Fig. 8B). This might be a feedback control in order to inhibit exaggerated elongation.

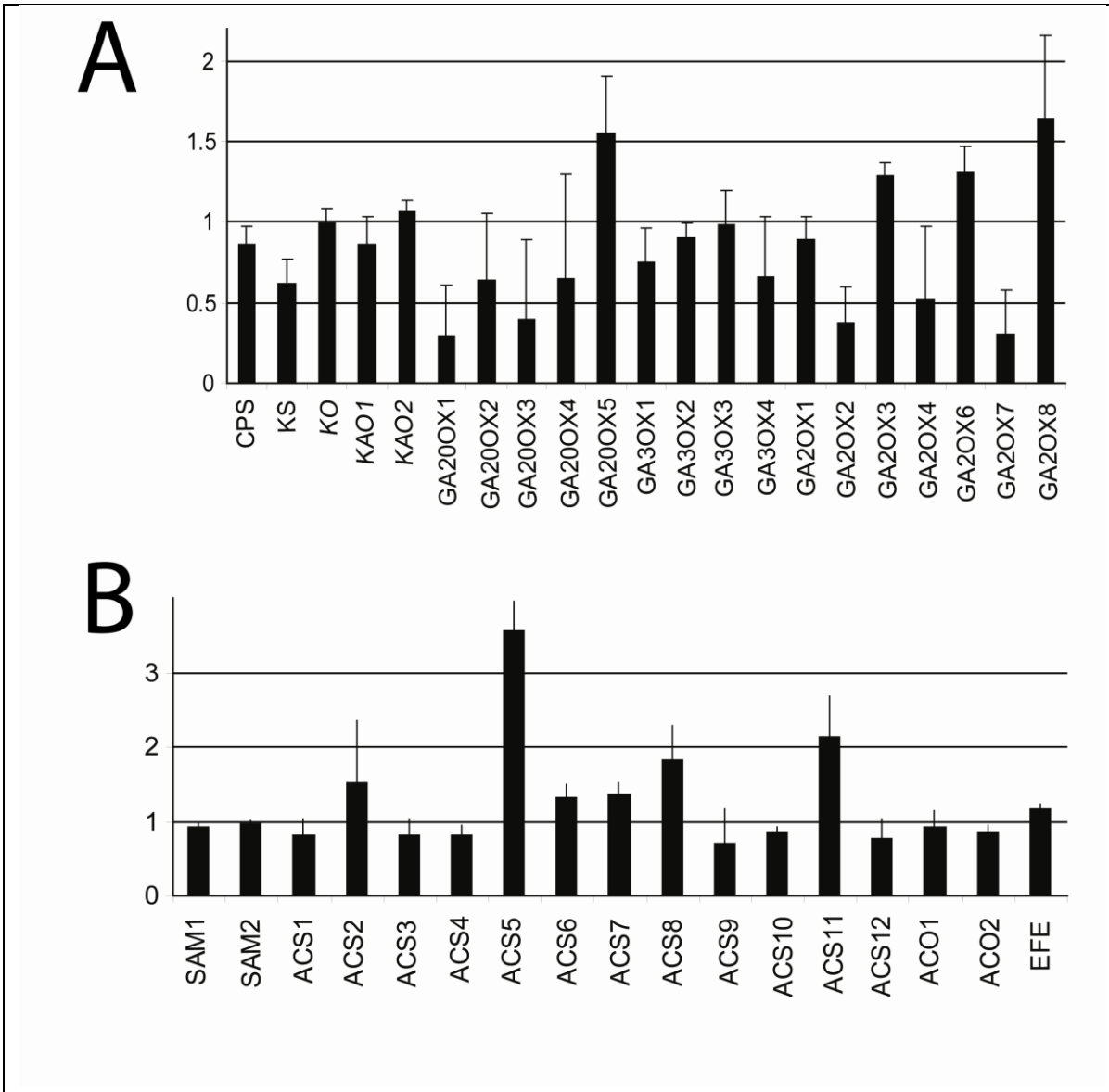

Figure 8 (A) Effect of ethylene treatment (5 ppm) on the transcription of GA metabolism genes. Values given are ratios of expression in treated/ expression in untreated plants. (B) Effect of GA3 (1 lM) treatment on the expression level of ethylene synthesis genes. Values given are the ratios of expression in treated/expression in untreated plants. Data were derived from ResponseViewer. Error bars represent the standard error (ethylene treatment: n=3; GA3 treatment: n=4).

4.5 Distribution and transport of ethylene and GA within the plant

4.5.1. Distribution and transport of ACC and ethylene

When plants are grown on media containing the ethylene precursor ACC, they show similar phenotypes to those treated with ethylene and mutants displaying constitutive ethylene signaling (Kieber *et al.*, 1993; Rodrigues- Pousada *et al.*, 1993). This indicates that ACC is taken up by the roots and transported all over the plant via the xylem (Finlayson *et al.*, 1991).

However, there may not always exist a correlation between the presence of ethylene and its precursor. As a gas, ethylene diffuses freely in air, but its diffusion in water is at least 4-fold lower than in air (Jackson, 1985). Hence, ethylene may accumulate in submerged plants to levels higher than 1 µl/l (Visser *et al.*, 1996). Some plants have adaptations to cope with waterlogging. Aerenchyma is an airy form of cortex found in roots of plants, which allows exchange of gases between the shoot and the root and is a means to allow oxygen to enter the root tissues (Jackson and Armstrong, 1999). In some species, ethylene enhances aerenchyma formation in adventitious roots (Jackson, 1985). Entrapment of ethylene by complete submergence causes accumulation of ethylene in the shoot, which in many semi-aquatic plants starts elongating to reach the water surface (Voesenek *et al.*, 1990; reviewed by Vriezen *et al.*, 2003). This elongation coincides with lower abscisic acid and higher GA production (reviewed by Vriezen *et al.*, 2003).

Within the canopy, ethylene concentrations are enhanced (Pierik *et al.*, 2004b) which may result from ethylene produced both by the plants (Finlayson *et al.*, 1998, Vandenbussche *et al.*, 2003) and by soil microbes (Arshad and Frankenberger, 1988). In tobacco, ethylene thus serves as a signal for elongation of internodes (Pierik *et al.*, 2005).

4.5.2. GA distribution and transport

From treatment with exogenous GA in the media, effects are found on whole *Arabidopsis* seedlings, indicating that the GA taken up by the roots is distributed in the shoot (Saibo *et al.*, 2003). Also, translocation of GAs in pea occurs mainly acropetally in shoots (Smith, 1993). The close association of some of the key GA biosynthesis genes with the vascular tissue may be an indication for the use of transport facilities within the plant. Indeed, xylem and phloem tissues were suggested as effective transport routes for GAs (Metzger and Zeevaart, 1980; Oden *et al.*, 1995). However, transport capacities and rates are different for individual GAs (Proebsting *et al.*, 1992; Smith, 1993; reviewed by Ross *et al.*, 2006) and, like the composition of the GA pool, reallocation of GA may be species dependent. In addition, translocation of precursors of bioactive GAs may occur, to specific parts of the plant where they are subsequently converted to bioactive GAs, by localized enzyme action (Smith, 1993). Interestingly, the GA precursor ent-kaurene can be released as a gas and hence, for instance, rescue deficits in GA (Otsuka *et al.*, 2004). At present, the biological relevance of this phenomenon is not understood. Yet it might be worthwhile to investigate whether situations in nature exist, as in the case for ethylene in shade avoidance, in which volatile ent-kaurene has an impact on growth.

4.6 Conclusion

The *in-silico* analysis at the organ level suggested that both ethylene and GA can be produced in almost the entire plant. However the different isoforms of the enzymes may account for specific responses to different environmental or developmental signals. Because of the apparent specificity of localization of synthesis of ethylene and GAs at the cell type and the tissue level, and the fact that these hormones can be distributed throughout the plant, they can both behave as ‘classical hormones’, being produced in one site and exerting their effect in another site. The *in-silico* analysis of cell-specific expression suggests different working areas of both hormones. As ethylene synthesis and signaling genes (at least those downstream

of EIN2) are primarily expressed in zone 3 (fast elongation and specialization zone), a higher ethylene synthesis and action may be expected in this zone. As several ethylene signaling genes are up-regulated by ethylene, a local higher ethylene production in zone 3 may stimulate ethylene signaling, causing the switch from elongation to differentiation, as suggested by Le *et al.* (2001).

The situation is different for GA, since in zone 3 GA breakdown seems to be more important than synthesis. In contrast, in the (pro)meristematic and transition zones, GA synthesis seems to be more prevalent. The GA signaling genes *RGA, GAI, RGL1, RGL2, RGL3*, and *SLY1* are all strongly expressed in the (pro)meristematic region of the root and (with the exception of *RGL3*) also expressed in the transition zone. Taken together, these data suggest that inhibition of GA signaling and its control is more important in the apical regions of the root.

Revelation of hormone gradients within a plant organ could underpin the importance of spatial distribution of hormone levels and their activity. At this moment, however, cell type-specific quantification of hormone content has not been achieved, yet future experiments using FACS technology and highly sensitive mass spectroscopy devices could enlighten us on the anatomical distribution of hormones. Such studies combined with well characterized reporter genes for each pathway will further indicate whether the signaling pathway is indeed active in a specific tissue.

4.7 Acknowledgements

This work was supported by a PhD fellowship to Jasper Dugardeyn from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and a post-doctoral fellowship to Filip Vandenbussche and a research grant (G.0313.05) to Dominique Van Der Straeten from the Fund for Scientific Research (FWO) Flanders. The authors thank Siobhan Brady and Rosangela Sozzani (Benfey laboratory, Duke University, Durham, NC, USA) for helpful discussion and revision of Fig. 4.

4.8 References

Aach, H., Bode, H., Robinson, DG. and Graebe, JE. (1997) ent-kaurene synthase is located in proplastids of meristematic shoot tissues. Planta **202**, 211-219.

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, JR. and Harberd, NP. (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science **311**, 91-94.

Achard, P., Vriezen, W.H., Van Der Straeten, D. and Harberd, N.P. 2003. Ethylene regulates *Arabidopsis* development via the modulation of DELLA protein growth repressor function. Plant Cell **15**, 2816-2825.

Adams, D.O. and Yang, S.F. 1979. Ethylene Biosynthesis - Identification of 1-Aminocyclopropane-1-Carboxylic Acid as an Intermediate in the Conversion of Methionine to Ethylene. Proceedings of the National Academy of Sciences of the United States of America **76**, 170-174.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker J.R. 1999. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science **284**, 2148-2152.

Alonso, J.M. and Stepanova, A.N. 2004. The ethylene signaling pathway. Science **306**, 1513-1515.

Alvey, L. and Harberd, N.P. 2005. DELLA proteins: integrators of multiple plant growth regulatory inputs? Physiologia Plantarum **123**, 153-160.

Arshad, M. and Frankenberger, W.T. 1988. Influence of Ethylene Produced by Soil Microorganisms on Etiolated Pea Seedlings. Applied and Environmental Microbiology **54**, 2728-2732.

Bovy, A.G., Angenent, G.C., Dons, H.J.M. van Altvorst, A.C. 1999. Heterologous expression of the *Arabidopsis* etr1-1 allele inhibits the senescence of carnation flowers. Molecular Breeding **5**, 301-308.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill ,A.J., Hoffman, N.E., Davis, K.R. and Gorlach J. 2001. Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. Plant Cell **13**, 1499-1510.

Capitani, G., Hohenester, E., Feng, L., Storici, P., Kirsch, J.F. and Jansonius, J.N. 1999. Structure of 1-aminocyclopropane-1-carboxylate synthase, a key enzyme in the biosynthesis of the plant hormone ethylene. Journal of Molecular Biology **294**, 745-756.

Chae, H.S., Faure, F. and Kieber, J.J. 2003. The eto1, eto2, and eto3 mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. Plant Cell **15**, 545-559.

Chang, C.R. 2003. Ethylene signaling: the MAPK module has finally landed. Trends in Plant Science **8**, 365-368.

Chen, Y.F., Randlett, M.D., Findell, J.L. and Schaller, G.E. 2002. Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of *Arabidopsis*. Journal of Biological Chemistry **277**, 19861-19866.

Chiang, H.H., Hwang, I. and Goodman H.M. 1995. Isolation of the *Arabidopsis* Ga4 Locus. Plant Cell **7**, 195-201.

De Grauwe, L., **Vriezen, W., Bertrand, S., Phillips, A., Vidal, A., Hedden, P. and Van Der Straeten D.** (2007) Interactions between gibberellin and ethylene signaling pathways in *Arabidopsis thaliana*. Planta 10.1007/s00425-007-0499-x.

De Paepe, A. and Van Der Straeten, D. 2005. Ethylene Biosynthesis and Signaling: An Overview. Vitamins and Hormones **72**, 399-430.

Dill, A., Thomas, S.G., Hu, J.H., Steber, C.M. and Sun T.P. 2004. The *Arabidopsis* F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell **16**, 1392-1405.

Finlayson, S.A., Foster, K.R. and Reid, D.M. 1991. Transport and Metabolism of 1-Aminocyclopropane-1-Carboxylic Acid in Sunflower (Helianthus-Annuus L) Seedlings. Plant Physiology **96**, 1360-1367.

Finlayson, S.A., Lee, I.J. and Morgan, P.W. 1998. Phytochrome B and the regulation of circadian ethylene production in Sorghum. Plant Physiology **116**, 17-25.

Foo, E., Ross, J.J., Davies, N.W., Reid, J.B. and Weller, J.L. 2006. A role for ethylene in the phytochrome-mediated control of vegetative development. Vol. 46, 911-921.

Friml, J. 2003. Auxin transport -- shaping the plant. Current Opinion in Plant Biology **6**, 7-12.

Fu, X.D. and Harberd, N.P. 2003. Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. Nature **421**, 740-743.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12**, 393-404.

Gagne, J.M., Smalle, J., Gingerich, D.J., Walker, J.M., Yoo, S.D., Yanagisawa, S. and Vierstra R.D. 2004. *Arabidopsis* EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proceedings of the National Academy of Sciences of the United States of America **101**, 6803-6808.

Gao, Z.Y., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.L., Kieber, J.J. and Schaller G.E. 2003. Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of *Arabidopsis* through participation in ethylene receptor signaling complexes. Journal of Biological Chemistry **278**, 34725-34732.

Gomez-Lim, M.A., Valdeslopez, V., Cruzhernandez, A. and Saucedoarias, L.J. 1993. Isolation and Characterization of a Gene Involved in Ethylene Biosynthesis from *Arabidopsis-Thaliana*. Gene **134**, 217-221.

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z.L., Powers, S.J., Gong, F., Phillips, A.L., Hedden, P., Sun, T.P. and Thomas, SG. 2006. Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. Plant Cell **18**, 3399-3414.

Guo, H.W. and Ecker, J.R. 2003. Plant responses to ethylene gas are mediated by SCF (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115**, 667-677.

Guzman, P. and Ecker, J.R. 1990. Exploiting the Triple Response of *Arabidopsis* to Identify Ethylene-Related Mutants. Plant Cell **2**, 513-523.

Hall, A.E. and Bleecker, A.B. 2003. Analysis of combinatorial loss-of-function mutants in the *Arabidopsis* ethylene receptors reveals that the ers1etr2 double mutant has severe developmental defects that are EIN2 dependent. Plant Cell **15**, 2032-2041.

Hedden, P. and Phillips, A.L. 2000. Gibberellin metabolism: new insights revealed by the genes. Trends in Plant Science **5**, 523-530.

Helliwell, C.A., Chandler, P.M., Poole, A., Dennis, E.S. and Peacock, W.J. 2001a. The CYP88A cytochrome P450, ent-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. Proceedings of the National Academy of Sciences of the United States of America **98**, 2065 2070.

Helliwell, C.A., Poole, A., Peacock, W.J. and Dennis, E.S. 1999. *Arabidopsis* ent-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. Plant Physiology **119**, 507-510.

Helliwell, C.A., Sullivan, J.A., Mould, R.M., Gray, J.C., Peacock, W.J. and Dennis H.S. 2001b. A plastid envelope location of *Arabidopsis* ent-kaurene oxidase links the platid and endoplasmatic reticulum steps of the gibberellin biosynthesis pathway. Plant Journal **28**, 201-208.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.H.G., Bleecker, A.B., Ecker, J.R. and Meyerowitz, E.M. 1998. EIN4 and ERS2 are members of the putative ethylene receptor gene family in *Arabidopsis*. Plant Cell **10**, 1321-1332.

Jackson, M.B. 1985. Ethylene and Responses of Plants to Soil Waterlogging and Submergence. Annual Review of Plant Physiology and Plant Molecular Biology **36**, 145-174.

Jackson, M.B. and Armstrong, W. 1999. Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. Plant biology, **1**, 274-287.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker J.R. 1993. Ctr1, a Negative Regulator of the Ethylene Response Pathway in *Arabidopsis*, Encodes a Member of the Raf Family of Protein-Kinases. Cell **72**, 427-441.

Le, J., Vandenbussche, F., Van Der Straeten, D. and Verbelen J.P. 2001. In the early response of *Arabidopsis* roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiology **125**, 519-522.

Lorenzo, O., Piquera, S.R., Sanchez-Serrano, J.J. and Solano R. 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15**, 165-178.

Metzger, J.D. and Zeevaart, J.A.D. 1980. Comparison of the Levels of 6 Endogenous Gibberellins in Roots and Shoots of Spinach in Relation to Photoperiod. Plant Physiology **66**, 679-683.

Mitchum, M.G., Yamaguchi, S., Hanada, A., Kuwahara, A., Yoshioka, Y., Kato, T., Tabata, S., Kamiya, Y. and Sun, T.P. 2006. Distinct and overlapping roles of two gibberellin 3-oxidases in *Arabidopsis* development. Plant Journal **45**, 804-818.

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y.C., Park, S.H., Ueguchi-Tanaka, M., Suzuki, H., Katoh, E., Iuchi, S., Kobayashi, M., Maeda, T., Matsuoka, M. and Yamaguchi, I.. 2006. Identification and characterization of *Arabidopsis* gibberellin receptors. Plant Journal **46**, 880-889.

Oden, P.C., Wang, Q., Hogberg, K.A. and Werner, M. 1995. Transport and Metabolism of Gibberellins in Relation to Flower Bud Differentiation in Norway spruce (Picea-Abies). Tree Physiology **15**, 451-456.

Olson, D.C., White, J.A., Edelman, L., Harkins, R.N. and Kende, H. 1991. Differential Expression of 2 Genes for 1-Aminocyclopropane-1-Carboxylate Synthase in Tomato Fruits. Proceedings of the National Academy of Sciences of the United States of America **88**, 5340-5344.

Olszewski, N., Sun, T.P. and Gubler, F. 2002. Gibberellin signaling: Biosynthesis, catabolism, and response pathways. Plant Cell **14**, S61-S80.

Ortega-Martinez, O., Pernas, M., Carol, R.J. and Dolan, L. 2007. Ethylene modulates stem cell division in the *Arabidopsis thaliana* root. Science **317**, 507-510.

Otsuka, M., Kenmoku, H., Ogawa, M., Okada, K., Mitsuhashi, W., Sassa, T., Kamiya, Y., Toyomasu, T. and Yamaguchi, S.. 2004. Emission of ent-Kaurene, a Diterpenoid Hydrocarbon Precursor for Gibberellins, into the Headspace from Plants. Vol. 45, 1129-1138.

Ouaked, F., Rozhon, W., Lecourieux, D. and Hirt, H. 2003. A MAPK pathway mediates ethylene signaling in plants. Embo Journal **22**, 1282-1288.

Phillips, S., Ward, D.A., Uknes, S., Appleford, N.E.J., Lang, E.T., Huttly, A.K., Gaskin, P., Graebe, J.E. and Hedden, P. 1995. Isolation and Expression of 3 Gibberellin 20-Oxidase Cdna Clones from *Arabidopsis*. Plant Physiology **108**, 1049-1057.

Pierik, R., Cuppens, M.L.C., Voesenek, L. and Visser, E.J.W. 2004a. Interactions between ethylene and gibberellins in phytochrome-mediated shade avoidance responses in tobacco. Plant Physiology **136**, 2928-2936.

Pierik, R., Millenaar, F.F., Peeters, A.J.M. and Voesenek, L. 2005. New perspectives in flooding research: The use of shade avoidance and *Arabidopsis thaliana*. Annals of Botany **96**, 533-540.

Pierik, R., Whitelam, G.C., Voesenek, L., de Kroon, H. and Visser, E.J.W. 2004b. Canopy studies on ethylene-insensitive tobacco identify ethylene as a novel element in blue light and plant-plant signaling. Plant Journal **38**, 310-319.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, S. and Genschik, P. 2003. EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F-box proteins: EBF1 and EBF2. Cell **115**, 679-689.

Proebsting, W.M., Hedden, P., Lewis, M.J., Croker, S.J. and Proebsting, L.N. 1992. Gibberellin Concentration and Transport in Genetic Lines of Pea - Effects of Grafting. Plant Physiology **100**, 1354-1360.

Ravanel, S., Gakiere, B., Job, D. and Douce, R. 1998. The specific features of methionine biosynthesis and metabolism in plants. Proceedings of the National Academy of Sciences of the United States of America **95**, 7805-7812.

Raz, V. and Ecker, J.R. 1999. Regulation of differential growth in the apical hook of *Arabidopsis*. Development **126**, 3661-3668.

Rodrigues-Pousada, R., Derycke, R., Dedonder, A., Vancaeneghem, W., Engler, G., Vanmontagu, M. and Van Der Straeten, D. 1993. The *Arabidopsis* 1-Aminocyclopropane-1-Carboxylate Synthase Gene-1 Is Expressed During Early Development. Plant Cell **5**, 897-911.

Ross J.J., Symons G.M., Abas L., Reid J.B., Luschnig C. 2006. Hormone distribution and transport. In: Hedden P, Thomas SG, eds. Plant Hormone Signaling, Vol. 24, Oxford, UK: Blackwell Publishing Ltd, 125-145.

Rottmann, W.H., Peter, G.F., Oeller, P.W., Keller, J.A., Shen, N.F., Nagy, B.P., Taylor, L.P., Campbell, A.D. and Theologis, A. 1991. 1-Aminocyclopropane-1-Carboxylate Synthase in Tomato Is Encoded by a Multigene Family Whose Transcription Is Induced During Fruit and Floral Senescence. Journal of Molecular Biology **222**, 937-961.

Ruzicka, K., Ljung, K., Vanneste, S,. Podhorska, R., Beeckman, T., Friml, J. and Benkova, E. 2007. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell **19**, 2197-2212.

Saibo, N.J.M., Vriezen, W.H., Beemster, G.T.S and Van Der Straeten, D. 2003. Growth and stomata development of *Arabidopsis* hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. Plant Journal **33**, 989-1000.

Sakai, H., Hua, J., Chen, Q.H.G., Chang, C.R., Medrano, L.J., Bleecker, A.B. and Meyerowitz, E.M. 1998. ETR2 is an ETR1-like gene involved in ethylene signaling in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **95**, 5812-5817.

Savaldi-Goldstein, S., Peto, C. and Chory, J. 2007. The epidermis both drives and restricts plant shoot growth. Nature **446**, 199-202.

Silverstone, A.L., Chang, C.W., Krol, E. and Sun, T.P. 1997. Developmental regulation of the gibberellin biosynthetic gene GA1 in *Arabidopsis thaliana*. Plant Journal **12**, 9-19.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y. and Sun, T.P. 2001. Repressing a repressor: Gibberellin-induced rapid reduction of the RGA protein in *Arabidopsis*. Plant Cell **13**, 1555-1565.

Smalle, J., Haegman, M., Kurepa, J., VanMontagu, M. and Van Der Straeten, D. 1997. Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. Proceedings of the National Academy of Sciences of the United States of America **94**, 2756-2761.

Smalle, J. and Van Der Straeten, D. 1997. Ethylene and vegetative development. Physiologia Plantarum **100**, 593-605.

Smith, V.A. 1993. Gibberellin Translocation in Pisum-Sativum L .3. Biological and Biochemical Consequences of the Le Mutation. Planta **191**, 158-165.

Solano, R., Stepanova, A., Chao, Q.M. and Ecker, J.R. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes & Development **12**, 3703-3714.

Starling, E.H. (1905) The Croonian Lectures. I. On the chemical correlation of the functions of the body. Lancet 166: 339-341.

Stepanova, A.N., Yun, J., Likhacheva, A.V. and Alonso, J.M. 2007. Multilevel interactions between ethylene and auxin in *Arabidopsis* roots. Plant Cell **19**, 2169-2185.

Suge, H., Nishizawa, T., Takahashi, H. and Takeda, K. 1997. Phenotypic plasticity of internode elongation stimulated by deep-seeding and ethylene in wheat seedlings. Plant Cell and Environment **20**, 961-964.

Sun, T.P., Goodman, H.M. and Ausubel, F.M. 1992. Cloning the *Arabidopsis* Ga1 Locus by Genomic Subtraction. Plant Cell **4**, 119-128.

Sun, T.P. and Kamiya, Y. 1994. The *Arabidopsis* Ga1 Locus Encodes the Cyclase Ent-Kaurene Synthetase-a of Gibberellin Biosynthesis. Plant Cell **6**, 1509-1518.

Swarup, R., Kramer, E.M., Perry, P., Knox, K., Leyser, H.M.O., Haseloff, J., Beemster, G.T.S., Bhalerao, R. and Bennett, M.J. 2005. Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nature Cell Biology **7**, 1057-1065.

Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G.T.S., Sandberg, G., Bhalerao, R., Ljung, K. and Bennett, M.J. 2007. Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. Plant Cell **19**, 2186-2196.

Taiz, L. and Zeiger, E. 2006. Plant Physiology: Sunderland (Mass.): Sinauer associates.

Tanimoto, E. 1987. Gibberellin-Dependent Root Elongation in Lactuca-Sativa - Recovery from Growth Retardant-Suppressed Elongation with Thickening by Low Concentration of Ga3. Plant and Cell Physiology **28**, 963-973.

Thomas, S.G., Phillips, A.L. and Hedden, P. 1999. Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proceedings of the National Academy of Sciences of the United States of America **96**, 4698-4703.

Tsuchisaka, A. and Theologis, A. 2004a. Heterodimeric interactions among the 1-amino-cyclopropane-1-carboxylate synthase polypeptides encoded by the *Arabidopsis* gene family. Proceedings of the National Academy of Sciences of the United States of America **101**, 2275-2280.

Tsuchisaka, A. and Theologis, A. 2004b. Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiology **136**, 2982-3000.

Vandenbussche, F., Vancompernolle, B., Rieu, I., Ahmad, M., Phillips, A., Moritz, T., Hedden, P. and Van Der Straeten, D. (2008.) Gibberellin independence of ethylene-stimulated *Arabidopsis* hypocotyl elongation in blue light. Journal of Experimental Botany

Vandenbussche, F. and Van Der Straeten, D.. 2004. Shaping the shoot: a circuitry that integrates multiple signals. Trends in Plant Science **9**, 499-506.

Vandenbussche, F. and Van Der Straeten, D. 2007. One for all and all for one: cross-talk of multiple signals controlling the plant phenotype. Journal of Plant Growth Regulation **26**, 178-187.

Vandenbussche, F., Vriezen, W.H., Smalle, J., Laarhoven, L.J.J., Harren, F.J.M. and Van Der Straeten, D. 2003. Ethylene and auxin control decreased light intensity. Plant Physiology **133**, 517-527.

Vandenbussche, F., Vriezen, W.H. and Van Der Straeten, D. 2006. Ethylene biosynthesis and signaling: a puzzle yet to be completed. In: Hedden P, Thomas SG, eds. Plant Hormone Signaling, Vol. 24, Oxford, UK: Blackwell Publishing Ltd, 125-145.

Van Der Straeten, D., Rodrigues-Pousada, R.A., Villarroel, R., Hanley, S., Goodman, H.M. and Van Montagu, M. 1992. Cloning, genetic mapping, and expression analysis of an *Arabidopsis thaliana* gene that encodes 1 aminocyclopropane 1 carboxylate synthase. Proc. Natl. Acad. Sci. USA **89**, 9969-9973.

Varbanova, M., Yamaguchi, S., Yang, Y., McKelvey, K., Hanada, A,. Borochov, R., Yu, F., Jikumaru, Y., Ross, J., Cortes, D., Ma, CJ., Noel, JP., Mander, L., Shulaev, V., Kamiya, Y., Rodermel, S., Weiss, D. and Pichersky, E. 2007. Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. Plant Cell **19**, 32-45.

Visser, E.J.W., Cohen, J.D., Barendse, G.W.M., Blom and C., Voesenek, L. 1996. An Ethylene-Mediated Increase in Sensitivity to Auxin Induces Adventitious Root Formation in Flooded Rumex palustris. Plant Physiology **112**, 1687-1692.

Voesenek, L., Pierik, P.J.M, Blom, C. and Sassen, M.M.A. 1990. Petiole Elongation in Rumex Species During Submergence and Ethylene Exposure - the Relative Contributions of Cell-Division and Cell Expansion. Journal of Plant Growth Regulation **9**, 13-17.

Vriezen, W.H., Achard, P., Harberd, N.P. and Van Der Straeten D. 2004. Ethylene-mediated enhancement of apical hook formation in etiolated *Arabidopsis thaliana* seedlings is gibberellin dependent. Plant Journal **37**, 505-516.

Vriezen, W.H., Zhou, Z.Y., Van Der Straeten, D. 2003. Regulation of submergence-induced enhanced shoot elongation in Oryza sativa L. Annals of Botany **91**, 263-270.

Yamaguchi, S., Sun, T.P., Kawaide, H. and Kamiya, Y. 1998. The GA2 locus of *Arabidopsis thaliana* encodes ent-kaurene synthase of gibberellin biosynthesis. Plant Physiology **116**, 1271-1278.

Yang, S.F. and Hoffman, N.E. 1984. Ethylene Biosynthesis and Its Regulation in Higher-Plants. Annual Review of Plant Physiology and Plant Molecular Biology **35**, 155-189.

Yang, Z., Tian, L.N., Latoszek-Green, M., Brown, D. and Wu, K.Q. 2005. *Arabidopsis* ERF4 is a transcriptional repressor capable of modulating ethylene and abscisic acid responses. Plant Molecular Biology **58**, 585-596.

Zhao, X.Y., Yu, X.H., Liu, X.M. and Lin, C.T. 2007. Light regulation of gibberellins metabolism in seedling development. Journal of Integrative Plant Biology **49**, 21-27.

Zhu, Y., Nomura, T., Xu, Y., Zhang, Y., Peng, Y., Mao, B., Hanada, A., Zhou, H., Wang, R., Li, P., Zhu, X., Mander, L.N., Kamiya, Y., Yamaguchi, S. and He, Z. 2006. ELONGATED UPPERMOST INTERNODE Encodes a Cytochrome P450 Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. Plant Cell, **18**, 442-456.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. Plant Physiology **136**, 2621-2632.

Chapter 5:

SLO2 is required for energy metabolism

Adapted from :

SLO2, a mitochondrial PPR protein affecting several RNA editing sites, is required for energy metabolism

Qiang Zhu,[a,1] Jasper Dugardeyn,[a,1,2] Chunyi Zhang,[a, 3] Mizuki Takenaka, [b] Kristina Kühn, [c] Christian Craddock,[d] Jan Smalle,[a,4] Michael Karampelias,[a,5] Jurgen Denecke,[e] Janny Peters[f], Tom Gerats[f], Axel Brennicke,[b] Peter Eastmond,[d] Etienne H. Meyer,[c] and Dominique Van Der Straeten[a,6]

[a1] These authors contributed equally to this work

The Plant Journal , 2012 ;71(5) : 836-849
DOI: 10.1111/j.1365-313X.2012.05036.x

Jasper Dugardeyn performed the original experiments for Figure 1, 2c, 2d, 3, 5b, 5c, 5e, 6 and wrote 50% of the first draft of the manuscript.

Pentatricopeptide repeat (PPR) proteins belong to a family of approximately 450 members in *Arabidopsis*, of which few were characterized. We identified loss of function alleles of *SLO2*, defective in a PPR protein belonging to the E+ subclass of the P-L-S subfamily. *slo2* mutants are characterized by retarded leaf emergence, restricted root growth, and late flowering. This phenotype is enhanced in absence of sucrose, suggesting a defect in energy metabolism. The *slo2* growth retardation phenotypes are largely suppressed by supplying sugars, increasing light dosage or the concentration of CO_2. The SLO2 protein is localized in mitochondria. We identified four RNA editing defects and reduced editing at three sites in *slo2* mutants. The resulting amino acid changes occur in four mitochondrial proteins belonging to complex I of the electron transport chain. Both complex I abundance and activity are highly reduced in the *slo2* mutants, as well as the abundance of complex III and IV. Moreover, ATP, NAD+, and sugar contents were much lower in the mutants. In contrast, the abundance of alternative oxidase was significantly enhanced. We propose that SLO2 is required for carbon energy balance in *Arabidopsis* by maintaining the abundance and/or activity of complexes I, III and IV of the mitochondrial electron transport chain.

5.1 Introduction

Pentatricopeptide repeat (PPR) proteins, which contain tandem arrays of a degenerate 35-amino-acid repeat, are uniquely amplified in plants (Lurin *et al.*, 2004; Andrés *et al.*, 2007; Schmitz-Linneweber and Small, 2008). In *Arabidopsis*, this family is composed of 450 members, and can be divided into two subfamilies based on the structure of the repeated motif, called P and PLS subfamilies. Members of the P subfamily contain the canonical P motif common to all eukaryotes, while members of the PLS subfamily contain the P motif, as well as two P motif-derived variants, the short (S) and long (L) motifs. Based on the presence of conserved domains in the C-terminal region, the PLS subfamily can be further divided into the PLS, E, E+ and DYW subgroups (Lurin *et al.*, 2004; Andrés *et al.*, 2007). In plants, PPR proteins are predominantly localized in plastids (19%) or mitochondria (54%) (Lurin *et al.*, 2004). So far only one nuclear PPR protein and one dual-targeted protein have been identified (Ding *et al.*, 2006; Hammani *et al.*, 2011b). Given the number and slight variation of sequence repeats, PPR proteins were proposed to function as gene-specific regulators of plant RNA metabolism (Lurin *et al.*, 2004). In plant cell organelles, PPR proteins mainly play roles in RNA stability, cleavage, splicing, and editing; while also being involved in translational initiation and regulation (Andrés *et al.*, 2007; Schmitz-Linneweber and Small, 2008). Some PPR proteins appear essential for plant growth and development, as supported by the embryo lethality or severe growth defects associated with loss of function mutants (de Longevialle *et al.*, 2007; Liu *et al.*, 2010; Sung *et al.*, 2010). Many PPR proteins function in RNA editing (Schmitz-Linneweber and Small, 2008). The biochemical effect of RNA editing in plants is most often a site specific C-to-U modification by cytosine deamination. (Shikanai, 2006). To date, all PPR proteins involved in plastid or mitochondrial RNA editing belong to the E and DYW subgroups of the PLS subfamily, with the exception of PPR596, which is in the P class (Doniwa *et al.*, 2010; Takenaka, 2010). In the plastid transcriptome, there are 34 editing sites (Tsudzuki *et al.*, 2001), while in mitochondria this number exceeds 500 (Giegé and Brennicke, 1999). In plastids, members of the E- and the DYW- class are implicated in RNA editing (Schmitz-Linneweber and Small, 2008; Yu *et al.*, 2009). In *Arabidopsis* and rice mitochondria, 14 RNA editing factors have been reported so far (Kim *et al.*, 2009; Zehrmann *et al.*, 2009; Sung *et al.*, 2010; Takenaka, 2010; Takenaka *et al.*, 2010; Tang *et al.*, 2010; Verbitskiy *et al.*, 2010; Hammani *et al.*, 2011a; Yuan and Liu, 2012).

The energy metabolism in plant cells encompasses mitochondria, plastids, and peroxisomes. Mitochondria are the main energy factories of the cell, performing oxidative phosphorylation driven by the electron transport chain (mETC). The electron transport chain of the cytochrome (classical) pathway is composed of 4 large complexes: complex I, complex II, complex III and complex IV. Complex I, an NADH dehydrogenase, is the first protein complex in the electron transport chain (ETC), which catalyzes the NADH oxidation while ubiquinone (UQ) is reduced; complex II is the only enzyme that participates both in the citric acid cycle and in the electron transport chain; complex III is also known as cytochrome c reductase, and oxidizes ubiquinol while reducing cytochrome c. Complex IV is a cytochrome c oxidase, the terminal oxidase of the classical ETC. Complex I is composed of at least 49 subunits in *Arabidopsis* (Klodmann *et al.*, 2010), the majority of which are encoded by nuclear genes. Dysfunction in complex I results in various phenotypes, such as increased respiration and decreased photosynthetic

efficiency, thereby causing growth and developmental defects (Gutierres *et al.*, 1997; Brangeon *et al.*, 2000; Sabar *et al.*, 2000; Pineau *et al.*, 2005; Garmier *et al.*, 2008; Keren *et al.*, 2009; Meyer *et al.*, 2009; Liu *et al.*, 2010; Sung *et al.*, 2010); or changes in stress resistance (Sugioka *et al.*, 1988; Sabar *et al.*, 2000; Dutilleul *et al.*, 2003; Meyer *et al.*, 2009; Yuan and Liu, 2012). With the exception of apocytochrome B which is encoded by the mitochondrial genome, complex III subunits are all nuclear genome encoded (Unseld *et al.*, 1997). Mutational analysis demonstrated that the ubiquinol-cytochrome c oxidoreductase activity of complex III is important for normal plant growth and stress response (des Francs-Small *et al.*, 2012). Complex IV is the terminal complex of the respiratory chain, composed around 12-13 subunits, while complex V is the ATP synthase complex, which comprises around 15 distinct subunits (Dudkina *et al.*, 2006). Besides the classical cytochrome pathway, plants also possess alternative NAD(P)H dehydrogenases in the first part of the respiratory chain and an alternative oxidase (AOX) in the latter (Millar *et al.*, 2011). *AOX* can be induced by environmental stress or factors that inhibit the respiratory chain (Millar *et al.*, 2001).

In this study, we characterize a PPR protein, named SLOW GROWTH 2 (SLO2). In *slo2* mutants, 7 editing changes were found which lead to 4 amino acid changes in subunits of complex I of the mitochondrial ETC, leading to a reduction of abundance and activity of complex I. Moreover, the abundance of complex III and complex IV were also reduced. We further demonstrate that *SLO2* plays a role in carbon and energy metabolism. To our knowledge, this is the only example of a single gene mutation leading to defects in 3 mitochondrial complexes.

5.2 Results

5.2.1. Isolation of the *slo2-1* mutant

We previously demonstrated that the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) enhances leaf emergence on low nutrient medium (LNM) (Smalle and Van Der Straeten, 1997). In search for ethylene mutants based on this observation, we identified the *slo2-1* mutant as one of the candidates by screening of a collection of 5000 T-DNA insertion lines (Feldmann, 1991) (Figure 1a and 1b). However, the subsequent characterization of *slo2-1* revealed only partial changes in ethylene sensitivity. In addition to the delayed leaf emergence of *slo2-1*, a delay at various stages throughout the life cycle was observed compared to the wild-type (Supplemental Table 1, DOI: 10.1111/j.1365-313X.2012.05036.x). Due to this general developmental delay the mutant was named *slow growth 2-1* (*slo2-1*). The *slo2-1* mutation segregates according to Mendelian law of genetics, and is recessive, thus a loss-of-function mutation.The *slo2-1* mutant was crossed (four times) into the Col-0 background, and phenotypic characteristics were preserved both in early and later development (Figure 1).

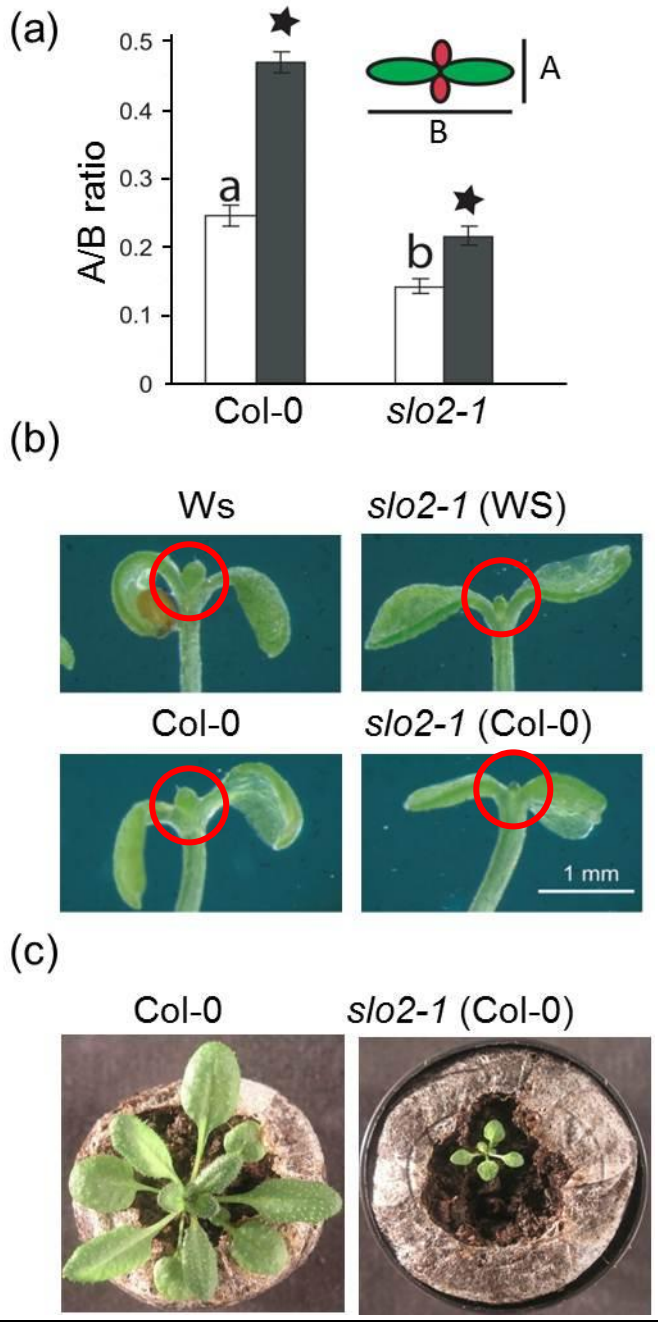

Figure 1. The *slo2-1* mutant shows reduced emergence of the first leaf pair

(a) Degree of leaf emergence (DLE; A/B) in 2-week-old seedlings grown on LNM in the presence (black) or absence (white) of 50 µM ACC. Stars reflect a significant difference between treated and control plants ($p<0,01$). Error bars represent standard error ($n = 50$)

(b) The *slo2-1* mutant displays a delayed leaf emergence. Close-up of leaf emergence of vertically grown 2-week-old Ws (wild-type), *slo2-1* in Ws background, Col-0 (wild-type) and *slo2-1* in Col-0 background. Seedlings were grown on LNM.

(c) Rosette phenotype of 3 weeks old plants grown in soil.

5.2.2. Map-based cloning of the *SLO2* gene

To uncover the molecular nature of the *slo2-1* mutation, we cloned the *SLO2* gene using a map-based cloning approach, since the mutation was not linked with the kanamycin resistance gene in the T-DNA. Details on the procedure are provided as supplementary information. The *SLO2* gene encodes a putative PPR protein with unknown function (*At2g13600*), The *slo2-1* mutant contains an in frame deletion of 21-bp. We identified a second mutant allele, designated *slo2-2*, in the SALK SIGNAL collection, *SALK_521900*, containing a T-DNA insertion at 878 bp downstream of the start codon (Figure 2b). *slo2-2* shows an evident reminiscence of the *slo2-1* mutant phenotype albeit less pronounced (Figure 2c, 2d, 3c and 3d). A third allele, *slo2-3* (Tilling T94087), harbors an EMS-induced point mutation (C to T) at position 247 downstream of ATG, causing a stop codon at amino acid 83 (Figure 2b). This mutation resulted in the strongest *slo2* phenotype (Figure 2c and 2d).

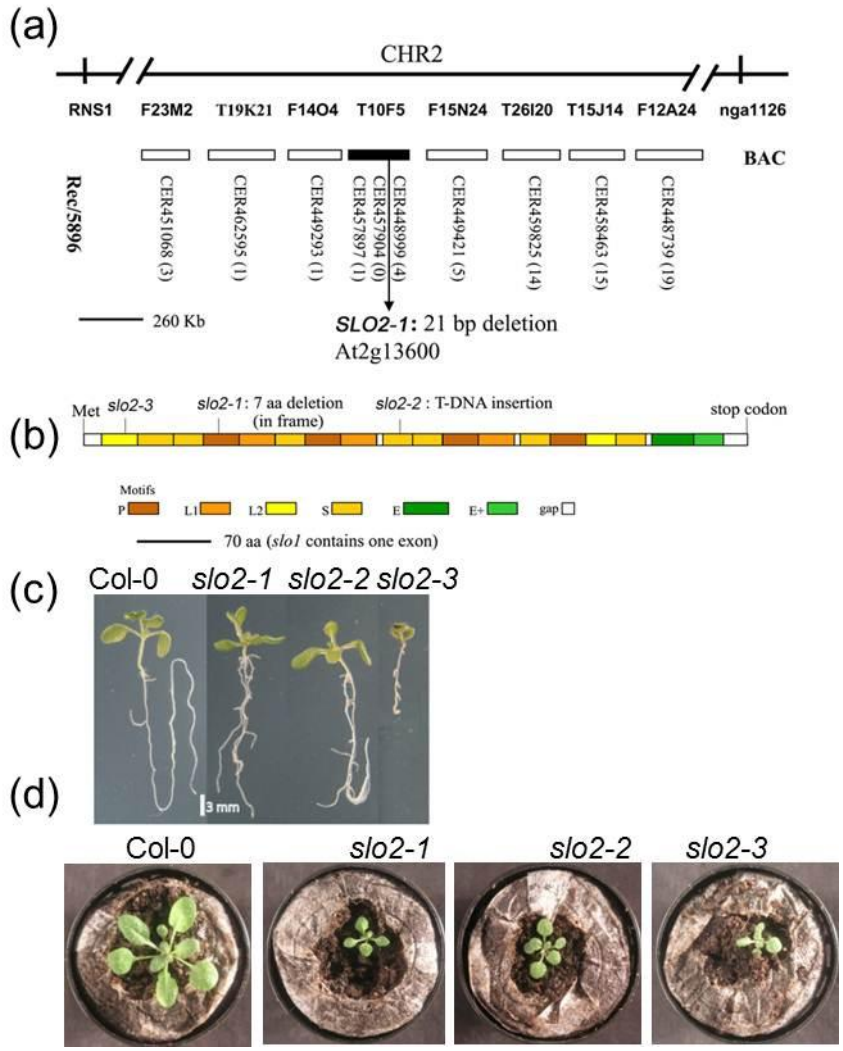

Figure 2. Map-based cloning of the *SLO2* gene

(a) Scheme of the map-based cloning of the *SLO2* gene, Detailed in supplementary data.

(b) Schematic representation of SLO2 protein motif structure and position of different *slo2* mutations. Detailed information for mutant alleles is included in supplementary data.

(c) Phenotypes of vertically grown 2-week-old wild-type seedlings and three alleles of *slo2*.

(d) Phenotypes of 3 week old wild-type and mutant plants grown on soil.

5.2.3. *SLO2* encodes a member of the Pentatricopeptide Repeat (PPR) Protein family

Basic Local Alignment Search Tool (BLAST) analysis identified *SLO2* as a member of the PPR family, belonging to the P-L-S subfamily. It consists of 7 PPR-like S , 5 PPR-like L , and 4 canonical P motifs with an E and E+ C-terminal extension (Lurin *et al.*, 2004) (Figure 2b). Unlike most plant PPR proteins, targeting prediction programs suggest inconclusive results on the subcellular localization of SLO2. A BLAST search of the full nucleotide sequence of *SLO2* against the complete *Arabidopsis* genome database did not result in closely similar genes (closest homologue is only 37% identical to SLO2). Given the low similarity of SLO2 with other PPR proteins and the evident phenotype exhibited by the *slo2* alleles (which is rare for PPR proteins), we conclude that *SLO2* encodes a unique PPR protein in the *Arabidopsis* and therefore probably carries unique functions.

5.2.4. Ectopic expression of *SLO2* complements the growth defects of *slo2* mutants

To further confirm that the phenotypes of *slo2* mutants were caused by a mutation in the *At2g13600* locus, we performed complementation experiments for *slo2-1* and *slo2-2* using the full length cDNA fused to GFP driven by its native promoter (*PSLO2:SLO2-GFP*) and the CaMV-35S promoter (*P35S:SLO2-GFP*), respectively. Both complementation lines showed reversal of the delay in leaf emergence and suppressed the reduced growth phenotypes in root and shoot (Figure 3a-d). The data above confirm that mutation of the *SLO2* gene is responsible for the mutant phenotype.

5.2.5. SLO2 protein is localized in mitochondria

To unequivocally determine the subcellular localization of SLO2, *P35S:SLO2-GFP* seedlings were stained with the mitochondria-specific marker mitotracker orange, and analysed with a confocal microscope. The results show that the fusion protein co-localizes with the mitochondrial marker (Figure 4a), suggesting a mitochondrial localization for SLO2.

To corroborate these results, we crossed the mitochondrial marker line (ATPase-mCherry) with *P35S:SLO2-GFP* transgenic plants, and surveyed the F1 progeny using confocal microscopy. The data confirmed that SLO2 resides in plant mitochondria (Figure 4b). Similar results were obtained in plant leaves, although the GFP signal was lower (data not shown).

Expression of SLO2 in leaves is further demonstrated in supplemental figure 2 of chapter 6, but localisation studies were performed in roots to avoid background fluorescence from chloroplasts.

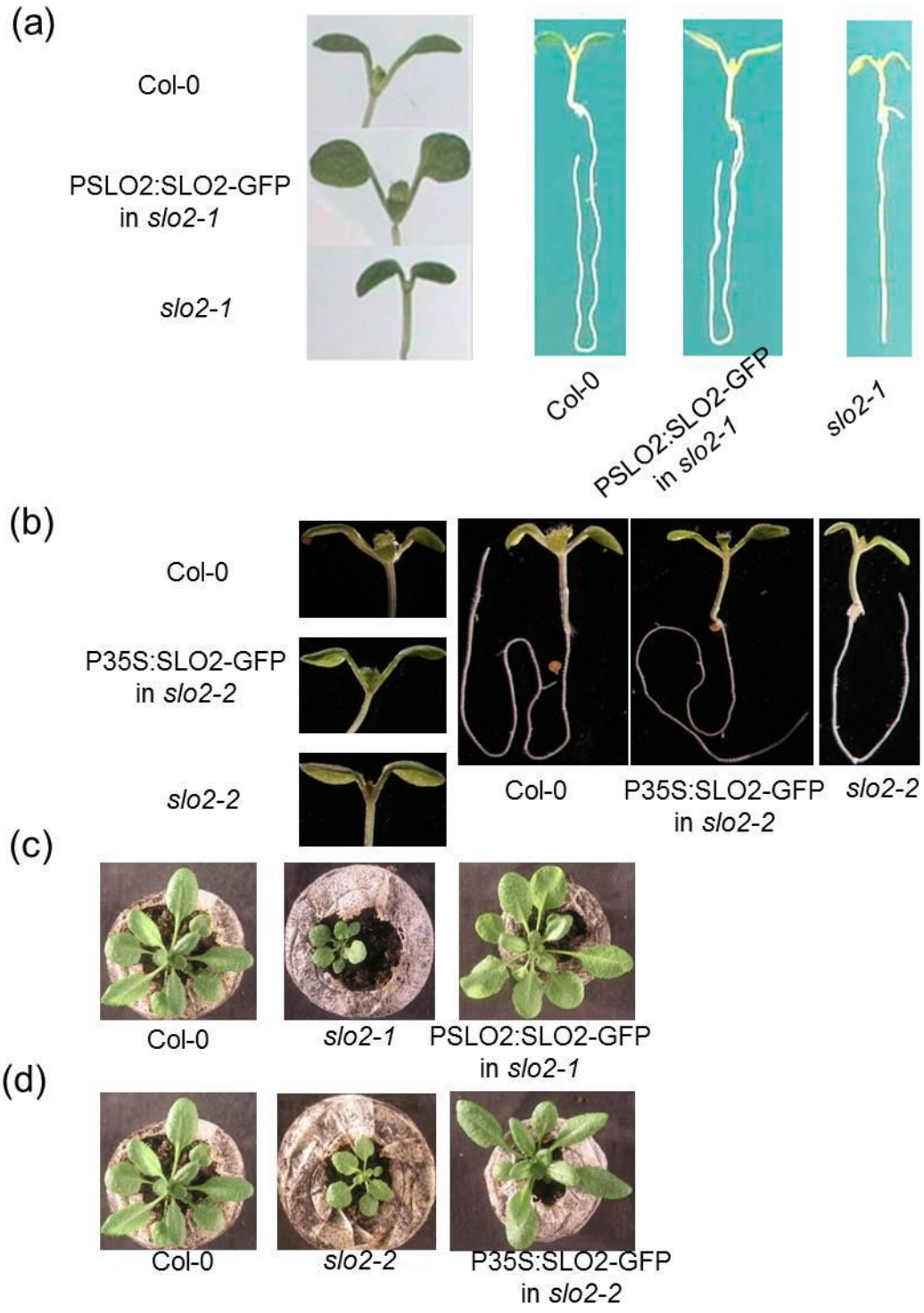

Figure 3. Complementation of *slo2* alleles with the wild-type *SLO2* gene

(a) Complementation of the *slo2-1* seedling phenotype using the *SLO2* native promoter. Phenotype of Col-0, *slo2-1* complemented with *pSLO2:SLO2-GFP* and *slo2-1*. Left: close-up of the shoot. Right: 7-day old seedlings.

(b) Complementation of the *slo2-2* seedling phenotype using the CaMV35S promoter.

(c) 4 weeks old plants of wild-type, *slo2-1*, *slo2-1*/*PSLO2:SLO2-GFP* complemented line.

(d) 4 weeks old plants of wild-type, *slo2-2*, *slo2-2*/*P35S:SLO2-GFP* complemented line.

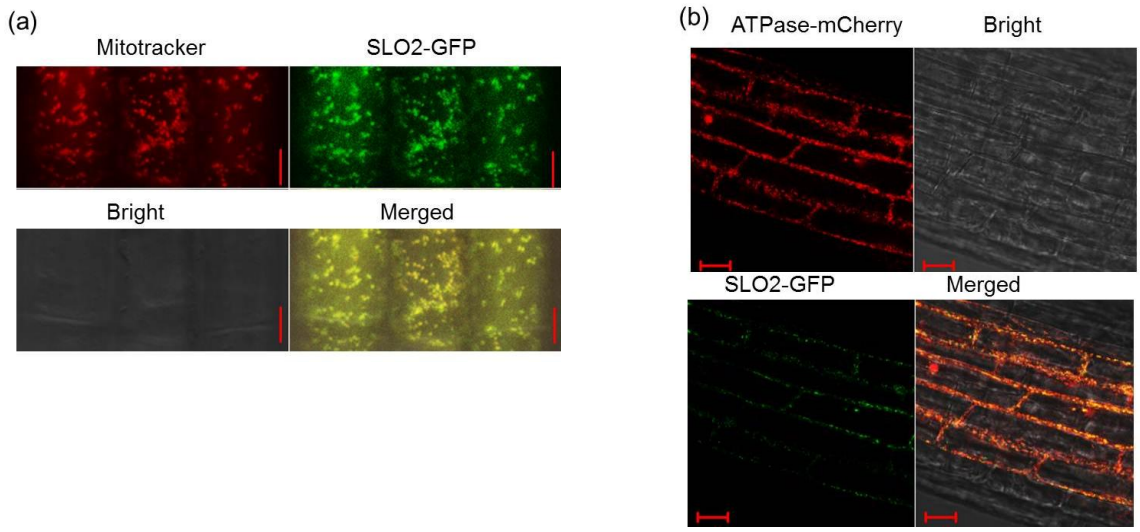

Figure 4. SLO2 protein is localized in mitochondria

(a) The root of transgenic plants carrying the *P35S:SLO2-GFP* construct were stained with mitotracker orange, and observed under a confocal microscope. Red: mitotracker orange; green: GFP; bright and (red, green and bright) merged field.

(b) The transgenic line carrying *P35S:SLO2-GFP* was crossed with a mitochondrial marker line carrying ATPase-mCherry. The root of F1 individuals was observed using a confocal microscope. Red: mCherry; green: GFP; bright and merged (red, green and bright) field.

5.2.6. *slo2* mutants are hypersensitive to sucrose

While lower levels of metabolizable sugars can stimulate seedling growth, high sugar concentrations have inhibitory effects (Rolland *et al.*, 2002). On medium without sucrose, *slo2* mutants showed a severe growth arrest (Figure 5a). However, the growth stimulation induced by 1% sucrose was remarkably higher in *slo2* alleles than in the wild-type (Figure 5a and 5b). Moreover, the early postembryonic growth was more strongly inhibited by 7% sucrose in *slo2* mutants (Figure 5a and 5b). To exclude an osmotic effect of sucrose, the response of *slo2* to mannitol and sorbitol, two non-metabolizable sugars, were tested. No difference in early post-germination development was observed. In addition, germination of *slo2* was delayed on sucrose in a dose-dependent manner, this effect being significantly stronger than in Col-0 (Figure 5c). Glucose and fructose also play a positive role in the post-germination growth of *slo2*, albeit to a lesser extent than sucrose (Figure S1).

We also tested the changes in *SLO2* gene expression in response to sucrose, in seedlings grown in the light and in darkness. As evidenced by *in situ hybridization*, the expression is enhanced by 1% sucrose in both conditions (Figure 5d). These results were corroborated by GUS histochemical analysis (Figure 5e).

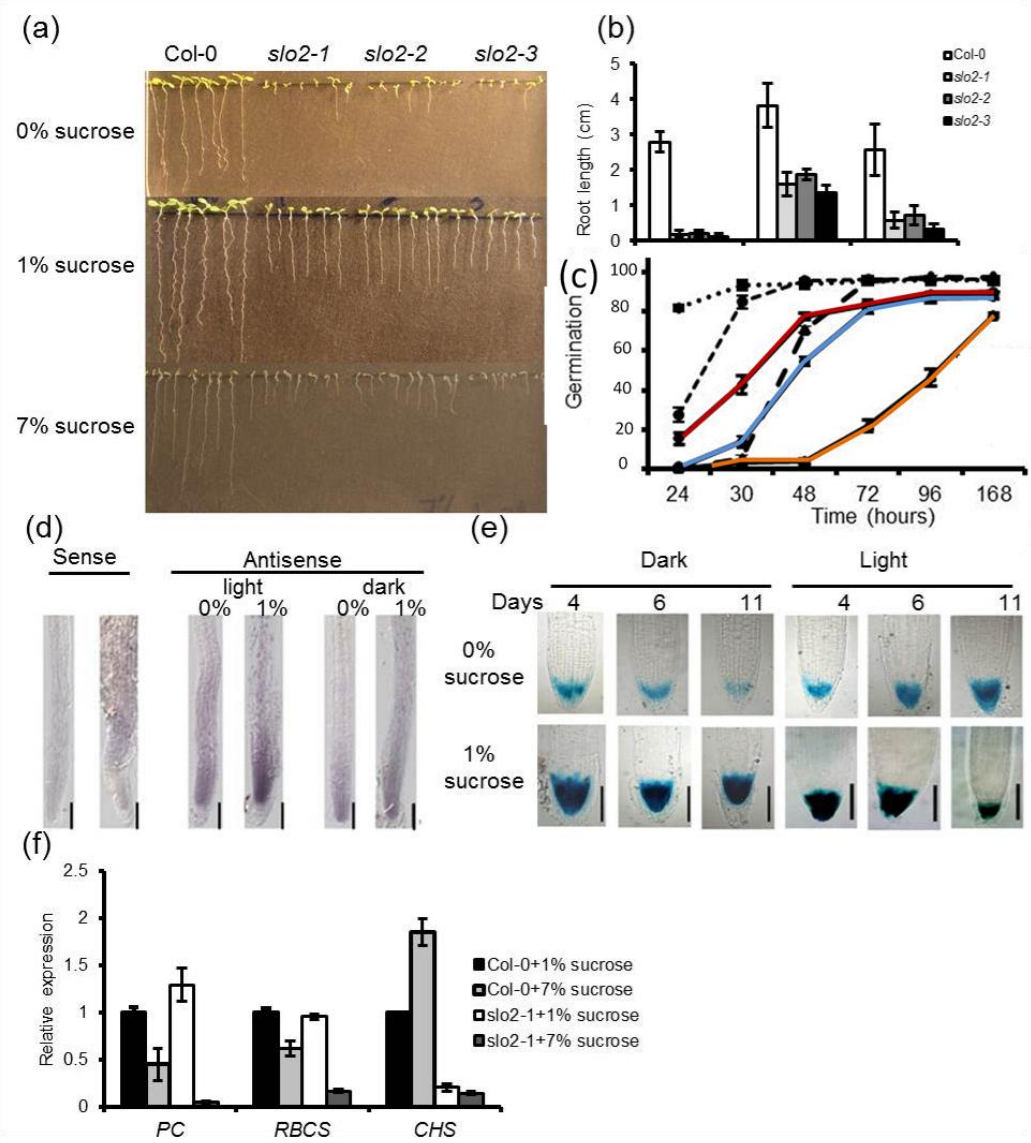

Figure 5. *slo2* mutants are hypersensitive to sucrose

(a) Effect of sucrose on *slo2* mutants. Seeds of wild-type and 3 *slo2* alleles were sown on ½ MS medium containing different concentrations of sucrose. Top: 0%; Middle: 1%; Lower: 7% sucrose. Pictures were taken after 7 days.

(b) Statistic analysis of root length from Figure (A)

(c) Delay in seed germination caused by sucrose is enhanced in *slo2-1* mutant. Data are averages ±SEM (n=3). Dotted line = WT 0% suc, short dashe line = WT .1% suc, long dashed line = WT 7% suc, red = *slo2*-1 0% suc, blue = *slo2-1* 1% suc, orange = *slo2-1* 7% suc

(d) Whole mount *In Situ Hybridization* for *SLO2* transcripts in roots. Six-day old individuals were hybridized with sense and antisense probes. Bar=100 µm.

(e) *SLO2* promoter driven expression of the GUS reporter gene in roots. Bar=100 µm.

(f) Effect of the *slo2* mutation on sugar-related gene expression. Expression of plastocyanin (*PC*), ribulose-1,5-bisphosphate carboxylase small subunit (*RBCS*) and chalcone synthase (*CHS*) in 6-day-old etiolated wild-type (Col-0) and *slo2-1* seedlings relative to the expression in the wild-type in the presence of 1% sucrose, obtained by quantitative PCR. Seedlings were grown on MS/2 medium containing 1 or 7% sucrose. To compensate for germination delay, *slo2-1* on 1% sucrose and wild-type on 7% sucrose were sown 1 day earlier; *slo2-1* on 7% sucrose was sown 2 days earlier than the wild-type on 1% sucrose.

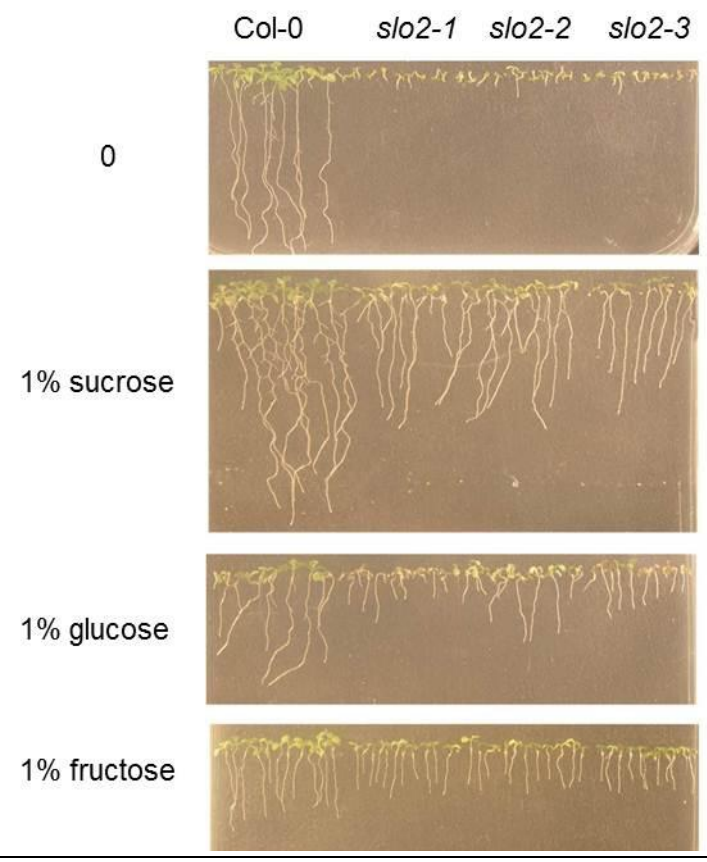

Figure S1 Effect of sucrose, glucose and fructose on *slo2* mutants. Seeds of wild-type and 3 *slo2* alleles were sown on ½ MS medium containing different concentrations of sucrose. Top: 0%; Middle: 1%; Lower: 7% sucrose. Pictures were taken after 7 days.

To obtain additional molecular evidence for the hypersensitivity of *slo2* to sucrose, we analyzed the expression levels of two sugar-repressed genes, ribulose-1,5-bisphosphate carboxylase small subunit (*RBCS*; (Cheng *et al.*, 1992; Sheen, 1994) and plastocyanin (*PC*) (Zhou *et al.*, 1998), and one sugar-induced gene, chalcone synthase (*CHS)* (Nemeth *et al.*, 1998) by quantitative RT-PCR. In the presence of 1% sucrose, the messenger levels of *RBCS* and *PC* in *slo2-1* were similar to that in Col-0; however, on medium containing 7% of sucrose, both genes were significantly stronger down-regulated in the mutant than in Col-0 (Figure 5f). In addition, induction of transcription of the hexokinase (HXK)-independent sugar-induced *CHS* gene (Sheen *et al.*, 1999) was completely abolished in *slo2* (Figure 5f). The results indicate that *SLO2* has strong effects on sugar signaling.

5.2.7. The *slo2* phenotype is dependent on the carbon status

Since the *slo2* mutant phenotype is affected by external sugars, we hypothesized that the slow growth of *slo2* may be directly linked to a disturbance of carbon/energy metabolism. We used three methods to test this point. First, we tested the effect of CO_2 fertilization on the growth of *slo2* mutants. Rubisco (RBC), which is a dual activity enzyme, is the main entrance for carbon into organic matter. The RBC carboxylation reaction occurs under high CO_2 and low O_2 conditions, while its oxygenation needs a high O_2 concentration (Holland, 2006). The *slo2* mutant phenotype was largely recovered under high CO_2 conditions (3000 ppm), reflected by the plant size (Figure 6a). Furthermore, CO_2 fertilization partially complemented the developmental delay in *slo2*. Under control conditions (450 ppm CO_2), *slo2-1* shows a developmental delay of 36 days compared to Col-0 (Figure 6b, Suppl. Table 1, DOI: 10.1111/j.1365-313X.2012.05036.x). Both genotypes take advantage of CO_2 fertilization

(1700 ppm), but the gap between them was reduced to 10 days only (Figure 6b). The same trend was noticed on the number of rosette leaves at initiation of flowering , proving that the restoration of flowering time is due to faster development (Figure 6c).

Secondly, we tested whether addition of an external carbon source could compensate the reduced carbon fixation or higher loss of CO_2 in *slo2* mutants. In the presence of sucrose, *slo2* alleles grew normally and could complete their life cycle (Figure 6d and 6e). In the absence of sucrose, at least 80% of the Col-0 plants and 2 complemented lines could complete their life cycle, while in the case of *slo2* mutants, this ratio is reduced to 0-12% depending on the allele (Figure 6d and 6e). These data support the fact that SLO2 is essential for completion of the life cycle in the absence of sucrose. A similar beneficial effect of sucrose (1%) addition was seen on leaf emergence and root growth of mutant plants (increase in root length 6-fold for *slo2*; 2,5-fold for Col-0).

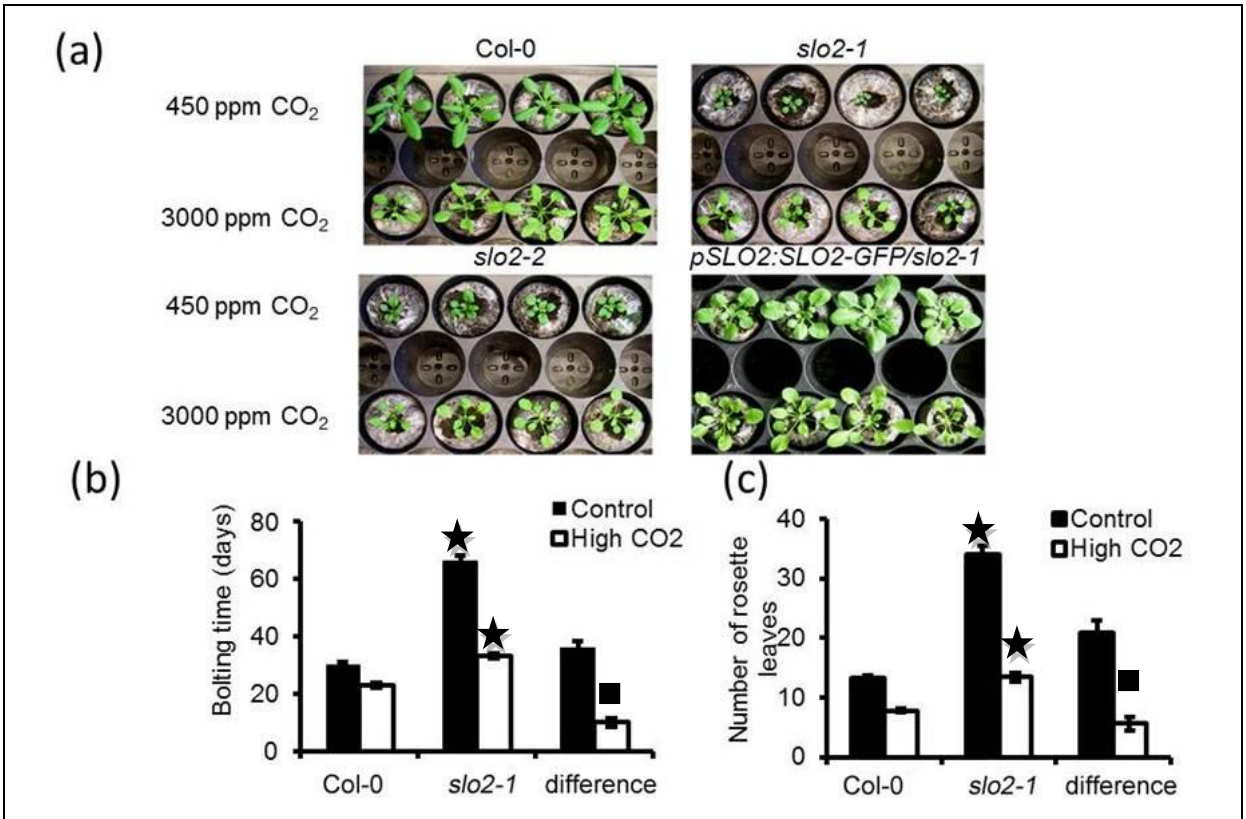

Figure 6. *SLO2* is involved in carbon and energy metabolism

(a) Effect of elevated CO_2 on *slo2* rosette. 7-day old seedlings were transferred into soil, and grew in atmosphere condition or with 3000 ppm CO_2. Pictures were taken 1 month later.

(b) Elevated CO_2 stimulates flowering in *slo2*. Bolting time of wild-type and *slo2-1* under normal and elevated CO_2 concentrations. Elevated CO_2 concentrations (white bars, 1700ppm CO_2) have a stronger stimulatory effect on the mean bolting time in *slo2-1* than in wild-type compared to control conditions (black bars, 450ppm CO_2). Stars reflect a significant difference (p<0,01) between Col-0 and *slo2-1* in the given condition. 'Difference' reflects the difference between the bolting time of wild-type and *slo2-1* in the respective conditions. Squares reflect a significant difference (p< 0,01) between the two conditions. Error bars represent standard error (n = 15).

(c) Elevated CO_2 stimulates flowering in *slo2*. Number of rosette leaves upon bolting in wild-type and *slo2-1*. Conditions and calculations are identical to (A).

A third way of investigating the effect of alteration of the carbon status on the mutant was by growing the plants under different light intensities and photoperiods. Plants were grown under five different light conditions and their bolting time was assessed (Supplemental Table S2, DOI: 10.1111/j.1365-313X.2012.05036.x). An increase in light dose (long days, high light) accelarates the growth and development of *slo2* mutants, the strongest positive effect being under continuous light. Overall, we conclude that in *slo2* mutants, addition of an external carbon source (CO_2 or sucrose) or an increased light dose largely compensates the growth retardation.

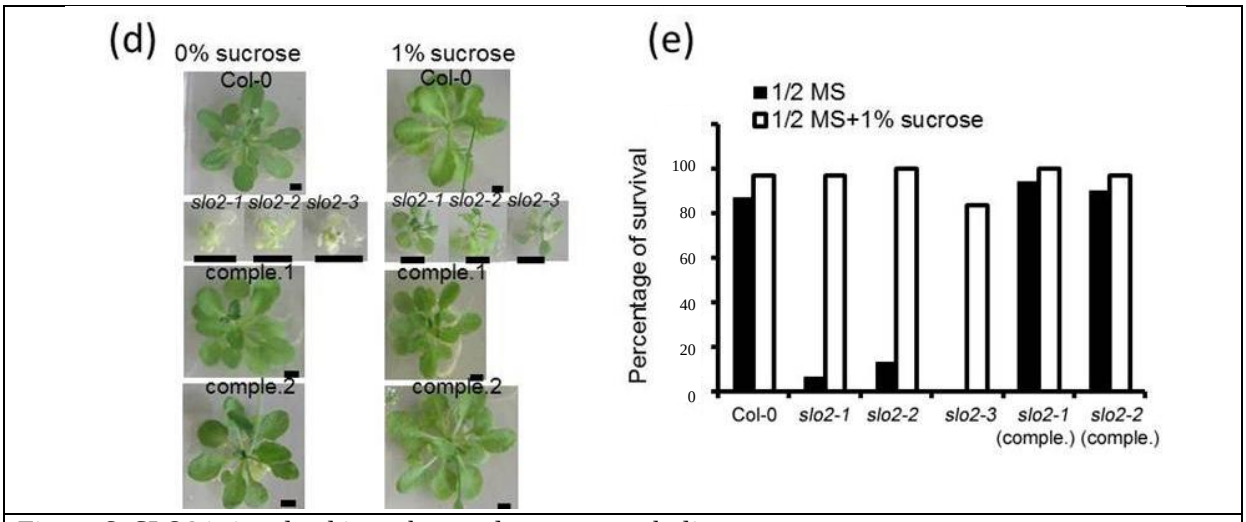

Figure 6. *SLO2* is involved in carbon and energy metabolism

(d) Phenotype of Col-0, 3 *slo2* mutants and 2 complemented lines on 0% and 1% sucrose. Scale bar corresponds to 1 cm.

(e) *slo2* mutants can not complete their lifecycle in the absence of an external carbon source. Plants were grown on MS/2 + 1% sucrose (white bars) or in the absence of sucrose (black bars) until flowering. The percentage of plants that could complete their life cycle is shown.

5.2.8. RNA editing changes in the *slo2* mutants

Since *SLO2* encodes an E type PPR protein, we tested the possibility that SLO2 might be involved in RNA editing. A multiplexed SNaPshot approach, monitoring 315 mitochondrial editing sites was used (Takenaka and Brennicke, 2009). The screen identified several editing sites affected in mitochondria of *slo2* mutants. One of these sites is *nad4L*-110, where the C to U editing was not detectable in mutant alleles (Figure 7). This editing event leads to a serine to leucine amino acid change (S37L) in the predicted NAD4L subunit protein of the NADH dehydrogenase (complex I). The editing deficiency of the mutant allele is restored in complemented *slo2-2* (containing *P35S:SLO2-GFP*; Figure 7).

All other editing defects were likewise observed similarly in both examined *slo2* alleles, such as the two adjacent C's in *mttB* (membrane targeting and translocation or *orfx*) at positions *mttB*-144 and *mttB*-145. The editing event at nucleotide 144 does not change the predicted amino acid (phenylalanine). As often observed for silent editing events, this site is edited to only about 50% in the steady state mRNA population in Col-0 plants. In both mutant alleles, editing at this site is not detectable. At nucleotide 145, editing changes the predicted amino acid residue from proline to serine (P49S) in the entire population of steady state transcripts. Like the preceding nucleotide, editing at this nucleotide is also lost in plants

homozygous for either of the two mutant *slo2* alleles (Figure 7). A third editing change occurs in *nad7* at site 739, the editing at this site leading to an amino acid change from leucine to phenylalanine. NAD7 is essential for complex I accumulation (Pineau *et al.*, 2005). The restoration at these sites in the complemented line confirms that SLO2 plays an essential role in these editing events.

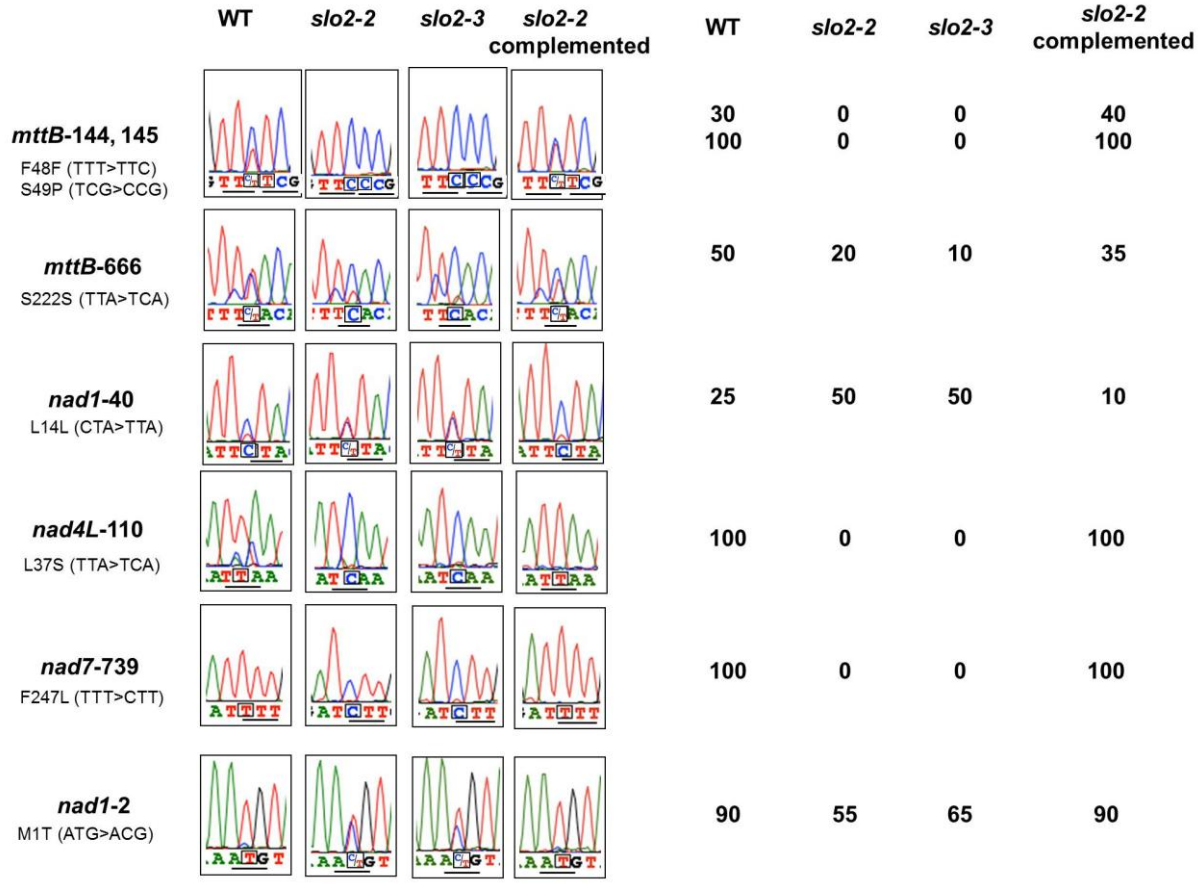

Figure 7. SLO2 is involved in multiple mitochondrial RNA editing events
RNA editing changes were detected using a multiplexed SNaPshot approach, the editing sites of the respective genes and the amino acid changes are listed. Sequencing chromatograms are displayed. The codons containing the editing sites are underlined, and the editing sites were marked with a square. The RNA editing efficiencies are shown on the right.

We also noticed some editing efficiency changes in several mRNAs, of which most lower the level of steady state editing in *slo2* mutants such as in the *mttB* RNA at site 666 (50% and 35% in wild-type and complemented line versus 20% and 10% in the *slo2-2* and *slo2-3* mutants, respectively) and *nad1* at site 2 (90% in the wild-type and complemented line versus about 60% in the mutants) (Figure 7). In one instance, an increase in the level of editing was observed, namely in *nad1* at site 40 (25% and 15% in wild-type and complemented line versus more than 50% in the mutants) (Figure 7). Among those sites with altered editing efficiency, some are silent, such as *mttB666* and *nad1-40*, while *nad1-2* is non-silent, with an amino acid substitution from threonine to methionine. Our results clearly show that the SLO2 protein is a mitochondrial RNA editing factor involved in several editing events.

5.2.9. Complex I, complex III and complex IV are reduced in *slo2* mutants

The NAD1, NAD7 and NAD4L proteins are components of the mitochondrial complex I, which may play a role in its assembly. A previous report showed that the absence of NAD7 directly leads to the lack of complex I in the CMSII mutant (Pineau *et al.*, 2005). As we find RNA editing changes in these genes, which lead to amino acid changes, we speculate that RNA editing defects in those sites may lead to mitochondrial dysfunction in *slo2* mutants. To test this hypothesis, we isolated mitochondrial membrane complexes from rosette leaves of Col-0, 3 *slo2* alleles, and the *slo2-1* complemented line and subjected these to blue native PAGE analysis. The result demonstrates that complex I abundance is highly reduced in all 3 *slo2* mutants compared with Col-0, and that this reduction is restored in the complemented line (Figure 8a). NADH dehydrogenase in-gel activity staining further indicated a strong reduction in complex I activity (Figure 8b). To investigate whether other complexes of the electron transport chain are affected in the mutants, we performed western blot using antibodies against subunits of complex III (cytochrome *c1*), IV (cytochrome oxidase II (COX II)) and V (α-ATPase). The results clearly showed that the level of complex III and complex IV was much reduced in the mutants, while being partially restored in the complemented line (Figure 8c). Moreover, western blot results from SDS-PAGE gel (Figure 8d) were consistent with the immunodetection results from blue native-gel electrophoresis (Figure 8e). Overall, our data indicate that SLO2 is necessary to sustain a proper level of complex I, complex III and complex IV in mitochondria.

5.2.10. *slo2* mutants contain less ATP/ADP, NAD^+ and sugars

In plants, complex I and complex III deficiency leads to a diminished proton translocation and a lower phosporylation efficiency. ATP is the major energy source in living cells, lack of ATP obviously having negative effects on plant growth and development. Based on the stunted growth of the mutants and the observed deficiency in mitochondrial electron transfer, we speculated that *slo2* mutants contain reduced energy pools. To test this hypothesis, we measured the ATP and ADP levels in Col-0, *slo2* mutants and in the complemented *slo2-2* line. A significant reduction in ATP, ADP and ATP/ADP ratio is seen in *slo2* mutants, while in the complemented line the levels were reverted to that in Col-0 (Figure 9). This suggests that *SLO2* is necessary to maintain the normal energy pool.

The pyridine nucleotides NAD^+ and NADH act as primary redox carriers in metabolism, and the balance of NADH/NAD^+ is critical for the central redox control and to prevent ROS generation (Shen *et al.,* 2006). In *slo2* mutants, a significantly higher NADH content and concomitantly decreased NAD^+ content were observed compared with that in Col-0 and the complemented *slo2-2* line (Figure 9). Consequently, the NADH/NAD^+ ratios of the mutants are much higher than that in Col-0. Thus, *SLO2* is necessary for maintaining a steady state cellular NADH/NAD^+ ratios.

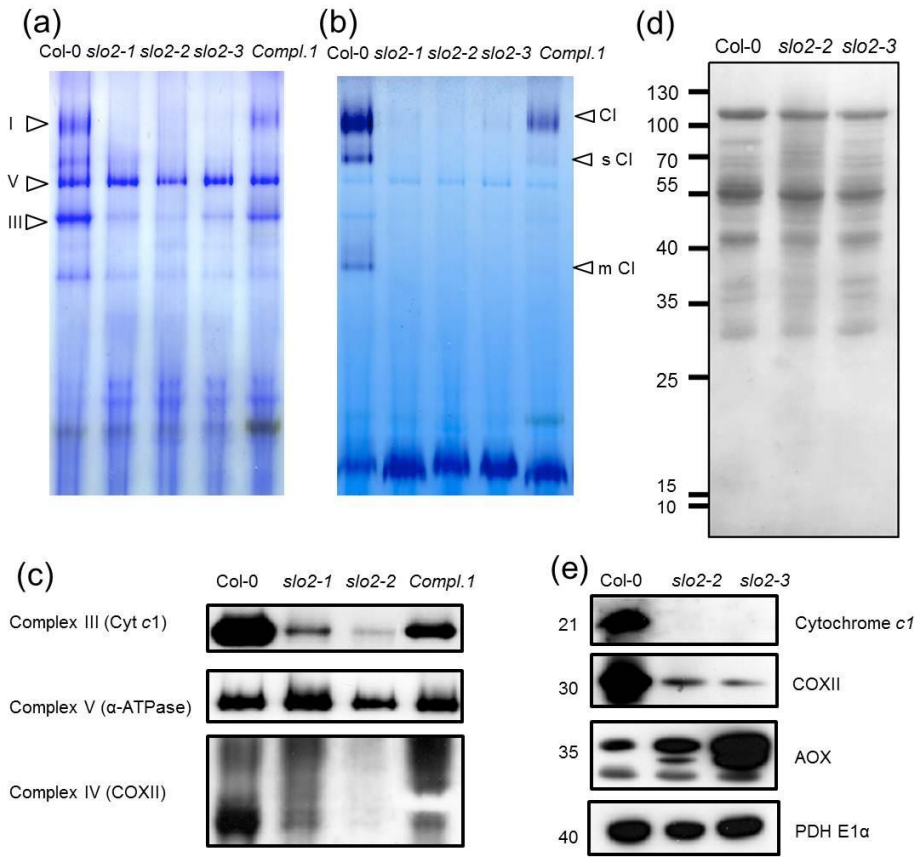

Figure 8. Complex I, complex III and complex IV are affected in the *slo2* mutants

(a) Coomassie-stained blue-native gel of mitochondrial membrane complexes from Col-0, 3 *slo2* mutants and the *slo2-1* complemented line. I, complex I; V, complex V; III, complex III.

(b) NADH dehydrogenase activity staining of a gel prepared as in (A). CI, complex I; s CI, smaller version of complex I; m CI, matrix arm of complex I.

(c) Western blot analysis of complex III, complex IV and complex V. Western blot was performed based on SDS-PAGE gel (figure 8d)

(d) Coomassie-stained gel of SDS-PAGE analysis of mitochondrial fractions from Col-0, *slo2-2* and *slo2-3*

(e) Western blot analysis of cytochrome *c1*, COXII and AOX. PDH E1α is used as a loading control. The molecular weight is indicated on the left. Western blot was performed based on SDS-PAGE gel (figure 8d)

During seed germination and seedling establishment, the storage reserves will be utilized. For example, triacylglycerides (TAG) will breakdown into sugars (such as sucrose, glucose and fructose), which act as the main carbon and energy resources. We noticed that in 3-day-old *slo2* mutant seedlings, the levels of sucrose, glucose and fructose are much reduced as compared to Col-0, while these changes were reversed in the complemented line (Figure 9). Our data suggest that *slo2* mutants contain less fuel sources for germination and seedling establishment.

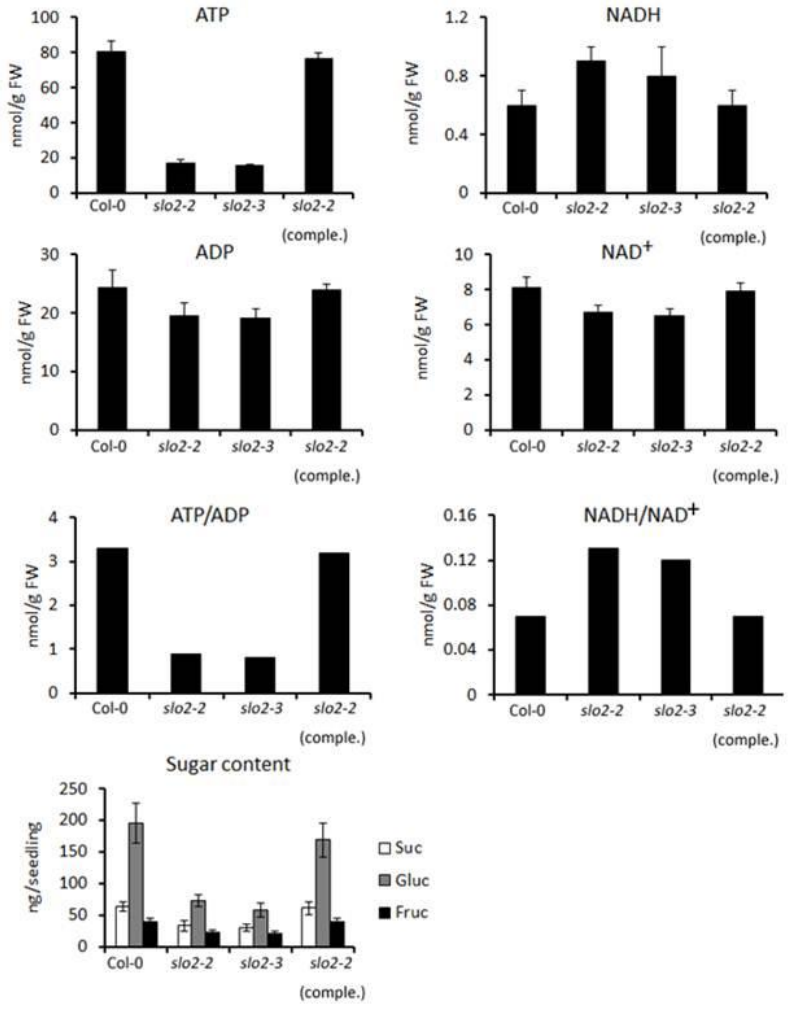

Figure 9. Metabolic changes in *slo2* mutants

5-day old seedlings grown on half strength MS medium containing 1% sucrose were used for ATP, ADP, NAD^+ and NADH analysis. 3-day old seedlings grown on half strength MS medium were used for sugar content analysis. Values are the mean ± SE of measurements on four repeats of 100 seedlings each.

5.3 Discussion

In this work we characterized the PPR protein SLO2 which plays a role in several mitochondrial RNA editing events, affecting complex I, complex III and complex IV, and as a result, the cellular energy and carbon status. Physiological and molecular analysis of three *slo2* alleles indicates that *SLO2* is important for normal plant growth and sugar response. Given that the *slo2-1* allele carries a deletion of 7AA in the first P domain, the latter may play an important role through stabilizing the SLO2 protein or to specifically interact with part of the nucleotide recognition sequence in either target RNAs or an essential protein interactor.

5.3.1. SLO2 is involved in multiple RNA editing events in mitochondria

Despite the lack of a recognizable mitochondrial targeting domain, the mature SLO2 protein is located in mitochondria and plays a specific role in mitochondrial RNA editing. To date, 14 RNA editing factors were identified. Seven factors are involved in RNA editing of single sites (Takenaka, 2010; Takenaka *et al.*, 2010; Verbitskiy *et al.*, 2011; Yuan and Liu,

2012), 5 have 2-4 changes in editing in *Arabidopsis* (Zehrmann *et al.*, 2009; Bentolila *et al.*, 2010; Sung *et al.*, 2010; Verbitskiy *et al.*, 2010; Hammani *et al.*, 2011a), while in rice, OGR1 controls 7 specific editing sites on 5 distinct mitochondrial transcripts (Kim *et al.*, 2009). Our analysis demonstrates that SLO2 is involved in 7 specific editing events on 4 distinct mitochondrial transcripts. As far as we know, this is the highest number of RNA editing changes ever identified in a single mutant in *Arabidopsis*. Interestingly, most of the identified mitochondrial editing factor (MEF) mutants do not exhibit obvious phenotypes under normal conditions (Zehrmann *et al.*, 2009; Takenaka *et al.*, 2010). In *Arabidopsis*, until now only three MEF mutants were reported to show growth retardation: *slo1* , *organelle transcript processing 87* (*otp 87)*, and *slow growth 1* (*slg 1)* (Sung *et al.*, 2010; Hammani *et al.*, 2011a; Yuan and Liu, 2012). *slo2* mutants shows growth retardation comparable to these three mutants, but leads to more editing defects. The editing changes in *slo2* result in amino acid changes in NAD4L (S37L), NAD1 (T1M), NAD7(L247F) and MTTB (P49S), which all are, with the exception of MTTB, important components of the NADH dehydrogenase complex I in the mitochondrial membrane. The amino acid changes resulting from RNA editing defects have strong impact on the abundance and function of complex I (Figure 8). Furthermore, our results clearly support a reduction of complex III and complex IV in *slo2* mutants (Figure 8), concomitant with a higher accumulation of AOX (Figure 8e). Hence, we propose that these RNA editing defects result in dysfunction of mitochondrial electron transfer chain complexes, contributing to the observed *slo2* phenotype.

5.3.2. SLO2 plays a unique role in the mitochondrial electron transfer chain

As stated above, defects in *slo2* probably result in the dysfunction of mitochondrial complex I, complex III and complex IV, affecting the mitochondrial electron transport. The question remains how a mutation in the *SLO2* gene can affect the other complexes of the mitochondrial ETR. We propose several possibilities. First, the possiblity that more RNA editing defects may exist in *slo2* mutants cannot be completely ruled out. In mitochondria, the number of RNA editing sites exceeds 500 (Giegé and Brennicke, 1999), while our multiplexed SNaPshot analysis monitored 315 mitochondrial editing sites. It is therefore reasonable to assume that a mutation in *slo2* may affect RNA editing of complex III and complex IV components, thereby leading to the dysfunction of complex III and complex IV. However, combining the results from SNaPshot analysis and cDNA sequencing (for sites that are not covered by SNaPshot analysis), we find no differences in editing of transcripts of mitochondrial genome encoded subunits of complex III and IV between wildtype and *slo2* mutants. This indicates that the observed decreases in complex III and IV are not related to altered RNA editing (Supplemental Table S3, DOI: 10.1111/j.1365-313X.2012.05036.x). Second, impaired metabolism caused by a defect in one mETC complex may alter the levels of other complexes. Several reports in non-plant species support this possibility. For instance, in mammalian species, the majority of complex I is associated with complex III and complex IV (Schägger and Pfeiffer, 2000). In *Caenorhabditis elegans*, mitochondrial complex I mutations lead to cytochrome c oxidase (complex IV) deficiency (Grad and Lemire, 2004), while in yeast, the levels of COX subunits COXI, II and III were also reduced in mutants that affect the assembly of complex IV (Glerum *et al.*, 1995; Shoubridge, 2001). However, this scenario is not supported by reports on *Arabidopsis* mutants with reduced or absent complex I

(i.e. *ndufs4, bir6, rug3*) in which other respiratory chain complexes were unaffected (Meyer *et al.*, 2009; Koprivova *et al.*, 2010; Kühn *et al.*, 2011). Similarly, *rpoTmp* mutants with primary defects in complex I and complex IV showed no reduction in complex III (Kühn *et al.*, 2009), and *wtf9* mutants with primary defects in complexes III and IV were unaffected in complex I (des Francs-Small *et al.*, 2012). Although complex III and complex IV are much reduced in *slo2*, the remaining proteins may be sufficient to sustain plant life under normal conditions. Meanwhile, it also opens the question of how these major changes in the mETC complexes are compensated in order to allow normal growth. Third, we identified 3 RNA editing changes in *mttB* (membrane targeting and translocation, a mitochondrial analog of the plastidial tatC protein), one of which results in an amino acid change in the MTTB protein (van der Merwe and Dubery, 2007). In bacteria, MTTB is an essential integral membrane protein which functions in membrane targeting and secretion of cofactor-containing proteins, such as iron-sulphur clusters (Weiner *et al.,* 1998). In higher plants, MTTB exhibits high similarity to its orthologue in bacteria, and may have a similar function in the mitochondrial membrane of plants (Sünkel *et al.*, 1994; Giegé and Brennicke, 2001). So it is plausible that the mutations in the MTTB protein which result from RNA editing defects in *slo2* mutants may inhibit the mitochondrial import of functional proteins for the formation of mETR complexes, such as nuclear encoded Fe-S containing proteins or cofactor containing redox proteins, possibly resulting in the reduced function of those complexes. Although the functional mechanism still needs to be investigated, our current study shows that *slo2* is an example in plants of a single gene mutation leading to comprehensive changes in three mETC complexes, yet remaining viable. To the best of our knowledge, no other PPR proteins have been characterized with such multiple effects on the mETC.

5.3.3. Loss of the SLO2 protein function leads to defects in plant growth and development

The status of mitochondrial electron transport and carbon dioxide fixation rate are two vital factors determining plant growth (Stitt, 1986; Griffin *et al.*, 2001). In plant mitochondria, the alternative respiration pathway is activated when the classical mETC is impaired (Juszczuk and Rychter, 2003), but this process produces significantly less ATP. Our results showed that the levels of ATP and ADP are much reduced, while the ATP/ADP ratio is lower in *slo2* mutants compared with that in Col-0 (Figure 9), suggesting that the plants have an impaired energy status. Clearly, energy shortage is probably the major cause of the growth defects in *slo2*. Carbon supply is another vital factor for plant growth. Our results showed that SLO2 is essential for survival of *Arabidopsis* in the absence of an external carbon source (Figure 6d and 6e). External carbon sources, such as CO_2, sugars or high light partially alleviate *slo2* defects, indicating disruption of the carbon balance in mutants. A lower endogenous energy level and net carbon fixation obviously negatively affect growth and development.

The $NADH/NAD^+$ ratio is vital for the central redox control and to prevent ROS accumulation (Shen *et al.*, 2006) Thus, the imbalance of $NADH/NAD^+$ in *slo2* mutants may lead to higher ROS accumulation. ROS play important roles in normal plant growth and development (Foreman *et al.*, 2003), and were proposed to have a pivotal role in environmental sensing and hormonal signaling (Swanson and Gilroy, 2010). Therefore, the observed severe growth defects of *slo2*, may also result from an enhanced level of H_2O_2.

In conclusion, we propose that SLO2 (directly or indirectly) modulates carbon and energy balance, thereby playing an essential role in a plant's life cycle under certain growth conditions. Future research will reveal the precise nature of the genes influenced by SLO2 as well as its interaction partners. This will help to further elucidate the roles of SLO2 in plant development. The observed defects probably resulting from an impaired mitochondrial ETC could be instrumental in unravelling the interorganellar network of mitochondria, plastids and peroxisomes.

5.4 Experimental procedures

5.4.1. Plant materials and growth conditions

Plants were grown as described previously (Smalle and Van Der Straeten, 1997). Unless stated otherwise, seeds were stratified for two days at 4°C. Growth conditions were 16h light/8h dark photoperiod, white fluorescence light (75 μM m^{-2} sec^{-1}), and 21°C. Plants on soil were grown in a growth chamber at 22°C. High light conditions were at a light intensity of 650 μmoles/m^2s, low light conditions were at a light intensity of 60 μmoles/m^2s. Long day conditions were 16h light/ 8h darkness; short day conditions were 8h light/ 16h darkness. In order to avoid seed batch effects, seeds harvested from plants grown simultaneously were used for analysis.

A collection of 5000 T-DNA insertion lines (Feldmann, 1991) and the Wassilewskija (Ws) wild-type of *Arabidopsis thaliana* (L.) Heynh. were obtained from the Nottingham *Arabidopsis* Stock Center and used for screening. SALK_021900 (*slo2-2*) was verified by PCR amplification using primers: forward 5'-TCTTTGATTGCGAAATCGCCT-3' and reverse: 5'-CGCATGCAGAAAGAACACCAA-3'. The primer specific to left border region of the construct which was used to generate the T-DNA insertion lines, is 5'-GCGTGGACCGCTTGCTGCAACT-3'. The Tilling line T94087 (*slo2-3*) which contains a T to C mutation leading to a stop codon at position 247, was verified by sequencing.

5.4.2. Map-based cloning of *SLO2*

The mapping population was generated by crossing *slo2* (Ws) to Col-0 or Ler. The position of *slo2* on the *Arabidopsis* genetic map was established by determining linkage between the *slo2* phenotype and simple sequence length polymorphism (SSLP) markers in F2 (Bell and Ecker, 1994). The segregation of polymorphic markers and mutant phenotype was analyzed using MAPMAKER 2.0 for Macintosh (Lander *et al.*, 1987) using the Kosambi mapping function. Fine-mapping was performed with InDel (Insertion/Deletion) markers released by TAIR-Cereon. The InDel primer sequences and the PCR fragment sizes (Col/Ler/Ws, in bp) are listed in the supplemental table S4 (DOI: 10.1111/j.1365-313X.2012.05036.x).

The forward primers were labeled with γ-^{33}p-ATP and the PCR products were separated and visualized on 6% acrylamide sequencing gel after exposure to phosphorimager cassettes on a Storm 820 phosphorimager (GE Healthcare).

5.4.3. Constructs and transgenic *Arabidopsis thaliana* plants

All constructs used for generating transgenic plants were made using the Gateway system (Invitrogen). PCR amplified fragments were first cloned into pDONR221 via BP reactions and then confirmed by sequencing, followed by LR reactions to obtain plant expression vectors. For the complementation experiment in *slo2-1* background, a 3.92 kb genomic fragment, including 1.67 kb of 5' upstream and 0.17 kb of 3' downstream regions, was amplified by PCR using primers 5'-TACGCTTCCAACACAACACG-3' and 5'-TCGTACAGCAACCGAAGATG-3', then cloned into destination vector *p*HGW (Karimi *et al.*, 2002). For the *PSLO2:GUS* transgenic line, the 1.67 kb promoter region was transcriptionally fused to *eGFP-GUS* using the pKGWFS7 vector using primers: 5'-

TACGCTTCCAACACAACACG-3' and 5'-CCATTATCGTCGTTTGCAGA-3' (Karimi *et al.*, 2005). For the *p35S:SLO2-GFP* construct, full-length *SLO2* cDNA was amplified using the primers 5'-ACCATGGCAACAAAATCATTTC-3' and 5'-ACATGGCGTTGTCCCAAAG-3', the verified fragment was used for C-terminal fusion with GFP under control of 35S promoter in vector pK7WGF2. All the binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90) or GV3101 by electroporation. The *slo2-1* and *slo2-2* alleles as well as wild-type plants were transformed using the floral-dip method (Clough and Bent, 1998).

The full-length cDNA was isolated using 5' and 3' rapid amplification of cDNA ends according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit; Clontech, Palo Alto, CA). The primers were located at 62891 to 62910 and 63689 to 63708 in the BAC clone T10F5, with a 778-bp overlap.

5.4.4. Quantitative RT-PCR analysis

Total RNA was extracted using Trizol® reagent (GIBCO/BRL, Gaithersburg, MD) and 5 µg RNA was further purified and concentrated using a DNA-free RNA kit (Zymo Research, Orange, CA). cDNA was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Quantitative RT-PCR was performed using a Cybergreen fluorescence-based assay kit (Platinum SBYR Green qPCR kit; Cat. No. 11733-046, Invitrogen). The PCR reactions were performed on a Rotor Gene 6, Corbett or Bio-rad MyiQ™2 Two-Color Real-Time PCR Detection System. At least two runs (technical repeats) were done for each data set and two to three biological repeats were performed. Data represent mean values and standard errors (SE). The primers are listed in supplemental Table S4 (DOI: 10.1111/j.1365-313X.2012.05036.x).

5.4.5. Subcellular localization

For the analysis of the subcellular localization of the SLO2 protein, transgenic *Arabidopsis* containing *P35S:SLO2-GFP* was stained with 250 nM Mitotracker orange for 1h. The root was visualized by confocal microscopy. To confirm the mitochondrial localization, transgenic plants containing *P35S:SLO2-GFP* construct were crossed with a mitochondrial marker line expressing ATPase-mCherry (kindly provided by D. Logan, University of Saskatchewan, Saskatoon). F_1 progeny was observed under a confocal microscope. GFP fluorescence was detected with excitation at 488 nm and emission at 525 nm; for the mito-tracker stain, fluorescence was detected with excitation at 543 nm and emission at 615 nm; for mCherry, fluorescence was detected with excitation at 568 nm and emission at 580 to 700 nm.

5.4.6. RNA editing analysis

Total RNA was extracted from 4-week-old *Arabidopsis* rosette leaves using RNeasy Mini Kit (QIAGEN company, Belgium), and was further purified and concentrated using a DNA-free RNA kit (Zymo Research, Orange, CA) according to manufacturers' protocol. Specific cDNAs were generated as described previously (Takenaka and Brennicke, 2007). The SNaPshot assay and RNA editing sites analysis were performed according to the established protocol (Takenaka and Brennicke, 2009). The editing defects were confirmed by sequencing specific RT-PCR products.

5.4.7. Blue native-PAGE and complex I activity assay

Eight-week-old plants grown in greenhouse conditions were used for isolation of mitochondria; Blue native PAGE of solubilised mitochondrial membranes, complex I activity assay and western blots were performed as described previously (Meyer *et al.*, 2009).

5.4.8. NAD^+, NADH, ATP, ADP and sugar measurements

ATP and ADP were extracted from 5-day-old seedlings using the TCA-ether extraction method (Jelitto *et al.*, 1992) and measured as described previously (Stitt *et al.*, 1989). 3-day old seedlings grown on half strength MS medium were used for sugar measurement. Sugar measurements were as described previously (Pritchard *et al.*, 2002). 5-day-old seedlings grown on half MS salts plus 1% sucrose were used for Pyridine nucleotides extraction and the concentration of NAD^+ and NADH determined as described by Shen and coworkers (Shen *et al.*, 2006).

5.4.9. Accession Number

Sequence data from this article can be found in the GenBank/EMBL data libraries or the *Arabidopsis* Genome Initiative database under accession number: *SLO2* (At2g13600).

5.5 Acknowledgements

The authors thank the Research Foundation Flanders (project G.0313.05) and Ghent University for financial support. J.D. is indebted to the IWT for a predoctoral fellowship. KK and EHM are supported by a Marie Curie International Reintegration Grant (PIRG256398)
Dr. David Logan (University of Saskatchewan) is kindly acknowledged for providing us the ATPase-mCherry line. QZ and DVDS thank Dr. Claire Lurin (URGV, Université UEVE, Evry) for helpful discussions and suggestions.

5.6 References

Andrés, C., Lurin, C. and Small, I.D. (2007) The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiol. Plant. **129**, 14-22.

Bell, C.J. and Ecker, J.R. (1994) Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics, **19**, 137-144.

Bentolila, S., Knight, W. and Hanson, M. (2010) Natural variation in *Arabidopsis* leads to the identification of REME1, a pentatricopeptide repeat-DYW protein controlling the editing of mitochondrial transcripts. Plant Physiol. **154**, 1966-1982.

Brangeon, J., Sabar, M., Gutierres, S., Combettes, B., Bove, J., Gendy, C., Chetrit, P., Des Francs-Small, C.C., Pla, M., Vedel, F. and De Paepe, R. (2000) Defective splicing of the first nad4 intron is associated with lack of several complex I subunits in the Nicotiana sylvestris NMS1 nuclear mutant. Plant J. **21**, 269-280.

Cheng, C.L., Acedo, G.N., Cristinsin, M. and Conkling, M.A. (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc. Natl Acad. Sci. USA, **89**, 1861-1864.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**, 735-743.

de Longevialle, A.F., Meyer, E.H., Andres, C., Taylor, N.L., Lurin, C., Millar, A.H. and Small, I.D. (2007) The pentatricopeptide repeat gene OTP43 is required for trans-splicing of the mitochondrial nad1 Intron 1 in *Arabidopsis thaliana*. Plant Cell, **19**, 3256-3265.

des Francs-Small, C.C., Kroeger, T., Zmudjak, M., Ostersetzer-Biran, O., Rahimi, N., Small, I. and Barkan, A. (2012) A PORR domain protein required for rpl2 and ccmF(C) intron splicing and for the biogenesis of c-type cytochromes in *Arabidopsis* mitochondria. The Plant journal : for cell and molecular biology, **69**, 996-1005.

Ding, Y.H., Liu, N.Y., Tang, Z.S., Liu, J. and Yang, W.C. (2006) *Arabidopsis* GLUTAMINE-RICH PROTEIN23 is essential for early embryogenesis and encodes a novel nuclear PPR motif protein that interacts with RNA polymerase II subunit III. Plant Cell, **18**, 815-830.

Doniwa, Y., Ueda, M., Ueta, M., Wada, A., Kadowaki, K. and Tsutsumi, N. (2010) The involvement of a PPR protein of the P subfamily in partial RNA editing of an *Arabidopsis* mitochondrial transcript. Gene, **454**, 39-46.

Dudkina, N.V., Heinemeyer, J., Sunderhaus, S., Boekema, E.J. and Braun, H.P. (2006) Respiratory chain supercomplexes in the plant mitochondrial membrane. Trends Plant Sci. **11**, 232-240.

Dutilleul, C., Driscoll, S., Cornic, G., De Paepe, R., Foyer, C.H. and Noctor, G. (2003) Functional mitochondrial complex I is required by tobacco leaves for optimal photosynthetic performance in photorespiratory conditions and during transients. Plant Physiol. **131**, 264-275.

Feldmann, K. (1991) T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. Plant J. **1**, 71-82.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., Davies, J.M. and Dolan, L. (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature, **422**, 442-446.

Garmier, M., Carroll, A.J., Delannoy, E., Vallet, C., Day, D.A., Small, I.D. and Millar, A.H. (2008) Complex I dysfunction redirects cellular and mitochondrial metabolism in *Arabidopsis*. Plant Physiol. **148**, 1324-1341.

Giegé, P. and Brennicke, A. (1999) RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. Proc. Natl Acad. Sci. USA, **96**, 15324-15329.

Giegé, P. and Brennicke, A. (2001) From gene to protein in higher plant mitochondria. C. R. Acad. Sci. III, **324**, 209-217.

Glerum, D.M., Koerner, T.J. and Tzagoloff, A. (1995) Cloning and characterization of COX14, whose product is required for assembly of yeast cytochrome oxidase. J. Biol. Chem. **270**, 15585-15590.

Grad, L.I. and Lemire, B.D. (2004) Mitochondrial complex I mutations in Caenorhabditis elegans produce cytochrome c oxidase deficiency, oxidative stress and vitamin-responsive lactic acidosis. Hum. Mol. Genet. **13**, 303-314.

Griffin, K.L., Anderson, O.R., Gastrich, M.D., Lewis, J.D., Lin, G., Schuster, W., Seemann, J.R., Tissue, D.T., Turnbull, M.H. and Whitehead, D. (2001) Plant growth in elevated CO2 alters mitochondrial number and chloroplast fine structure. Proc. Natl Acad. Sci. USA, **98**, 2473-2478.

Gutierres, S., Sabar, M., Lelandais, C., Chetrit, P., Diolez, P., Degand, H., Boutry, M., Vedel, F., de Kouchkovsky, Y. and De Paepe, R. (1997) Lack of mitochondrial and nuclear-encoded subunits of complex I and alteration of the respiratory chain in Nicotiana sylvestris mitochondrial deletion mutants. Proc. Natl Acad. Sci. USA, **94**, 3436-3441.

Hammani, K., Colas des Francs-Small, C., Takenaka, M., Tanz, S.K., Okuda, K., Shikanai, T., Brennicke, A. and Small, I. (2011a) The pentatricopeptide repeat protein OTP87 is essential for RNA editing of nad7 and atp1 transcripts in *Arabidopsis* mitochondria. J. Biol. Chem. **286**, 21361-21371.

Hammani, K., Gobert, A., Hleibieh, K., Choulier, L., Small, I. and Giegé, P. (2011b) An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation. Plant Cell, **23**, 730-740.

Holland, H.D. (2006) The oxygenation of the atmosphere and oceans. Philos. Trans. R. Soc. Lond., B, Biol. Sci. **361**, 903-915.

Jelitto, T., Sonnewald, U., Willmitzer, L., Hajirezeai, M. and Stitt, M. (1992) Inorganic pyrophosphate content and metabolites in potato and tobacco plants expressing E. coli pyrophosphatase in their cytosol. Planta, **188**, 238-244.

Juszczuk, I.M. and Rychter, A.M. (2003) Alternative oxidase in higher plants. Acta. Biochim. Pol. **50**, 1257-1271.

Kühn, K., Carrie, C., Giraud, E., Wang, Y., Meyer, E.H., Narsai, R., des Francs-Small, C.C., Zhang, B., Murcha, M.W. and Whelan, J. (2011) The RCC1 family protein RUG3 is required for splicing of nad2 and complex I biogenesis in mitochondria of *Arabidopsis thaliana*. Plant J. **67**, 1067-1080.

Kühn, K., Richter, U., Meyer, E.H., Delannoy, E., de Longevialle, A.F., O'Toole, N., Borner, T., Millar, A.H., Small, I.D. and Whelan, J. (2009) Phage-type RNA polymerase RPOTmp performs gene-specific transcription in mitochondria of *Arabidopsis thaliana*. Plant Cell, **21**, 2762-2779.

Karimi, M., De Meyer, B. and Hilson, P. (2005) Modular cloning in plant cells. Trends Plant Sci. **10**, 103-105.

Karimi, M., Inze, D. and Depicker, A. (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. **7**, 193-195.

Keren, I., Bezawork-Geleta, A., Kolton, M., Maayan, I., Belausov, E., Levy, M., Mett, A., Gidoni, D., Shaya, F. and Ostersetzer-Biran, O. (2009) AtnMat2, a nuclear-encoded maturase required for splicing of group-II introns in *Arabidopsis* mitochondria. RNA, **15**, 2299-2311.

Kim, S.R., Yang, J.I., Moon, S., Ryu, C.H., An, K., Kim, K.M., Yim, J. and An, G. (2009) Rice OGR1 encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria. Plant J. **59**, 738-749.

Klodmann, J., Sunderhaus, S., Nimtz, M., Jansch, L. and Braun, H.P. (2010) Internal architecture of mitochondrial complex I from *Arabidopsis thaliana*. Plant Cell, **22**, 797-810.

Koprivova, A., des Francs-Small, C.C., Calder, G., Mugford, S.T., Tanz, S., Lee, B.R., Zechmann, B., Small, I. and Kopriva, S. (2010) Identification of a pentatricopeptide repeat protein implicated in splicing of intron 1 of mitochondrial nad7 transcripts. J. Biol. Chem. **285**, 32192-32199.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E. and Newberg, L.A. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, **1**, 174-181.

Liu, Y., He, J., Chen, Z., Ren, X., Hong, X. and Gong, Z. (2010) ABA overly-sensitive 5 (ABO5), encoding a pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3, is involved in the abscisic acid response in *Arabidopsis*. Plant J. **63**, 749-765.

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., Lecharny, A., Le Ret, M., Martin-Magniette, M.L., Mireau, H., Peeters, N., Renou, J.P., Szurek, B., Taconnat, L. and Small, I. (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell, **16**, 2089-2103.

Meyer, E.H., Tomaz, T., Carroll, A.J., Estavillo, G., Delannoy, E., Tanz, S.K., Small, I.D., Pogson, B.J. and Millar, A.H. (2009) Remodeled respiration in ndufs4 with low phosphorylation efficiency suppresses *Arabidopsis* germination and growth and alters control of metabolism at night. Plant Physiol. **151**, 603-619.

Millar, A.H., Sweetlove, L.J., Giegé, P. and Leaver, C.J. (2001) Analysis of the *Arabidopsis* mitochondrial proteome. Plant Physiol. **127**, 1711-1727.

Millar, A.H., Whelan, J., Soole, K.L. and Day, D.A. (2011) Organization and regulation of mitochondrial respiration in plants. Annu. Rev. Plant Biol. **62**, 79-104.

Nemeth, K., Salchert, K., Putnoky, P., Bhalerao, R., Koncz-Kalman, Z., Stankovic-Stangeland, B., Bako, L., Mathur, J., Okresz, L., Stabel, S., Geigenberger, P., Stitt, M., Redei, G.P., Schell, J. and Koncz, C. (1998) Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in *Arabidopsis*. Genes Dev. **12**, 3059-3073.

Pineau, B., Mathieu, C., Gerard-Hirne, C., De Paepe, R. and Chetrit, P. (2005) Targeting the NAD7 subunit to mitochondria restores a functional complex I and a wild type phenotype in the Nicotiana sylvestris CMS II mutant lacking nad7. J. Biol. Chem. **280**, 25994-26001.

Pritchard, S.L., Charlton, W.L., Baker, A. and Graham, I.A. (2002) Germination and storage reserve mobilization are regulated independently in *Arabidopsis*. Plant J. **31**, 639-647.

Rolland, F., Moore, B. and Sheen, J. (2002) Sugar sensing and signaling in plants. Plant Cell, **14 Suppl**, S185-205.

Sünkel, S., Brennicke, A. and Knoop, V. (1994) RNA editing of a conserved reading frame in plant mitochondria increases its similarity to two overlapping reading frames in Escherichia coli. Mol. Gen. Genet. **242**, 65-72.

Sabar, M., De Paepe, R. and de Kouchkovsky, Y. (2000) Complex I impairment, respiratory compensations, and photosynthetic decrease in nuclear and mitochondrial male sterile mutants of Nicotiana sylvestris. Plant Physiol. **124**, 1239-1250.

Schägger, H. and Pfeiffer, K. (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J. **19**, 1777-1783.

Schmitz-Linneweber, C. and Small, I. (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci.**13**, 663-670.

Sheen, J. (1994) Feedback control of gene expression. photosynth. Res. **39**, 427-438.

Sheen, J., Zhou, L. and Jang, J.C. (1999) Sugars as signaling molecules. Curr Opin Plant Biol, **2**, 410-418.

Shen, W., Wei, Y., Dauk, M., Tan, Y., Taylor, D.C., Selvaraj, G. and Zou, J. (2006) Involvement of a glycerol-3-phosphate dehydrogenase in modulating the NADH/NAD+ ratio provides evidence of a mitochondrial glycerol-3-phosphate shuttle in *Arabidopsis*. Plant Cell, **18**, 422-441.

Shikanai, T. (2006) RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol. Life Sci. **63**, 698-708.

Shoubridge, E.A. (2001) Cytochrome c oxidase deficiency. Am. J. Med. Genet. **106**, 46-52.

Smalle, J. and Van Der Straeten, D. (1997) Ethylene and vegetative development. Physiol. Plant. **100**, 593-605.

Stitt, M. (1986) Limitation of photosynthesis by carbon metabolism. I. Evidence for excess electron transport capacity in leaves carrying out photosynthesis in saturating light and CO2. Plant Physiol. **81**, 1115-1122.

Stitt, M., Lilley, R.M., Gerhardt, R. and Heldt, H.W. (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. In Methods in Enzymology (Sidney Fleischer, B.F. ed: Academic Press, pp. 518-552.

Sugioka, K., Nakano, M., Totsune-Nakano, H., Minakami, H., Tero-Kubota, S. and Ikegami, Y. (1988) Mechanism of O2- generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems. Biochim. Biophys. Acta. **936**, 377-385.

Sung, T.Y., Tseng, C.C. and Hsieh, M.H. (2010) The SLO1 PPR protein is required for RNA editing at multiple sites with similar upstream sequences in *Arabidopsis* mitochondria. Plant J. **63**, 499-511.

Swanson, S. and Gilroy, S. (2010) ROS in plant development. Physiol. Plant, **138**, 384-392.

Takenaka, M. (2010) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the nad7 transcript in mitochondria of *Arabidopsis*. Plant Physiol. **152**, 939-947.

Takenaka, M. and Brennicke, A. (2007) RNA editing in plant mitochondria: assays and biochemical approaches. Methods Enzymol. **424**, 439-458.

Takenaka, M. and Brennicke, A. (2009) Multiplex single-base extension typing to identify nuclear genes required for RNA editing in plant organelles. Nucleic Acids Res. **37**, e13.

Takenaka, M., Verbitskiy, D., Zehrmann, A. and Brennicke, A. (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J. Biol. Chem. **285**, 27122-27129.

Tang, J., Kobayashi, K., Suzuki, M., Matsumoto, S. and Muranaka, T. (2010) The mitochondrial PPR protein LOVASTATIN INSENSITIVE 1 plays regulatory roles in cytosolic and plastidial isoprenoid biosynthesis through RNA editing. Plant J. **61**, 456-466.

Tsudzuki, T., Wakasugi, T. and Sugiura, M. (2001) Comparative analysis of RNA editing sites in higher plant chloroplasts. J. Mol. Evol. **53**, 327-332.

Unseld, M., Marienfeld, J.R., Brandt, P. and Brennicke, A. (1997) The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nat. Genet. **15**, 57-61.

van der Merwe, J.A. and Dubery, I.A. (2007) Expression of mitochondrial tatC in Nicotiana tabacum is responsive to benzothiadiazole and salicylic acid. J.Plant Physiol. **164**, 1231-1234.

Verbitskiy, D., Hartel, B., Zehrmann, A., Brennicke, A. and Takenaka, M. (2011) The DYW-E-PPR protein MEF14 is required for RNA editing at site matR-1895 in mitochondria of *Arabidopsis thaliana*. FEBS Lett. **585**, 700-704.

Verbitskiy, D., Zehrmann, A., van der Merwe, J.A., Brennicke, A. and Takenaka, M. (2010) The PPR protein encoded by the LOVASTATIN INSENSITIVE 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J. **61**, 446-455.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A. and Turner, R.J. (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell, **93**, 93-101.

Yu, Q.B., Jiang, Y., Chong, K. and Yang, Z.N. (2009) AtECB2, a pentatricopeptide repeat protein, is required for chloroplast transcript accD RNA editing and early chloroplast biogenesis in *Arabidopsis thaliana*. Plant J. **59**, 1011-1023.

Yuan, H. and Liu, D. (2012) Functional disruption of the PPR protein SLG1 affects mitochondrial RNA editing, plant development, and responses to abiotic stresses in *Arabidopsis*. Plant J. **70**, 432-444.

Zehrmann, A., Verbitskiy, D., van der Merwe, J.A., Brennicke, A. and Takenaka, M. (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell, **21**, 558-567.

Zhou, L., Jang, J.C., Jones, T.L. and Sheen, J. (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proc. Natl Acad. Sci. USA, **95**, 10294-10299.

Chapter 6: SLO2 participates in multiple stress and hormone responses

Adapted from:

The *Arabidopsis* PPR RNA editing factor SLO2 participates in multiple stress and hormone responses.

ZHU, Q., DUGARDEYN, J., ZHANG, C., MUHLENBOCK, P., VALCKE, R., DE CONINCK, B., ODEN, S., KARAMPELIAS, M., CAMMUE, B., PRINSEN, E. and VAN DER STRAETEN, D.

Molecular plant, accepted for publication

Jasper Dugardeyn performed the original experiments for Figure1, 2a, 2c, 2d, 3a, 3b, 6 and 10 and wrote 30% of the first draft of the manuscript

Recently, we reported that the novel mitochondrial RNA editing factor SLO2 is essential for mitochondrial electron transport, and vital for plant growth through regulation of carbon and energy metabolism. Here, we show that mutation in *SLO2* causes hypersensitivity to ABA and insensitivity to ethylene, suggesting a link with stress responses. Indeed, *slo2* mutants are hypersensitive to salt and osmotic stress during the germination stage, while adult plants show increased drought and salt tolerance. Moreover, *slo2* mutants are more susceptible to *Botrytis cinerea* infection. An increased expression of nuclear encoded stress-responsive genes, as well as mitochondrial encoded *NAD* genes of complex I and genes of the alternative respiratory pathway, was observed in *slo2* mutants; further enhanced by ABA treatment. In addition, H_2O_2 accumulation and altered amino acid levels were recorded in *slo2* mutants. We conclude that SLO2 is required for plant sensitivity to ABA, ethylene, biotic and abiotic stress. Although two stress-related RNA editing factors were reported very recently, this study demonstrates a unique role of SLO2, and further supports a link between mitochondrial RNA editing events and stress response.

6.1 Introduction

When encountering stress, plants adapt to the adverse conditions through diverse survival mechanisms. Many signaling molecules including plant hormones (particularly abscisic acid (ABA), ethylene, jasmonate (JA) and salicylic acid (SA)) and reactive oxygen species (ROS) play crucial roles in stress-related pathways through controlling the expression of stress-responsive genes (Baena-Gonzalez and Sheen, 2008). Of these factors, ABA is at the cross-road of biotic and abiotic stress responses (Lee and Luan, 2012), and evidence indicates that it may play both negative and positive roles in plant defense (Fan *et al.*, 2009). In addition, ethylene, JA and SA play a clear role in biotic stress signaling upon pathogen infection (Mauch-Mani and Mauch, 2005). Under most stress conditions, ROS increase as a response to stress (Jaspers and Kangasjarvi, 2010). Mitochondria are a major source for cellular ROS (Mittler *et al.*, 2004). Of the 4 complexes that comprise the classical mitochondrial electron transport chain (mETC: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *bc1* complex), complex IV (cytochrome *c* oxidase)), complex I, II and III are the primary sites of superoxide production (Noctor *et al.*, 2007).

Besides the classical mETC, plant mitochondria also possess alternative NAD(P)H dehydrogenases in the first and an alternative oxidase (AOX) in the latter part of the pathway (Millar *et al.*, 2011). The NAD(P)H dehydrogenases operate in parallel with complex I (Rasmusson *et al.*, 2004). Plant mitochondria contain at least 7 alternative NAD(P)H dehydrogenases, which can be divided into 3 subfamilies: *NDA* (*NDA1* and *NDA2*), *NDB* (*NDB1-NDB4*), and *NDC* (*NDC1*) (Michalecka *et al.*, 2003). Electrons from ubiquinone (UQ) to oxygen can also be transferred through AOX, thus reducing the formation of ROS (Millar *et al.*, 2001). The expression of NAD(P)H dehydrogenases and AOX is enhanced by biotic and abiotic stresses (Rasmusson *et al.*, 2004).

Besides the nuclear genome, plant chloroplasts and mitochondria also have their own independent genomes (Timmis *et al.*, 2004). During the maturation of organellar mRNA, a number of processes occur, including RNA editing, splicing, and cleavage (Stern *et al.*, 2010). Pentatricopeptide repeat (PPR) proteins play a major role therein (Saha *et al.*, 2007; Schmitz-Linneweber and Small, 2008). They are characterized by tandem arrays of a degenerate 35-amino-acid repeat (Lurin *et al.*, 2004; Schmitz-Linneweber and Small, 2008). In *Arabidopsis*, there are 450 members in the PPR family, a large majority of which are predicted to be localized in mitochondria or chloroplasts and involved in RNA metabolism, including RNA editing (Lurin *et al.*, 2004; Schmitz-Linneweber and Small, 2008). RNA editing is a process that alters cytidine (C) to uridine (U) in specific sites of mitochondria or plastids in flowering plants; the reverse reaction (from U to C) is rare (Shikanai, 2006). The first RNA editing factor was identified in chloroplasts in 2005 (Kotera *et al.*, 2005), while only recently being discovered in mitochondria (Zehrmann *et al.*, 2009). It was speculated that PPR proteins can recognize *cis* elements localized 5-20 nucleotides upstream of the editing sites, and that one PPR factor can be involved in a single or multiple RNA editing events (Okuda and Shikanai, 2012). Recently, a code for sequence-specific RNA recognition by PPR tracts has been proposed (Barkan *et al.*, 2012). To date, a number of PPR proteins that act as mitochondrial RNA editing factors were identified in *Arabidopsis* and rice (Murayama *et al.*, 2012; Sung *et al.*, 2010; Takenaka, 2010; Takenaka *et al.*, 2010; Verbitskiy *et al.*, 2011; Verbitskiy *et al.*,

2010; Yuan and Liu, 2012; Zehrmann *et al.*, 2012; Zehrmann *et al.*, 2010; Zehrmann *et al.*, 2009; Zhu *et al.*, 2012a). SLG1 (slow growth 1) and AHG11 (ABA hypersensitive germination 11), reported to be necessary for RNA editing of respectively *NAD3* and *NAD4* transcripts of complex I, are both involved in plant development and in response to abiotic stress (Murayama *et al.*, 2012; Yuan and Liu, 2012).

Recently, we characterized the PPR protein SLO2, which acts as a mitochondrial RNA editing factor, and plays a vital role in *Arabidopsis* growth through regulation of energy metabolism (Zhu *et al.*, 2012a). *slo2* mutants exhibit retarded leaf emergence, restricted root and shoot growth, and late flowering. These phenotypes are suppressed by supplying sugars, increasing light dosage or CO_2 concentration. Mutation in *SLO2* leads to 7 RNA editing changes, with RNA editing defects in sites *mttB-144*, *mttB-145*, *nad4L-110*, *nad7-739* resulting in 4 amino acid changes in polypeptides, and RNA editing efficiency changes in *mttB-666*, *nad1-2*, *nad1-40* (Zhu *et al.*, 2012a). In addition, the abundance of complex I, III and IV are much reduced in *slo2* mutants.

Given the function of SLO2, the presumed ROS increase upon mETC dysfunction, and ROS involvement in stress response, we examined the role of SLO2 in plant stress tolerance. First, we demonstrated that mutation in *SLO2* leads to altered response to the plant hormones ethylene and ABA, as well as to biotic and abiotic stress. Furthermore, we showed that *slo2* mutants suffer from internal oxidative stress, reflected by H_2O_2 accumulation. Consequently, broad changes in the nuclear transcriptome related both to growth and stress responses were identified in *slo2*. In addition, after ABA treatment, *slo2* mutants exhibited various phenotypic changes, and alterations in the expression pattern of nuclear and mitochondrial genes compared to wild-type. Our results indicate that SLO2 is necessary for maintaining plant response to ethylene, ABA, biotic and abiotic stress, and plays a unique role therein.

6.2 Results

6.2.1. Expression of the *SLO2* gene

In-situ hybridization data revealed *SLO2* expression in different tissues and at different developmental stages (Supplemental Figure 1, digitally available in Zhu *et al.*, 2013). The strongest expression of *SLO2* was found in the first pair of true leaves (Supplemental Figure 1A, digitally available in Zhu *et al.*, 2013). In roots, *SLO2* was expressed in the meristematic zone of primary roots, and in emerging lateral roots, suggesting a possible role for SLO2 in root growth (Supplemental Figure 1B, digitally available in Zhu *et al.*, 2013).

Furthermore, histochemical analysis of *pSLO2::GUS* transgenic plants revealed GUS activity in the root tip of young seedlings (Supplemental Figure 1C, digitally available in Zhu *et al.*, 2013). In flowers, staining was detected in the upper part of the inflorescence stem, the pedunculi, the replum of the ovary, as well as in developing siliques and mature anther filaments (Supplemental Figure 1C, digitally available in Zhu *et al.*, 2013).

As a first indication for hormone and stress responsiveness of *SLO2*, we analyzed microarray data using Genevestigator software (Hruz *et al.*, 2008). The results indicated that *SLO2* expression is not strongly responsive to biotic (*Pseudomonas syringae*), or abiotic stresses (cold, drought, salt, wounding, osmotic and oxidative stresses) nor to hormone treatments (ABA, 1-aminocyclopropane-1-carboxylate (ACC), gibberellic acid (GA), indole-3-acetic acid (IAA), MeJA, SA) (Data not shown). To verify the reliability of these data, we selected some stress and hormone treatments (ABA, NaCl, JA, mannitol, methyl viologen (MV)), and checked *SLO2* transcription levels using real-time RT-PCR. Our data confirmed that *SLO2* is not responsive to any of these treatments at the transcriptional level (Supplemental Figure 1D, digitally available in Zhu *et al.*, 2013).

6.2.2. Loss-of-function of *SLO2* reduces sensitivity to ethylene

The *slo2-1* mutant was selected in a screen for ethylene-insensitivity based on delayed leaf emergence (DLE) on low nutrient medium (Zhu *et al.*, 2012a). Ethylene effects on dark-grown seedlings are known as the "triple response", which consists of inhibition of elongation and radial swelling of the stem, as well as absence of a normal geotropic response (Knight and Crocker, 1913).We further investigated the triple response of *slo2* mutants. In control conditions, the root and hypocotyl length of wild-type were longer than that of *slo2* mutants. After application of 4 µM ACC, the root and hypocotyl length of *slo2* alleles was inhibited, albeit that the relative inhibition was lower than that in the wild-type (Figure 1A, B). No differences were observed in the exaggeration of the apical hook after ACC treatment (Supplemental Figure 2, digitally available in Zhu *et al.*, 2013). We also monitored the phenotype of light-grown plants. First, we measured the cotyledon and leaf expansion (Figure 1C). In the absence of ACC, the first true leaves of *slo2-1* were smaller than the WT, while in the presence of ACC, cotyledons and true leaves were consistently larger than the wild-type. Similar results were obtained when seedlings were grown in the presence of 100 ppb ethylene (Figure 1D). Both at the shoot and the root level, a stronger inhibition of growth was observed in the wild-type as opposed to *slo2* mutants. When grown on medium with or without 50 µM ACC in the light, ACC effectively inhibited root growth, but the relative inhibition was

stronger in wild-type than in *slo2* mutant alleles (Figure 1E, F). We conclude that mutation in *SLO2* reduces the ethylene response.

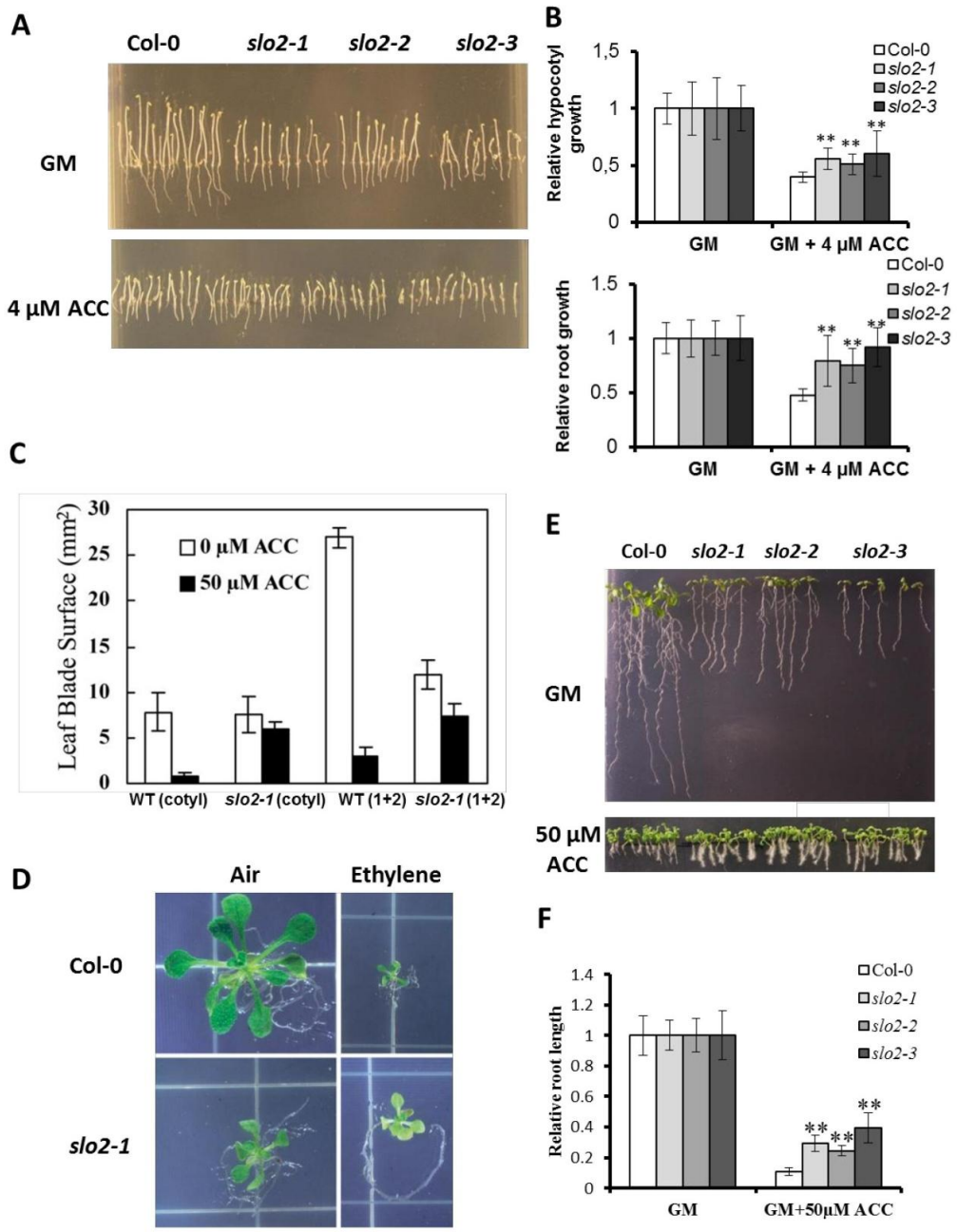

Figure 1. Ethylene response of *slo2* mutants

A, Col-0 and *slo2* mutants grown on growth medium (GM) with or without ACC for 5 days in dark condition.

B, Relative qualitative analysis of hypocotyl and root length from (A). Data are averages ±SD (n>10), **, $p<0.01$.

C, Leaf blade surface area of cotyledons (cotyl) and first pair of true leaves (1+2) from 3-week-old *slo2-1* and wild-type plants grown on medium with or without 50 µM ACC. Data are averages ±SE (n=10).

D, Phenotypes of 3-week-old Col-0 and *slo2-1* plants continuously grown in air or 100 ppb ethylene.

E, 7-day-old light-grown Col-0 and *slo2* seedlings on medium without or with 50µM ACC

F. Relative root length from seedlings in panel E. Data are averages ±SD (n>10). **, $p<0.01$.

6.2.3. *slo2* mutants are hypersensitive to ABA at the germination and early seedling stage

ABA and ethylene function antagonistically, as most ABA-insensitive mutants show enhanced sensitivity to ethylene and vice versa (Gazzarrini and McCourt, 2001). In addition, our previous report demonstrated that loss-of-function of *SLO2* causes hypersensitivity to sucrose (Zhu *et al.*, 2012a). Several lines of evidence support the connections between sucrose and ABA signaling (Dekkers *et al.*, 2008). Therefore, we tested the effect of ABA on *slo2* using different approaches. Germination of *slo2* on 0.5 µM ABA was delayed compared to that of Col-0 (Figure 2A). In addition, the post-germination growth of *slo2* mutants confirmed the hypersensitivity to ABA reflected by a delay in seedling development (Figure 2B, C). ABA does not only inhibit germination and post-germination growth, but also root growth at a later stage.

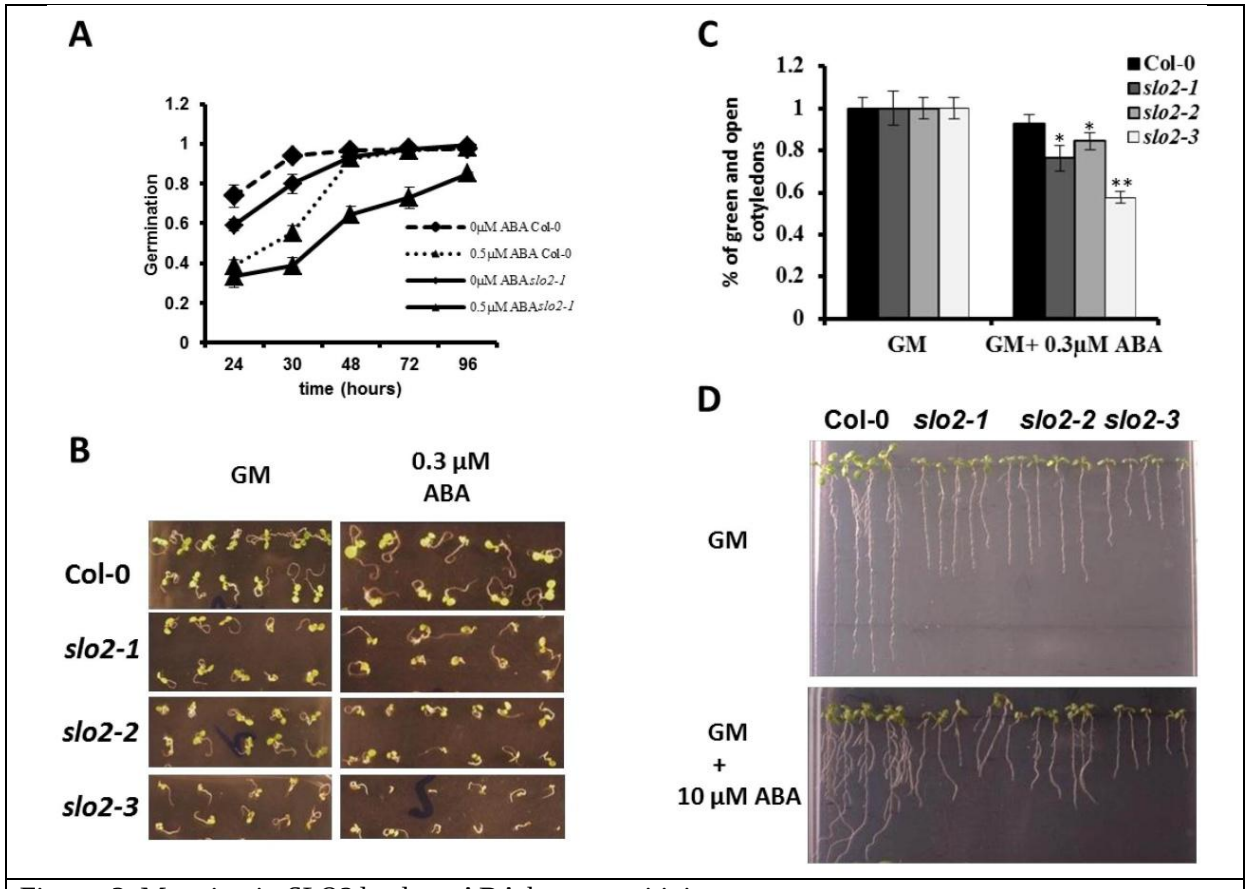

Figure 2. Mutation in *SLO2* leads to ABA hypersensitivity

A, Germination time courses of Col-0 and *slo2-1* mutant. Average values ± SD from 3 independent experiments are presented.

B, Growth status of Col-0 and *slo2* mutants on growth medium with or without 0.3 µM ABA. Pictures were taken 7-days after germination.

C. Statistical analysis of the percentage of green and open cotyledons shown in panel B. Data represent the mean ± SD of 3 independent experiments, each with n>40. *, $p<0.05$.

D, Root growth assay of Col-0 and *slo2* mutants on medium with or without 10 µM ABA. 3-day old seedlings grown on germination medium were transferred to medium with or without 10 µM ABA. Pictures were taken after 7 days.

The root growth of *slo2* mutants was more inhibited than that of the wild-type (Figure 2 D, 2E). This was further confirmed by a root-bending assay revealing a more severe inhibition of seedling root elongation in the mutants (Figure 2F, 2G). ABA hypersensitivity of *slo2* mutants may result from an elevated ABA level (Pandey *et al.*, 2005). Indeed, we found that *slo2* mutant seeds contain more ABA (Supplemental Figure 3, digitally available in Zhu *et al.*, 2013), partially explaining the ABA hypersensitivity of *slo2* mutants during germination and early seedling growth stage. Overall, our results demonstrate that mutation in *SLO2* causes ABA hypersensitivity in *Arabidopsis*.

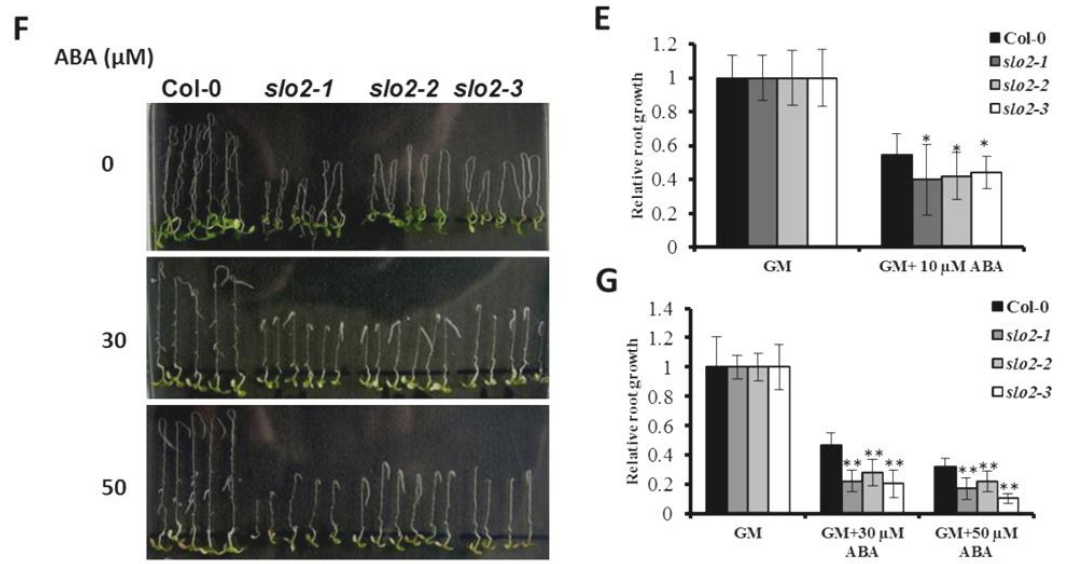

Figure 2. Mutation in *SLO2* leads to ABA hypersensitivity

E, Relative root growth of seedlings shown in panel (D). Relative root growth reflects the root length in the presence of ABA expressed as a percentage of root length in the absence of ABA. Standard errors are presented (n > 40). Data represent the mean ± SD of 3 independent measurements. *, $p<0.05$.

F, Root bent assay. 4-day-old seedlings were transferred to medium with or without ABA, and plants were grown reversely for 7 days.

G. Relative root growth of seedlings shown in panel (F). Relative root growth reflects the root length in the presence of ABA expressed as a percentage of the root length in the absence of ABA. Data represent the mean ± SD of 3 independent experiments, each with n>40. *, $p<0.05$.

6.2.4. *slo2* mutants have an altered sensitivity to various abiotic stresses

ABA plays an important role in plant responses to abiotic stress (Zhu, 2002). Therefore, we tested the effect of NaCl and sorbitol on the *slo2* growth. On control medium, *slo2* seedlings exhibit much shorter roots than the wild-type. On medium containing salt or sorbitol, *slo2* mutants showed hypersensitivity reflected in impaired shoot growth and root length (Figure 3A). A detailed germination time-course under salt and osmotic stress further confirmed these observations. Under control conditions, nearly all *slo2* mutants had germinated on day 5 (i.e. 3 days later than the wild-type), while on medium containing 100 mM NaCl, the germination of *slo2* mutants was much more inhibited. Wild-type seedlings had nearly all germinated at day 5, while three days later, the majority of *slo2* seedlings had not (91% for wild-type at day 5, 32%-

54% for 3 *slo2* mutants at day 8). On medium containing 300 mM sorbitol, the germination pattern was similar as on NaCl-containing medium (Figure 3B).

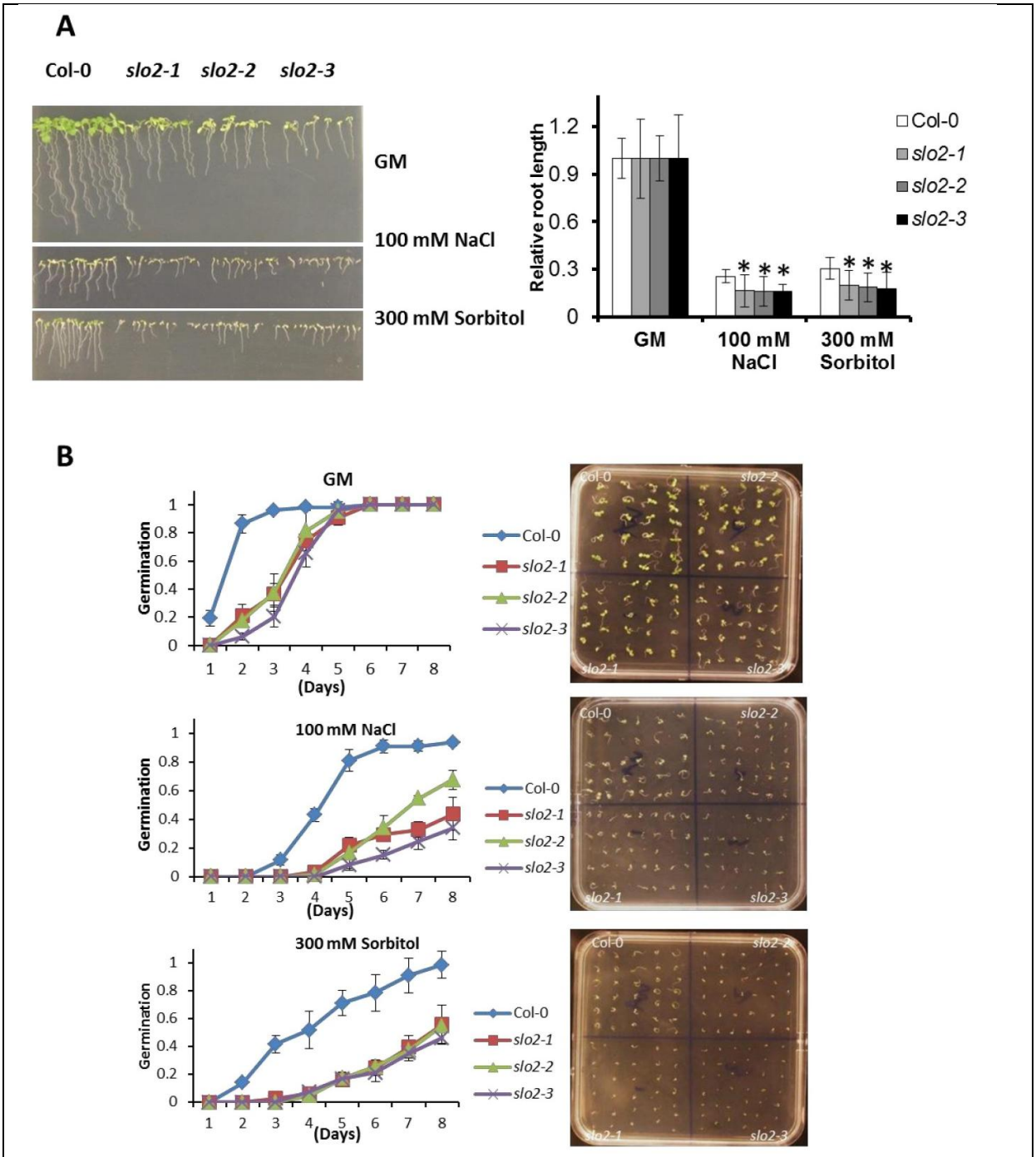

Figure 3. *slo2* mutants are hypersensitive to salt and osmotic stress at germination and early growth stage.

A, Seedling growth of *slo2* is inhibited by salt and osmotic stress. Col-0 and 3 *slo2* alleles were grown on medium with or without stress factors for 7 days. Right panel: relative root growth, reflecting root length in the presence of NaCl or sorbitol expressed as a percentage of root length in the absence of NaCl or sorbitol. Values are means ± SD (n ≥15). *, $p<0.05$

B, Germination time course assay. Col-0 and 3 *slo2* mutants were grown on medium with or without stress factors. Values are means ± SD from three independent experiments. The seedling growth status is shown on the right

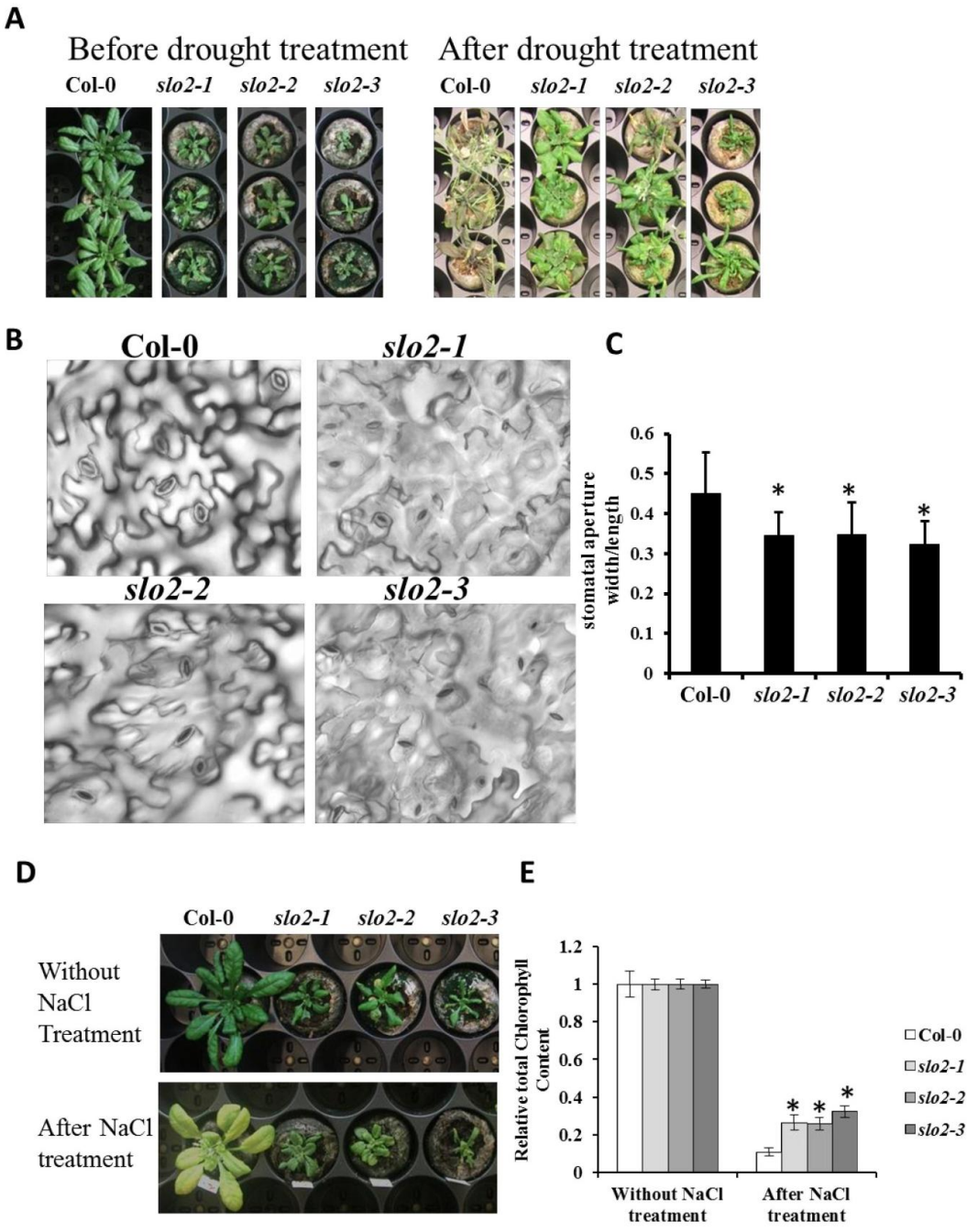

Figure 4. Drought and salt tolerance of Col-0 and *slo2* mutants

A, Growth status of Col-0 and 3 *slo2* alleles before and after drought treatment. 1-month-old plants were grown in soil in the same container, withheld from water for 10 days.

B, Stomatal aperture of Col-0 and 3 *slo2* alleles. lower epidermis from 1-month-old plants were peeled off and used for microscopic observation.

C, Statistical analysis of stomatal aperture. Data are mean ratios of width to length ± SD (n>30). *, $p<0.05$.

D, Growth status of Col-0 and 3 *slo2* alleles with or without salt treatment. 1-month-old plants were watered with 100 mM NaCl for 4 days, 200 mM NaCl for 4 days, and finally 300 mM NaCl for 6 days.

E, Relative chlorophyll content measurement, which means chlorophyll level in the presence of NaCl expressed as a percentage of chlorophyll level in the absence of NaCl. *, $p<0.05$.

Based on the results above, we hypothesized that *SLO2* mutation may alter drought or salt tolerance. To test this, a drought tolerance assay was performed using plants of Col-0 and 3 *slo2* alleles of the same age. After interruption of watering for 10 days, all Col-0 plants were wilted and dead, while the 3 *slo2* alleles survived and appeared healthy (Figure 4A). Moreover, in control conditions, the stomatal apertures in the 3 *slo2* alleles were reduced as compared to Col-0 (Figure 4B and 4C), though the stomatal densities were increased (supplemental Figure 4A, digitally available in Zhu *et al.*, 2013). Water loss experiments demonstrated that Col-0 and *slo2* mutants show similar water loss curves (Supplemental Figure 4B, digitally available in Zhu *et al.*, 2013), probably because the significant reduction of stomatal aperture in *slo2* mutants is compensated by the increased stomatal density. We also performed a drought tolerance assay on Col-0, *slo2-1* and *slo2-2* plants with similar size, obtained upon staggered growth (*slo2-3* which is extremely retarded in growth, was not included). All genotypes showed a similar response to the decrease in soil water content (Supplemental Figure 5, digitally available in Zhu *et al.*, 2013).These results correlate well with previous reports on complex I mutants *slg1*, *ndufa1* and *ndufs4* (Meyer *et al.*, 2009; Yuan and Liu, 2012). We further investigated whether the *SLO2* mutation altered salt tolerance. Upon watering with a solution containing up to 300 mM NaCl, Col-0 became visibly chlorotic while the *slo2* alleles retained their healthy appearance until 6 days after treatment (Figure 4D). Measurement of chlorophyll content indicated a decrease in *slo2* mutants that was less pronounced than in the wild-type (9-fold decrease for the wild-type, versus 4-fold for *slo2* mutants; Figure 4E). Hypersensitivity to ABA, salt and osmotic stress upon seed germination, combined with an increased tolerance to abiotic stress in adult plants, was also observed in other mutants, such as *slg1* (Yuan and Liu, 2012), *sad1* (*supersensitive to ABA and drought*) (Xiong *et al.*, 2001a), or *fiery1* (*fry1)* (Xiong *et al.*, 2001b). These results clearly demonstrate that SLO2 is implicated in plant tolerance to abiotic stress.

6.2.5. Salt and osmotic stress affect mutant seed germination through an ABA-dependent process

Plants are known to counter stresses by ABA-dependent and ABA-independent pathways (Cutler *et al.*, 2010; Zhu, 2002). Thus, *slo2* hypersensitivity to osmotic or salt stress may be mediated through an ABA-dependent or an ABA-independent pathway. Our previous experiments suggest ABA dependence. To confirm this, we applied the ABA biosynthesis inhibitor norflurazon (NF) upon germination of *slo2* in response to osmotic or salt stress (Laluk *et al.*, 2011). Application of 100 µM NF did not affect the germination of either Col-0 or *slo2* mutants at day 5 and day 8 (nearly 100%) (Figure 5). However, on NaCl containing medium, ~80% of the wild-type germinated on day 5, while for *slo2* mutants, this number ranged from 7%-21% (Figure 5). After application of NF to the medium, the germination rate of *slo2* was largely reverted at day 5 (*slo2-1*, 50%; *slo2-2*, 59%; *slo2-3*, 24%). A similar effect of NF was observed on medium containing sorbitol. These results further confirmed that salt and osmotic stress exert their inhibitory effects on germination of *slo2* through an ABA-dependent pathway.

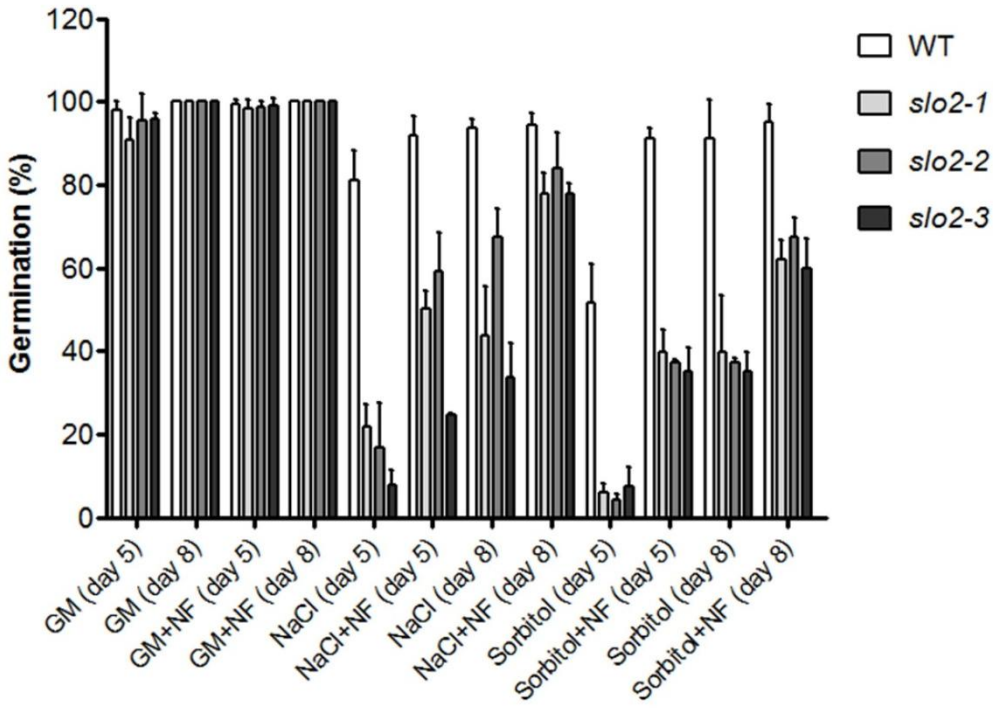

Figure 5. Effect of the ABA biosynthesis inhibitor NF (norflurazone) on germination of *slo2* mutants under NaCl and osmotic stress

Germination of Col-0 and *slo2* mutant seeds on medium containing 100 mM NaCl or 300 mM sorbitol with or without 100 µM NF. Seed germination rate was scored at day 5 and day 8 after transfer to the growth chamber, respectively. Data are mean ratios of width to length ± SD (n>100). *, $p<0.05$.

6.2.6. *slo2* mutants are more susceptible to *Botrytis cinerea*

ABA and ethylene are involved in both biotic and abiotic stress responses (Baena-Gonzalez and Sheen, 2008; Cutler *et al.*, 2010). Exogenous ABA can increase plant susceptibility to the necrotrophic pathogen *B. cinerea* (Audenaert *et al.*, 2002). Since *slo2* mutants are hypersensitive to ABA, we investigated their susceptibility to *B. cinerea*. Interestingly, all *slo2* alleles showed enhanced susceptibility to *B. cinerea*, indicated by the increased necrosis at infection sites, and quick leaf decomposition. Moreover this phenotype was reversed in the complemented lines (Figure 6A). This observation was confirmed in an independent disease assay by determining the percentage of infected leaves at several days post-inoculation (Figure 6B). These results show that mutation in *SLO2* does not only affect abiotic stress responses, but also affects plant tolerance to necrotrophic pathogens, like *B. cinerea*.

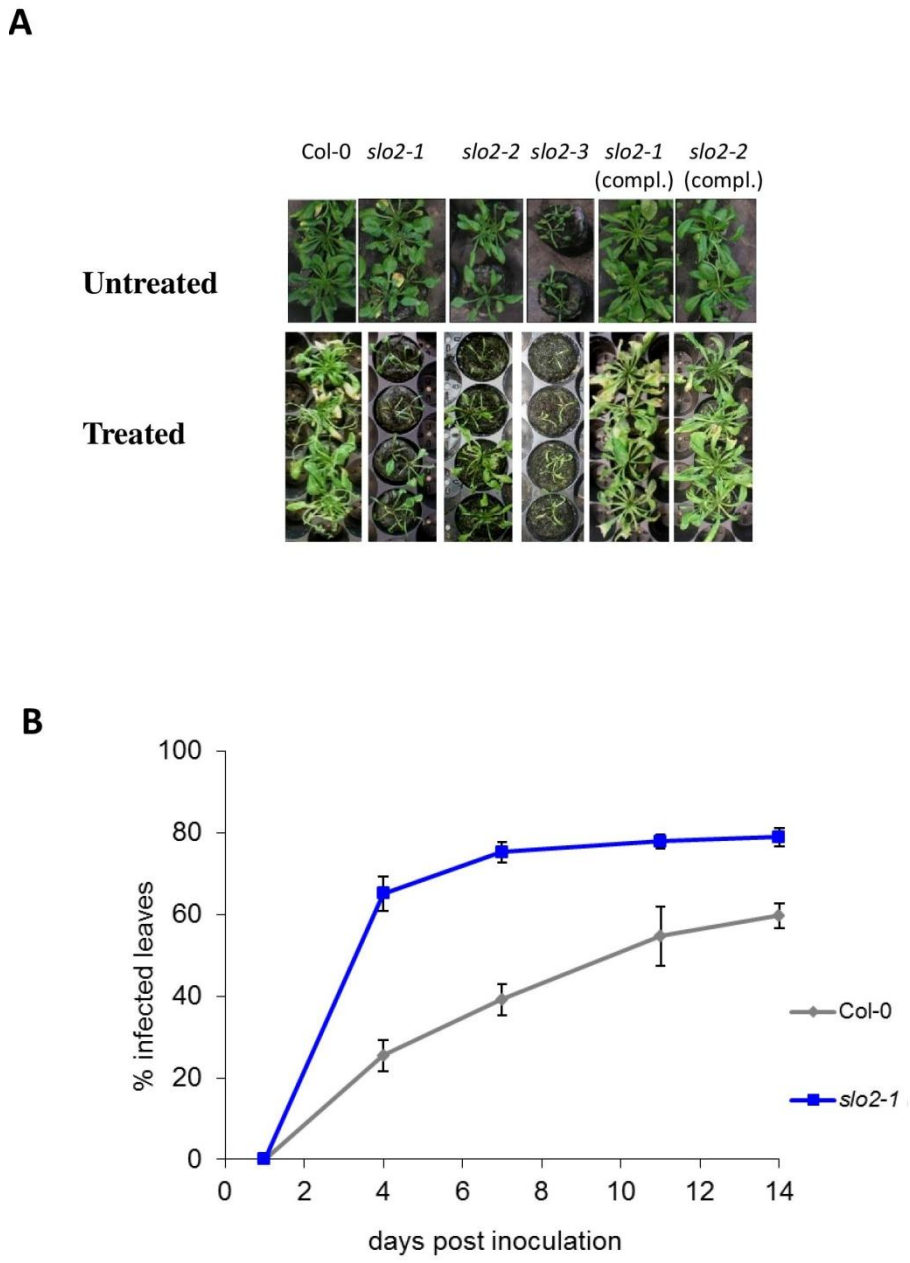

Figure 6. *slo2* mutants exhibit increased susceptibility to *Botrytis cinerea* infection

A, Disease symptoms of Col-0, 3 *slo2* mutants and 2 complemented lines. Plants were sprayed with 1×10^4 *B. cinerea* spores/mL in PDB, or mock-treated with PDB. Pictures were taken 14 days after inoculation.

B, Statistical analysis of *Botrytis* infected leaves. Plants were sprayed with a concentration of 5×10^7 *B. cinerea* spores/mL in PDB solution, and percentage infected leaves were scored at different time points. Wild-type and *slo2-1* mutant in Col-0 background are shown. Three independent experiments with about 60 leaves per line per experiment were performed with similar results. Data represent mean values and standard errors (SE).

6.2.7. Expression profiles of ABA responsive genes

The above-mentioned results indicated that *SLO2* may be linked to the ABA-dependent stress signaling pathway. Many genes responding to ABA and/or abiotic stress have been used as markers to monitor stress response pathways, such as abscissic acid response element

(ABRE) binding factor 3 (*ABF3*), ABRE binding factor 4 (*ABF4*), C-repeat-binding factor 1/dehydration responsive element-binding factor 1b (*CBF1/DREB1b*) and dehydration responsive element-binding factor 2b (*DREB2b*) (Yamaguchi-Shinozaki and Shinozaki, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). To examine whether the expression of ABA/stress responsive genes might be affected by loss-of-function of *SLO2*, we compared the expression levels of *ABF3, ABF4, CBF1/DREB1b, RAB18* and *DREB2b*, in wild-type and *slo2* mutants. Under control conditions, the transcriptional levels of these genes in *slo2* mutants were comparable to those in the wild-type (or higher, as in the strong allele *slo2-3*; Zhu *et al.*, 2012a). Their levels were increased in both wild-type and *slo2* mutants after ABA treatment (Figure 7). However, in response to 6 or 12 h of ABA treatment, their expression was much higher in the mutants compared to the wild-type. We observed a similar expression pattern for neoxanthine *cis*-epoxy-dioxygenase (*NCED3)*, which plays a key role in ABA biosynthesis (Figure 7). We concluded that, in general, *slo2* mutants have enhanced expression of ABA and stress related genes in response to ABA signaling.

6.2.8. Expression levels of mitochondrial complex I subunit genes are elevated in *slo2* mutants

Our previous results illustrated that *slo2* mutants display a phenotype reminiscent of complex I defects, and that both the abundance and activity of complex I are much reduced in the mutants (Zhu *et al.*, 2012a). In the mitochondrial genome, 9 *nad* genes encode subunits of the NADH dehydrogenase of complex I: *nad1, nad2, nad3, nad4, nad4L, nad5, nad6, nad7* and *nad9* (Rasmusson *et al.*, 1998). We investigated the expression of these transcripts in response to ABA. Without ABA, mutation in *SLO2* led to an enhanced expression of all *nad* genes (except for *NAD2* in *slo2-3* and for *NAD6* in *slo2-2)* as compared to the wild-type (Figure 8). Moreover, after 6 h and/or 12 h of treatment, the expression levels in *slo2* were much higher in at least one time point compared with Col-0. These results demonstrated not only that proper function of SLO2 is linked to a feedback control on expression of mitochondrial complex I *nad* genes under control conditions, but also after ABA treatment, indicative for a role linked to stress.

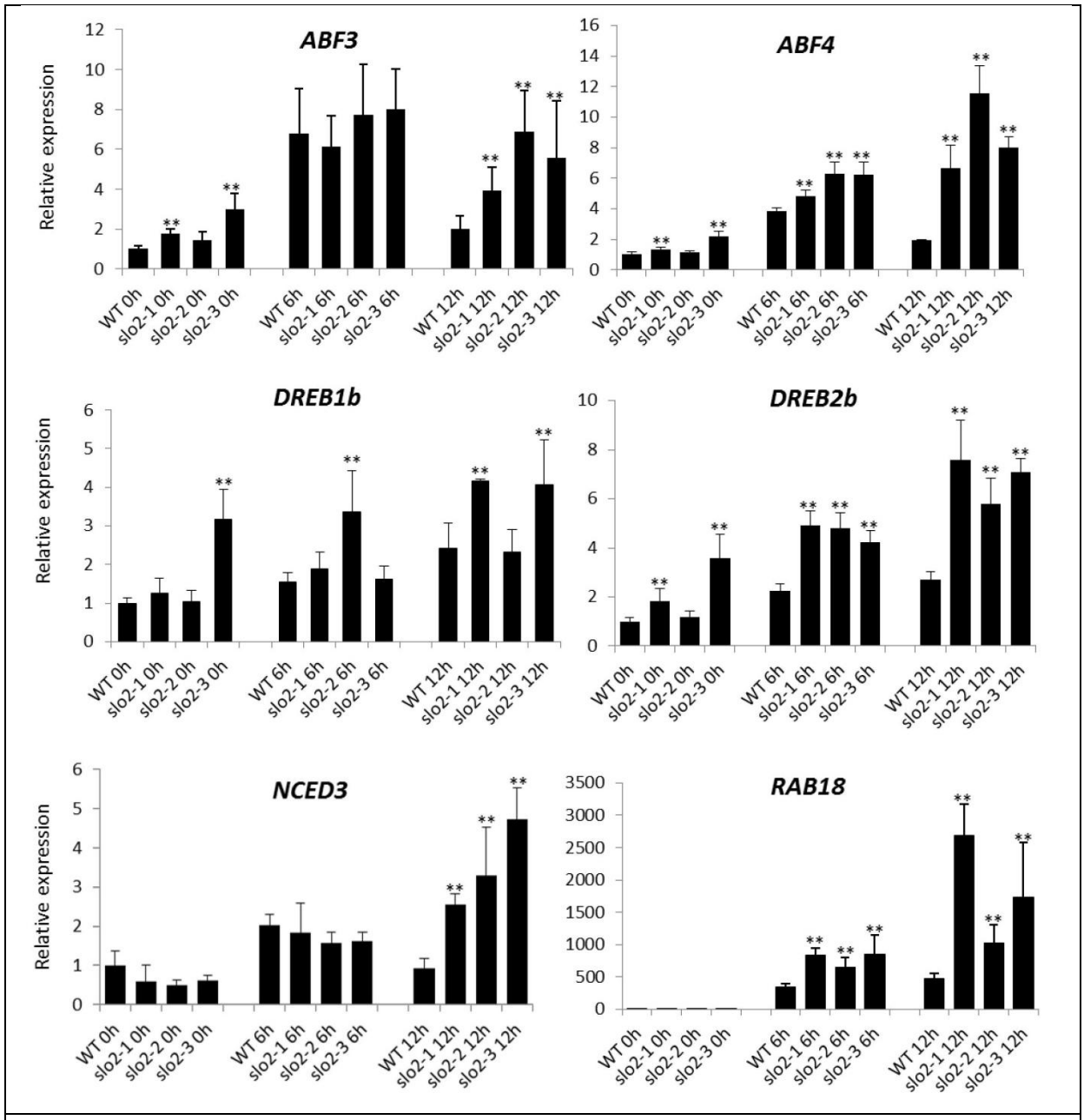

Figure 7. Expression profiles of ABA responsive genes

Expression of ABA-responsive genes after ABA treatment. 7-day old seedlings were incubated with 100 μM ABA for different time as indicated. The gene expression levels in Col-0 at time point 0 were set as 1. Three biological repeats were performed. Data represent mean values ± SD. *, $p<0.05$; **, $p<0.01$

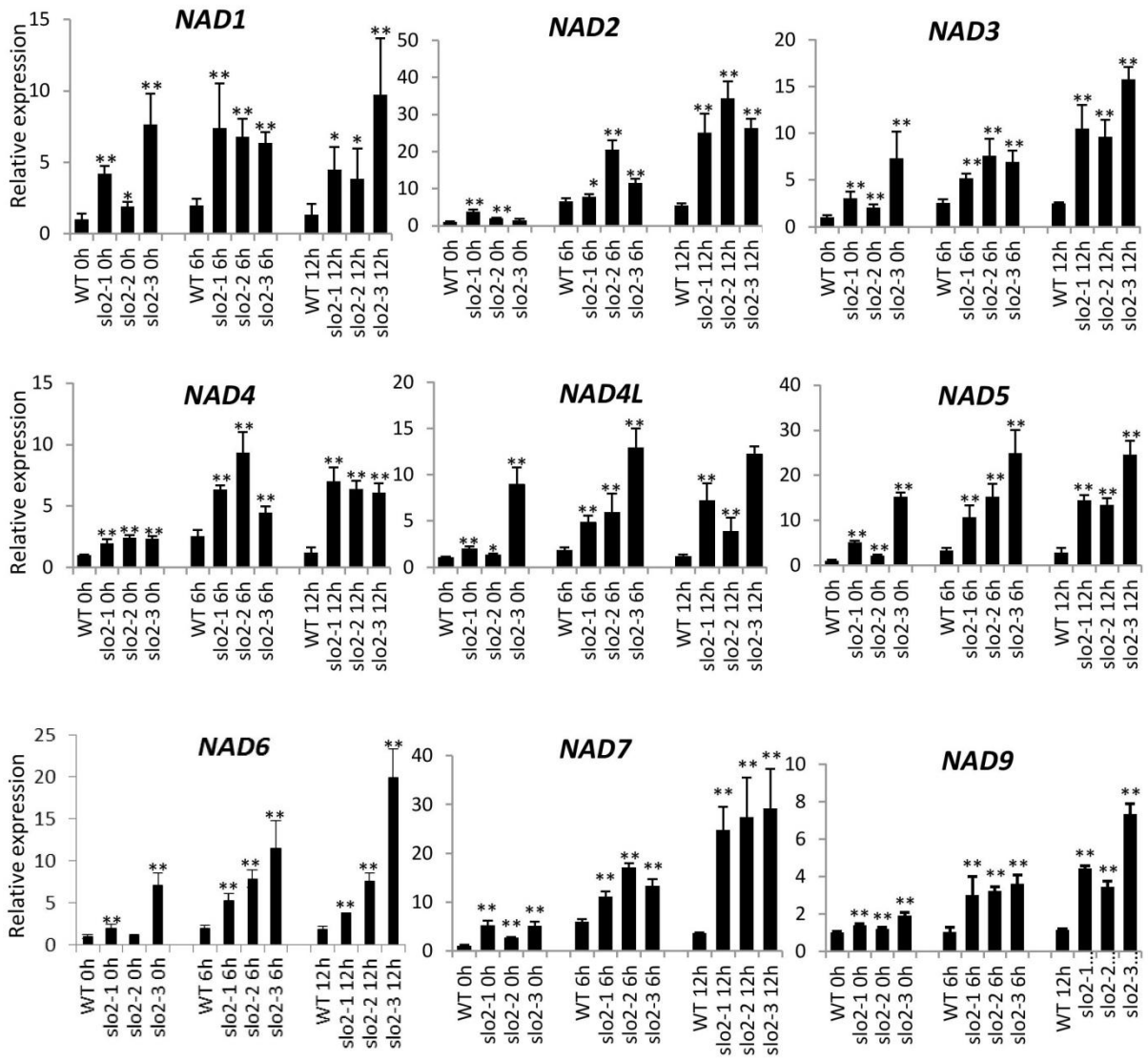

Figure 8. Expression levels of mitochondria complex-I subunit genes in Col-0 and *slo2* mutants
Expression of mitochondria complex-I subunit genes in Col-0 and *slo2* mutants after ABA treatment. 7-day-old seedlings were incubated with 100 μM ABA for different time periods as indicated. The gene expression levels in Col-0 at time point 0 were set as 1. Three biological repeats were performed. Data represent mean values ± SD. *, $p<0.05$; **, $p<0.01$

6.2.9. Expression profiles of alternative respiratory pathway transcripts are strongly up-regulated in *slo2* in response to ABA

Plants have two respiratory pathways in mitochondria: the cytochrome pathway and the alternative pathway (Rasmusson *et al.*, 2004). Electron transport in the cytochrome pathway is coupled with ATP synthesis, while being largely uncoupled in the alternative pathway (Moller, 2001). The latter can be induced by inhibition of the cytochrome pathway (Moller, 2001). Our previous data showed that loss of function of *SLO2* leads to the dysfunction of the mitochondrial electron transfer chain, related to a pronounced reduction of abundance of complex I, III, and IV, with concomitant increase in AOX (Zhu *et al.*, 2012b). Under control conditions, the levels of *AOX1a,* AOX1d, NDB2, and NDB4 transcripts in all slo2 alleles were much higher than in Col-0 (except for AOX1d in *slo2-1* and *slo2-2* and for NDB2 in *slo2-1*),

while for NDA1 and NDA2, the transcript levels did not change much in 7-day-old seedlings. AOX1a is considered as a marker for the mitochondrial retrograde pathway, and its expression is directly regulated by ABI4 (Giraud *et al.*, 2009). Recent reports indicate that ABA controls the expression pattern of alternative pathway genes (Laluk *et al.*, 2011; Liu *et al.*, 2010). Therefore, we also tested the expression of alternative NAD(P)H and AOX genes in *slo2* genotypes in response to ABA treatment. In the absence of ABA, alternative oxidase genes and *NDB4* were up-regulated in most *slo2* mutant seedlings, while the expression of other alternative NAD(P)H dehydrogenase genes was comparable with that in wild-type seedlings. Upon ABA treatment, all the alternative pathway genes were up-regulated in wild-type and in *slo2* mutants, albeit much stronger in the mutants (Figure 9).

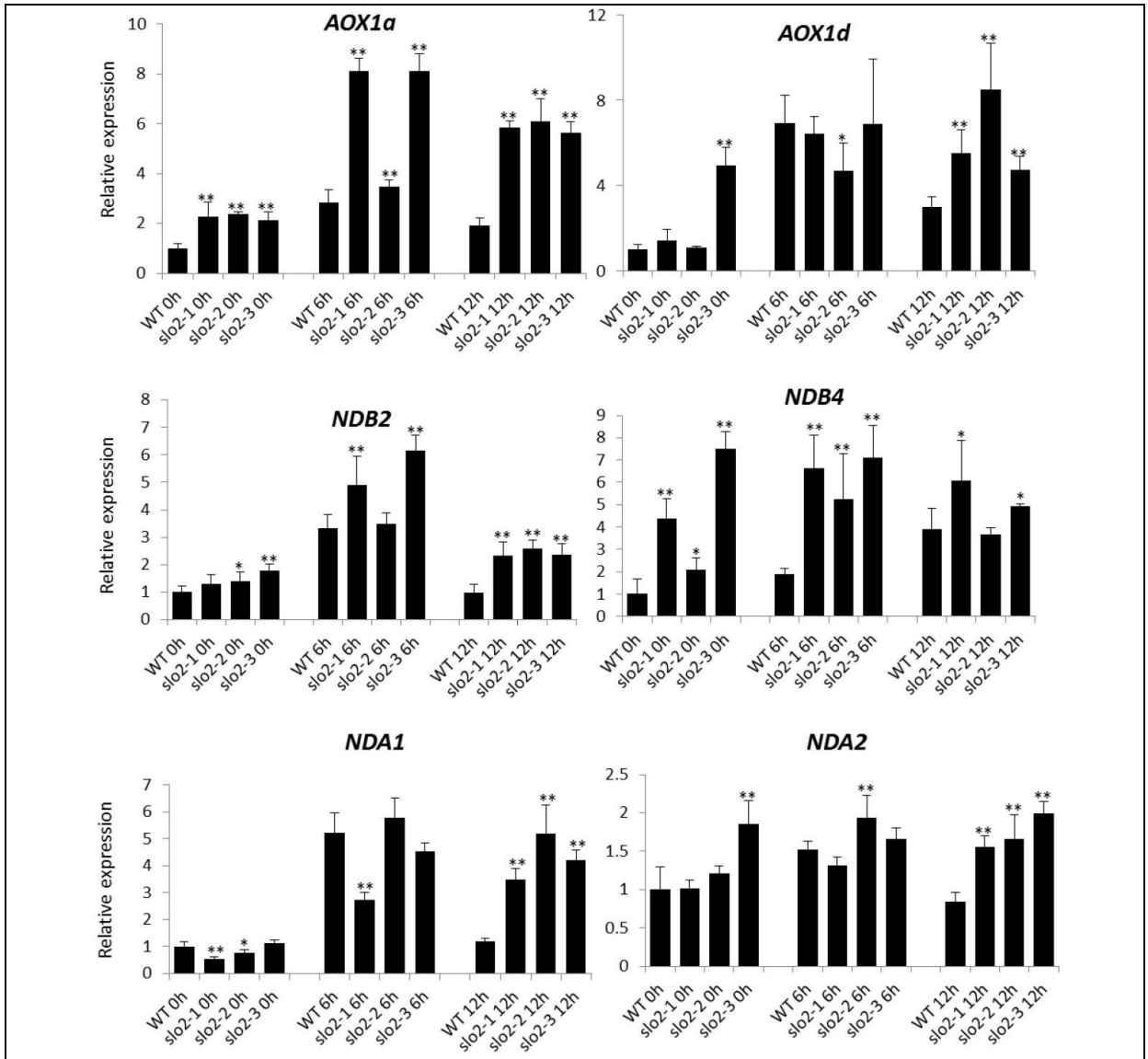

Figure 9. Expression profiles of alternative respiratory pathway transcripts in response to ABA

Expression of alternative respiratory pathway genes in Col-0 and *slo2* mutants after ABA treatment. 7-day-old seedlings were incubated with 100 μM ABA for different time as indicated. The gene expression levels in Col-0 at time point 0 were set as 1. Three biological repeats were performed. Data represent mean values ± SD. *, $p<0.05$; **, $p<0.01$

6.2.10. *slo2* mutants accumulate reactive oxygen species (ROS)

Inhibition of the mETC leads to H_2O_2 accumulation (Liu *et al.*, 2010). Since mutation of *SLO2* causes a drastic reduction of complex I, III and IV of the mETC (Zhu *et al.*, 2012a), we assumed that ROS levels may be elevated in mutants. Indeed, 2,7-dichlorofluorescein diacetate (H_2DCF-DA) staining showed a much more intense signal in the *slo2* mutants compared with wild-type and two complemented lines (for the *SLO2* expression level, see supplemental Figure 6, digitally available in Zhu *et al.*, 2013), indicating increased ROS levels in the mutants (Figure 10A). Furthermore, quantitative analysis of H_2O_2 levels using Amplex Red corroborated these observations (Figure 10B). To confirm these data, we stained rosette leaves of Col-0 and 3 *slo2* alleles with diaminobenzidine (DAB, for H_2O_2) and nitroblue tetrazolium (NBT, for superoxide), respectively. Overall, the results indicate increases in both ROS species in the mutants, and correlate well with the above-mentioned observations (Supplemental Figure 7, digitally available in Zhu *et al.*, 2013).

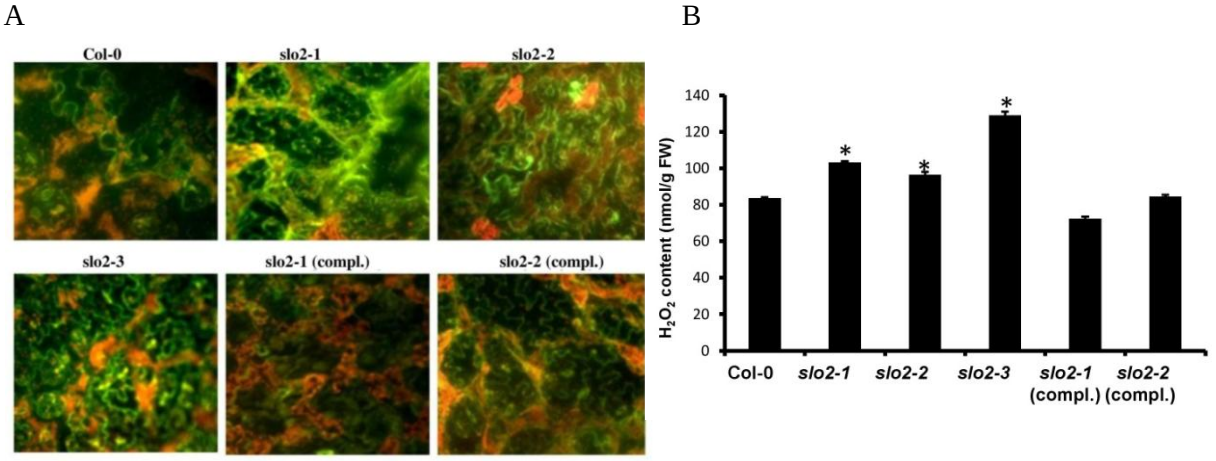

Figure 10. *slo2* mutants have an enhanced level of H_2O_2.
A, leaves of *slo2* mutants contain a higher concentration of H_2O_2. 4-week-old wild-type, *slo2* mutants and two complemented lines were stained with DCFH-DA method and viewed under a fluorescence microscope.
B, Quantitative measurement of H_2O_2 content in rosettes of Col-0, 3 *slo2* alleles and two complemented lines. Data represent the mean ± SD of 3 independent measurements. *, $p<0.05$.

6.2.11. Amino acid changes in *slo2* mutants

Dysfunction of the mitochondrial electron transport chain alters cellular metabolism, including glycine metabolism (Juszczuk *et al.*, 2012). Previous studies on *cytoplasmic male sterile II* (*CMSII*), *NADH dehydrogenase fragment S subunit 4* (*ndufs4*) and *nuclear maturase 1* (*nMAT1*) mutants indicate that the mitochondrial complex I is indeed essential to sustain amino acid homeostasis (Dutilleul *et al.*, 2003; Hager *et al.*, 2010; Keren *et al.*, 2012; Meyer *et al.*, 2009). To investigate the physiological effects of *SLO2* mutation on amino acid metabolism, we assayed the amino acid content by GC-MS (Baker *et al.*, 2006). Our results showed that in all *slo2* alleles the content of several amino acids is higher than in Col-0, while being restored to wild-type levels in the complemented *slo2-2* line. Significant enhancement of the levels of alanine, glycine, valine, isoleucine, serine, proline, asparagine, glutamine, ornithine, histidine, and tyrosine were recorded, while the level of aspartic acid was decreased

compared to Col-0 and the complemented lines (Figure 11). These results corroborate the findings on the above-mentioned complex I mutants.

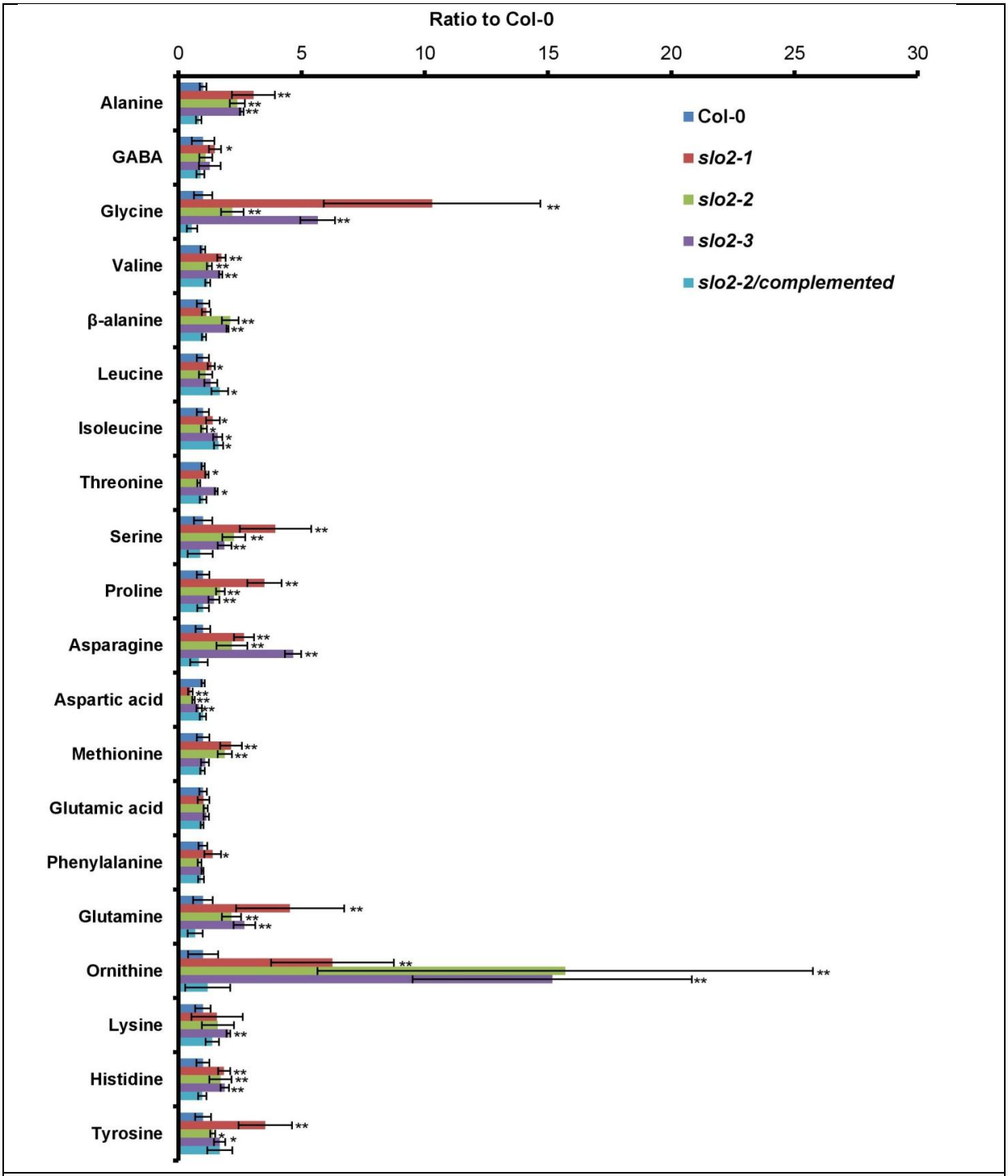

Figure 11. Amino acid profiling.
4-week old plants were used for amino acid extraction. Three biological repeats with 2 technical repeats were performed. Data represent mean values ± SD. *, $p<0.05$; **, $p<0.01$.

6.2.12. Global gene expression changes in *slo2* mutants

To further compare the transcript profiles of the wild-type and *slo2*, we performed an ATH1 microarray analysis (22810 probe sets representing approximately 24000 genes) on

mRNA from rosette leaves of 1-month-old Col-0 and *slo2-3* plants. After normalization of the hybridization signals, and using a threshold fold change>2 and a significance level $p<0.05$ (Rosenwasser *et al.*, 2011), we identified 778 genes with an altered transcription level in the mutant, 216 of which were down-regulated and 562 up-regulated (Supporting Information Table S1, digitally available in Zhu *et al.*, 2013). Thus, mutation in *SLO2* results in extensive changes in gene expression.

We selected 7 differentially expressed genes related to different aspects of mitochondrial metabolism to confirm the reliability of our microarray data by qRT-PCR, including *AT3G22370* (alternative oxidase 1a), *AT1G32350* (alternative oxidase 1D), *AT2G22500* (mitochondrial carrier protein), *AT3G17240* (lipoamide dehydrogenase precursor), *AT4G31810* (mitochondrial Enoyl-CoA Hydratase), *AT4G22490* (lipid transfer protein type 6), *AT4G05020* (NAD(P)H dehydrogenase B2). The results indicate a close correlation between the microarray data and qRT-PCR data (Supplemental Figure 8, digitally available in Zhu *et al.*, 2013).

To discover functionally related patterns, the selected genes were clustered according to their gene ontology classification using AgriGO (Du *et al.*, 2010). Down-regulated genes were mainly related to stress responses, including biotic stress and abiotic stress (osmotic, drought, salt, temperature and wounding stress). Moreover, a large number of these genes are responsive to chemical stimuli, including hormones (abscisic acid, cytokinin and gibberellin) (Supplemental Figure 10A, Supplemental Table 2, digitally available in Zhu *et al.*, 2013). Likewise, of the 562 up-regulated genes, a large number of genes respond to applied stimuli, including: biotic stress (fungus, bacterium, virus, and insect attack); abiotic stress (salt, drought, osmotic, wound, temperature, hypoxia, oxidative, and light stress); and chemical stimuli (ABA, SA, carbohydrate, chitin). In addition, many genes are involved in metabolic processes, such as protein amino acid phosphorylation, SA biosynthesis and metabolism, toxin and indole metabolism, as well as cell wall macromolecule catabolic processes (Supplemental Figure 10B, Supplemental Tables 3 and 4, digitally available in Zhu *et al.*, 2013).

Furthermore, the microarray data provide molecular support for the slow growth phenotype of *slo2*, as well as the involvement of SLO2 in the control of carbon and energy metabolism. We found 44 significantly changed genes involved in plant development, 5 in electron transport, 25 in carbohydrate response, and 6 in light response (Supplemental Table 5, digitally available in Zhu *et al.*, 2013). Finally, based on results derived from the TAIR website, the *slo2* mutation leads to multiple changes in different cell compartments (Supplemental Table 6, digitally available in Zhu *et al.*, 2013).

Previously, four transcripts were identified as hallmarks for general oxidative stress response (Gadjev *et al.*, 2006). These genes are at least 4-fold up-regulated in *slo2* (Supplemental Table 7, digitally available in Zhu *et al.*, 2013), which is in line with the observed H_2O_2 accumulation in *slo2* mutants. However, of the 152 genes proposed to embody the *Arabidopsis* antioxidant network (Mittler *et al.*, 2004; Mittler *et al.*, 2011), only 12 were up-regulated more than 2-fold in *slo2* mutants (Supplemental Table 8, digitally available in Zhu *et al.*, 2013). This indicates that, although *slo2* mutants are suffering from oxidative stress, their anti-oxidative system is not fully activated. This raises the question on the role of complex I in

the oxidative stress response. Altogether, these results show that loss-of-function of *SLO2* leads to global gene expression changes, mainly in stress response and metabolic processes;

6.3 Discussion

In this study, we showed that loss-of-function of *SLO2* results in an altered sensitivity to ABA and ethylene, and altered tolerance to salt and osmotic stresses. Although the functional mechanism remains to be investigated, it can be inferred that SLO2 is required to maintain sensitivity to ABA, ethylene, biotic and abiotic stress.

SLO2 mutation leads to severe changes in 3 complexes of the mETC and an enhanced NADH/NAD+ ratio (Zhu *et al.*, 2012a), thereby contributing to the production of ROS (Figure 10). ROS can damage cellular components, but it can also act as a signal transduction factor (Apel and Hirt, 2004). Under stress conditions, plants activate cascades of stress responsive genes to survive adverse conditions. Our microarray results reveal expression changes in many stress-responsive genes in *slo2* (Supplemental Figure 10, digitally available in Zhu *et al.*, 2013), probably reflecting an internal stress condition. Stress responsive genes can be expressed through ABA-dependent or ABA-independent pathways (Chinnusamy *et al.*, 2004). The ABA biosynthesis inhibitor NF could effectively rescue the stress hypersensitivity of *slo2*, indicating an enhancement of the endogenous ABA level in the mutants (Figure 5), supported by ABA measurements (Supplemental Figure 3, digitally available in Zhu *et al.*, 2013), which could contribute to the induction of ABA and stress-responsive genes (Murayama *et al.*, 2012). We speculate that the impediment of the mETC caused by *SLO2* mutation results in ROS accumulation, leading to an internal stress and concomitant ABA accumulation. In *Arabidopsis*, the NCED family contains 9 genes, of which five (NCED2,3,5,6,9) were reported to play a major role in ABA synthesis (Lefebvre *et al.*, 2006; Tan *et al.*, 2003). Although under normal conditions, the expression of *NCED3* in *slo2* mutants is comparable to Col-0 (Figure 7), other NCED isoforms may contribute to the enhancement of ABA in *slo2* mutants. ABA hypersensitivity was also observed in the PPR mutants *ahg11, pgn* (*pentatricopeptide repeat protein for germination on NaCl*) and *ppr40* (Laluk *et al.*, 2011; Murayama *et al.*, 2012; Zsigmond *et al.*, 2008). However, we cannot rule out the possibility that *SLO2* may be directly involved in ABA biosynthesis or degradation.

As reported for other complex I mutants, such as *ndufa1*, *ndufs4* (Meyer *et al.*, 2009), and *slg1* (Yuan and Liu, 2012), *SLO2* mutation also altered plant response to environmental stress, probably because of changes in the mitochondrial ETC. Our results clearly showed that *slo2* mutants increased tolerance to drought and salt stress compared to Col-0 plants of the same age (Figure 4). However, Col-0 and *slo2* mutant plants of similar size showed comparable responses to drought stress (Supplemental Figure 5, digitally available in Zhu *et al.*, 2013). Thus, the drought tolerance phenotype in *slo2* may reflect differences in the water uptake from soil (lower water use rate), and/or an altered transpiration rate related to the delayed growth, rather than an effective tolerance to water deficit. Similar results were shown in *ndufa1, ndufs4* and *slg1* mutants (Meyer *et al.*, 2009). We speculate that the reduction of stomatal aperture observed in *slo2* (Figure 4B and 4C) is compensated by the increased stomatal density in the mutants (Supplemental Figure 4A, digitally available in Zhu *et al.*, 2013). Water loss rates of wild type and mutants were indeed not significantly different (Supplemental Figure 4B, digitally available in Zhu *et al.*, 2013). Therefore, the altered stress tolerance in *slo2* mutants may mainly derive

from the increased proline content (Figure 11) and perhaps other metabolites. Combined with the microarray and amino acid profiling results (Figures 11and 12), we conclude that activation of stress-responsive genes and thereby metabolic changes, such as increased proline levels mainly contribute to the stress- tolerance phenotype of *slo2* mutants. Further investigations may reveal the possible role of SLO2 on stomatal development and function.

Since SLO2 is localized in mitochondria and involved in RNA metabolism, we checked the expression of mitochondria-related genes. Under normal growth conditions, all components of complex I subunit genes including *NAD1, NAD2, NAD3, NAD4, NAD4L, NAD5, NAD6, NAD7* and *NAD9*, and genes involved in alternative pathways such as *AOX1a, NDB2*, and *NDB4* were higher expressed in *slo2* mutants than in Col-0. Although mutation in *SLO2* leads to a blockage of the mETC, the expression level of *NAD1-9* genes was increased. We speculate that the enhanced mitochondrial transcript levels may result from a feedback effect in response to the blockage of the mETC rather than being a direct consequence of *SLO2* mutation. Since even in the absence of ABA the expression of most of the NAD genes is upregulated in slo2, it is possible that like this the plants are trying to compensate for the lacking functional subunits of the mitochondrial electron transport chain. We can however not rule out, that this higher expression could also be generated by an endogenous higher responsiveness to ABA (as shown in figure 8).

Similar results were observed in other mitochondrial complex I mutants, such as *abo5* and *rug3* (Kühn *et al.*, 2011; Liu *et al.*, 2010).In addition, after ABA treatment, those genes were more strongly induced in *slo2* mutants (Figure 8, Figure 9). Altogether, these results suggest that SLO2 plays a critical role in mitochondrial gene expression.

Several reports from complex I related mutants (such as *CMSII*, *nMat1* and *ndufs4*) suggested that the mitochondrial complex I is necessary for amino acid homeostasis (Dutilleul *et al.*, 2003; Hager *et al.*, 2010; Keren *et al.*, 2012; Meyer *et al.*, 2009). Amino acid profiling in *slo2* confirms this (Figure 11). Our results clearly indicate that *slo2* mutants contain higher levels of alanine, glycine, valine, beta-alanine, isoleucine, serine, proline, asparagine, glutamine, ornithine, histidine, and tyrosine (Figure 11), which play vital roles in nitrogen metabolism and in the photorespiratory cycle (Keys *et al.*, 1978). Under stress conditions, many plant species accumulate high levels of proline, which serves as an adaptive response (Verbruggen and Hermans, 2008), and as an important regulator of cellular ROS balance (Szabados and Savoure, 2010). *slo2* mutants accumulate proline to levels 2-4 times higher than in the wild-type, which may contribute to their increased tolerance to salt and drought stress. In photosynthetic tissues, serine and glycine are synthesized through the photorespiration pathway (Somerville and Somerville, 1983). Despite the fact that several complex I related mutants disrupt amino acid homeostasis (Keren *et al.*, 2012; Nakagawa and Sakurai, 2006), glycine and serine do not show substantial changes, indicating that disruption of complex I is not sufficient to explain the increase of glycine and serine observed in *slo2*. The increased level of these two amino acids could indicate that mutation of *SLO2* results in a higher photorespiration rate, which was experimentally supported (Supplemental Figure 9, digitally available in Zhu *et al.*, 2013). However, its function in this process remains to be investigated.

Up to now, a few PPR proteins were reported to be involved in stress responses, such as *ABA overly-sensitive 5* (*ABO5*), *genomes uncoupled 1* (*GUN1*), *lovastatin insensitive 1* (*LOI1*), *PPR protein like* (*PPRL*), *PGN*, *SLG1*, *ahg11* and *PPR40* (Katiyar-Agarwal *et al.*, 2006;

Koussevitzky *et al.*, 2007; Laluk *et al.*, 2011; Liu *et al.*, 2010; Murayama *et al.*, 2012; Tang *et al.*, 2010; Yuan and Liu, 2012; Zsigmond *et al.*, 2008). Of these PPR proteins, only *SLG1* and *AHG11* act as an RNA editing factor and are involved in ABA and abiotic stress responses (Murayama *et al.*, 2012). *slo2* and *slg1* mutants share many similarities, such as slow growth and development, RNA editing changes, lowered levels of mitochondrial complex I, and changes in response to ABA and abiotic stress tolerance. However, *slo2* on one hand, and *slg1* and *ahg11* on the other hand, also show striking differences. First, mutation of *SLO2* affects multiple editing sites, while *slg1* and *ahg11* are affecting editing of single sites, in respectively *NAD3* and *NAD4* (Murayama *et al.*, 2012; Yuan and Liu, 2012). Second, mutation in *SLO2* does not only cause complex I but also complex III and IV defects, indicating the unique role of SLO2 (Yuan and Liu, 2012; Zhu *et al.*, 2012a). Third, *slo2* mutants also exhibited insensitivity to ethylene, and increased susceptibility to *B. cinerea* (Figure 1 and Figure 6). Moreover, mutation of *AHG11* does not result in a visible phenotype under normal growth conditions, and its function is restricted to seed germination and seedling growth (Murayama *et al.*, 2012), while SLO2 is necessary for normal plant growth and stress responses at all developmental stages. Together with the enhanced ROS levels in *slo2* mutants (Figure 10A and 10B), these findings indicate that SLO2 has a broader function than SLG1 and AHG11. Our data suggest that SLO2 influences ROS, ABA and ethylene pathways. In summary, although its functional mechanism is still unknown, SLO2 appears to play a unique role in plant stress response. As for *slg1*and *ahg11*, our study suggests a link between mitochondrial RNA editing factors and plant responses to biotic and abiotic stress and further supports the view that compromised mitochondrial functioning resulting from defective mitochondrial RNA editing alters plant stress responses.

In most cases, blocking the mitochondrial electron chain leads to improved stress tolerance, such as previously reported for complex I mutants *slg1*, *ndufs4*, *atcib22* (B22 subunit of *Arabidopsis* complex I) (Han *et al.*, 2010; Meyer *et al.*, 2009; Yuan and Liu, 2012). However, some complex I mutants showed hypersensitivity to stress conditions, such as lowered heat stress tolerance in *css1*(*changed sensitivity to cellulose synthesis inhibitors 1*) (Nakagawa and Sakurai, 2006), and increased sensitivity to cold and osmotic stress in *fro1*(*frostbite1*) mutants (Lee *et al.*, 2002). *slo2* mutants are more tolerant to abiotic stress (Figure 4) while being hypersensitive to biotic stress (Figure 6). Since our microarray data indicate that the anti-oxidative stress system is not fully activated in *slo2* (Supplemental Table S8, digitally available in Zhu *et al.*, 2013), the altered stress tolerance may not be directly related to changes in ROS metabolism. It is possible that ROS acts as a signal molecule that triggers a more complex stress-related gene expression network, thereby resulting in differences in stress tolerance.

In summary, *SLO2* mutation leads to an impediment of the mETC, resulting in accumulation of H_2O_2, causing alterations in biotic and abiotic stress response. Although it is clear that SLO2 is required for normal stress response, we cannot rule out the possibility that SLO2 is involved in the regulation thereof. Our future work will aim at the identification of binding partners of SLO2, such as specific RNA or proteins, in order to advance our understanding of its molecular function. This may further shed light on the involvement of PPR proteins in plant stress response.

6.4 Experimental procedures

6.4.1. Plant growth and treatments

slo2 alleles and complemented lines were described in Zhu *et al.*, (2012a). Seed germination and plant growth were performed as reported previously (Zhu *et al.*, 2012a). Seeds were stratified for 2 days at 4 °C. Growth conditions were a 16 h light/8 h dark photoperiod, white fluorescence light (75 μM m^{-2} sec^{-1}), and 21°C. Plants on soil were grown in a growth chamber at 22°C. ABA and abiotic stress treatments were performed as described previously (Pandey *et al.*, 2005). 4-week-old plants were used for drought and salt treatments, for drought treatments, watering was stopped for 10-14 days; for salt treatments, plants were watered with increasing concentrations of NaCl of 100, 200 and 300 mM at 3-day intervals over 12 days (Chen *et al.*, 2012).

6.4.2. Germination assays

Germination time was obtained by daily monitoring of the percentage of germination of a population of around 100 seeds as described previously (Pandey *et al.*, 2005). The vertical growth assays were performed in a similar manner. Plant growth was monitored and photographed after 7 days.

6.4.3. Quantitative RT-PCR analysis

RNA extraction and qRT-PCR analysis were performed as described (Zhu *et al.*, 2012). Briefly, RNA was extracted using the RNeasy Mini Kit (Qiagen company), then purified using a DNA-free RNA kit (Zymo Research, Orange, CA) according to manufacturers' protocol. qRT-PCR was performed using a Cybergreen fluorescence-based assay kit according to the manufacturer's instructions. PCR reactions were performed on a Rotor Gene 6, Corbett or Bio-rad MyiQ™2 Two-Color Real-Time PCR Detection System. Two to three biological repeats were performed. The primers are listed in Supporting Information Table S11 (digitally available in Zhu *et al.*, 2013).

6.4.4. ABA determinations

ABA quantification on 5 mg seeds was done as previously described (Prinsen *et al.*, 1995).

6.4.5. Expression pattern analysis

In situ hybridization were performed according to the protocol described previously (Brewer *et al.*, 2006). The *pSLO2:GUS* transgenic line was described previously (Zhu *et al.*, 2012a). Histochemical staining for GUS activity was performed as described previously (Jefferson *et al.*, 1987).

6.4.6. Photorespiration measurements

Gas exchange analysis was made using an LC-pro+ portable photosynthesis system (ADC BioScientific Limited). Calculations were made based on the method described by (Parsons *et al.*, 1997). Photorespiration was analyzed by measuring the post-illumination CO_2 burst (PIB) under photorespiratory conditions as described in (Kebeish *et al.*, 2007). Plants were put under high light and the net CO_2-flux was measured. When the CO_2-flux was stable the light was switched off and the measurement continued. The peak that is measured after switching

to darkness, is an indicator of the rate of CO_2 produced during photorespiration before darkening since the intermediates for the photorespiratory cycle are produced during illumination (Cousins *et al.*, 2008; Hoefnagel *et al.*, 1998). The photorespiration rates were expressed on the basis of rosette surface area. The rosette area was determined using a standardized area analysis in ImageJ of grayscale digital images.

6.4.7. Chlorophyll and stomatal aperture measurements

Chlorophyll was measured according to an established protocol (Lichtenthaler, 1987). Stomatal aperture was measured with ImageJ software on images taken on a stereo microscope (Stemi SV11, Carl Zeiss Inc.) with an Olympus C5050 camera.

6.4.8. ROS detection

The 7^{th} and 8^{th} leaf of 4-weeks-old mutants and Col-0 were used for DCFH-DA staining as described previously (Lorrain *et al.*, 2004). Fluorescence was observed using a fluorescence microscope Zeiss Axiovert 200. For quantitative measurement of H_2O_2, rosette leaves from 3-4 week old plants were extracted, and H_2O_2 content was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen) following the manufacturer's instructions. Two independent biological experiments with triple technical repeats were performed. For NBT staining, leaves were incubated with 0.5 mg/ml NBT prepared in 10 mM potassium phosphate buffer pH 7.8 for 3 h, and subsequently treated with 90% ethanol at 65 °C for 15 min to remove chlorophyll (Ramel *et al.*, 2009). For DAB staining, leaves were incubated in 1 mg/ml DAB solution, pH 3.8 as described previously (Ramel *et al.*, 2009), chlorophyll was removed as mentioned for NBT.

6.4.9. *Botrytis* infection

Four-week-old soil-grown *A. thaliana* plants were sprayed with a suspension of $1x10^4$ or $5x10^7$ *Botrytis cinerea* spores/mL in 12 g/L potato dextrose broth (PDB; BD Biosciences, San Jose, California, USA), until droplet run-off (De Coninck *et al.*, 2010). Disease symptoms were scored visually by determining the percentage infected leaves at several days post inoculation.

6.4.10. Microarray analysis

Rosette leaves from wild-type and *slo2-3* plants grown under long-day conditions for 4 weeks were used for RNA isolation. Total RNA was isolated using an RNeasy Mini Plant Kit, quality control tests were performed prior to hybridization. *Arabidopsis* ATH1 genome arrays were performed at NASC's International Affymetrix facility and scanned according to the manufacturer's protocol. Three replicates were run for each sample to ensure experimental reliability.

CEL files were provided by NASC. Raw data were normalized by the RMA method using RMAexpress software, and all data were processed using TMEV software (Saeed *et al.*, 2006). Fold change and associated *p* value of each gene were obtained; $p \leq 0.05$ and at least 2-fold change were used as cut-offs to identify the statistically significant up- and down-regulated genes as described previously (Lin *et al.*, 2011). Selected genes were used for further annotation analysis. The functional annotation analysis was done using the TAIR GO annotation (http://www.*Arabidopsis*.org/tools/bulk/go/), and AgriGO (Du *et al.*, 2010).

6.4.11. Amino acid analysis

Freeze-dried rosette leaves from 4-week old plants were used for amino acid extraction, derivatization and analysis, according to the methods described in Baker *et al.* (2006), except that each sample consisted of 15mg of dry material.

6.4.12. Accession number

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank databases under the following accession number: *SLO2* (At2g13600).

6.5 Acknowledgements

The authors thank the Research Foundation Flanders (project G.0313.05 granted to DVDS) and Ghent University for financial support. J.D. is indebted to the IWT for a predoctoral fellowship. B.D.C is indebted to FWO-Vlaanderen for a postdoctoral fellowship (FWO/12A7213N).The authors also wish to acknowledge Jindrich Peiren and Daniëlle Janssens from the Laboratory of Microbiology of Ghent University (LM-UGent), for their help in freeze-drying the samples for amino acid analysis. The amino acid analysis was kindly performed by the MeT-RO metabolomics service at Rothamsted Research (UK).

6.6 References

Apel, K., and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**, 373-399.

Audenaert, K., De Meyer, G.B., and Hofte, M.M. (2002) Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol **128**, 491-501.

Baena-Gonzalez, E., and Sheen, J. (2008) Convergent energy and stress signaling. Trends Plant Sci **13**:474-482.

Baker, J.M., Hawkins, N.D., Ward, J.L., Lovegrove, A., Napier, J.A., Shewry, P.R., and Beale, M.H. (2006) A metabolomic study of substantial equivalence of field-grown genetically modified wheat. Plant Biotechnol J **4**:381-392.

Barkan, A., Rojas, M., Fujii, S., Yap, A., Chong, Y.S., Bond, C.S., and Small, I. (2012) A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. PLoS Genet **8**, e1002910.

Brewer, P.B., Heisler, M.G., Hejatko, J., Friml, J., and Benkova, E. (2006) In situ hybridization for mRNA detection in *Arabidopsis* tissue sections. Nat Protoc **1**, 1462-1467.

Chen, J.H., Jiang, H.W., Hsieh, E.J., Chen, H.Y., Chien, C.T., Hsieh, H.L., and Lin, T.P. (2012) Drought and salt stress tolerance of an *Arabidopsis* glutathione S-transferase U17 knockout mutant are attributed to the combined effect of glutathione and abscisic acid. Plant physiology **158**, 340-351.

Chinnusamy, V., Schumaker, K., and Zhu, J.K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot **55**, 225-236.

Cousins, A.B., Pracharoenwattana, I., Zhou, W., Smith, S.M., and Badger, M.R. (2008) Peroxisomal malate dehydrogenase is not essential for photorespiration in *Arabidopsis* but its absence causes an increase in the stoichiometry of photorespiratory CO2 release. Plant Physiol **148**, 786-795.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol **61**, 651-679.

De Coninck, B.M.A., Sels, J., Venmans, E., Thys, W., Goderis, I.J.W.M., Carron, D., Delauré, S.L., Cammue, B.P.A., De Bolle, M.F.C., and Mathys, J. (2010) *Arabidopsis thaliana* plant defensin AtPDF1.1 is involved in the plant response to biotic stress. New Phytologist **187**, 1075-1088.

Dekkers, B.J., Schuurmans, J.A., and Smeekens, S.C. (2008) Interaction between sugar and abscisic acid signalling during early seedling development in *Arabidopsis*. Plant Mol Biol **67**, 151-167.

D**u, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z.** (2010) agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Research **38**, W64-70.

Dutilleul, C., Driscoll, S., Cornic, G., De Paepe, R., Foyer, C.H., and Noctor, G. (2003) Functional mitochondrial complex I is required by tobacco leaves for optimal photosynthetic performance in photorespiratory conditions and during transients. Plant Physiol **131**, 264-275.

Fan, J., Hill, L., Crooks, C., Doerner, P., and Lamb, C. (2009) Abscisic acid has a key role in modulating diverse plant-pathogen interactions. Plant Physiol **150**, 1750-1761.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inze, D., Mittler, R., and Van Breusegem, F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in *Arabidopsis*. Plant Physiol **141**, 436-445.

Gazzarrini, S., and McCourt, P. (2001) Genetic interactions between ABA, ethylene and sugar signaling pathways. Current Opinion in Plant Biology **4**, 387-391.

Giraud, E., Van Aken, O., Ho, L.H., and Whelan, J. (2009) The transcription factor ABI4 is a regulator of mitochondrial retrograde expression of ALTERNATIVE OXIDASE1a. Plant Physiol **150**, 1286-1296.

Hager, J., Pellny, T.K., Mauve, C., Lelarge-Trouverie, C., De Paepe, R., Foyer, C.H., and Noctor, G. (2010) Conditional modulation of NAD levels and metabolite profiles in Nicotiana sylvestris by mitochondrial electron transport and carbon/nitrogen supply. Planta **231**, 1145-1157.

Han, L., Qin, G., Kang, D., Chen, Z., Gu, H., and Qu, L.J. (2010) A nuclear-encoded mitochondrial gene AtCIB22 is essential for plant development in *Arabidopsis*. Journal of genetics and genomics = Yi chuan xue bao 37:667-683.

Hoefnagel, M.H.N., Atkin, O.K., and Wiskich, J.T. (1998) Interdependence between chloroplasts and mitochondria in the light and the dark. biochim Biophys Acta **1366**, 235-255.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinformatics 2008, 420747.

Jaspers, P., and Kangasjarvi, J. (2010) Reactive oxygen species in abiotic stress signaling. Physiol Plant 138, 405-413.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6**, 3901-3907.

Juszczuk, I.M., Szal, B., and Rychter, A.M. (2012) Oxidation-reduction and reactive oxygen species homeostasis in mutant plants with respiratory chain complex I dysfunction. Plant Cell Environ **35,** 296-307.

Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Jr., Zhu, J.K., Staskawicz, B.J., and Jin, H. (2006) A pathogen-inducible endogenous siRNA in plant immunity. Proc Natl Acad Sci U S A **103**, 18002-18007.

Kebeish, R., Niessen, M., Thiruveedhi, K., Bari, R., Hirsch, H.J., Rosenkranz, R., Stabler, N., Schonfeld, B., Kreuzaler, F., and Peterhansel, C. (2007) Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in *Arabidopsis thaliana*. Nat Biotechnol **25**, 593-599.

Keren, I., Tal, L., des Francs-Small, C.C., Araujo, W.L., Shevtsov, S., Shaya, F., Fernie, A.R., Small, I., and Ostersetzer-Biran, O. (2012) nMAT1, a nuclear-encoded maturase involved in the trans-splicing of nad1 intron 1, is essential for mitochondrial complex I assembly and function. Plant J **71**, 413-426.

Keys, A.J., Bird, I.F., Cornelius, M.J., Lea, P.J., Wallsgrove, R.M., and Miflin, B.J. (1978) Photorespiratory nitrogen cycle. Nature **275**, 741-743.

Knight, L.I., and Crocker, W. (1913*)* Toxicity of Smoke. Botanical Gazette **55**, 337-371.

Kotera, E., Tasaka, M., and Shikanai, T. (2005*)* A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature **433**, 326-330.

Koussevitzky, S., Nott, A., Mockler, T.C., Hong, F., Sachetto-Martins, G., Surpin, M., Lim, J., Mittler, R., and Chory, J. (2007*)* Signals from chloroplasts converge to regulate nuclear gene expression. Science **316**, 715-719.

Kühn, K., Carrie, C., Giraud, E., Wang, Y., Meyer, E.H., Narsai, R., des Francs-Small, C.C., Zhang, B., Murcha, M.W., and Whelan, J. (2011*)* The RCC1 family protein RUG3 is required for splicing of nad2 and complex I biogenesis in mitochondria of *Arabidopsis thaliana*. Plant J **67**, 1067-1080.

Laluk, K., Abuqamar, S., and Mengiste, T. (2011*)* The *Arabidopsis* Mitochondria-Localized Pentatricopeptide Repeat Protein PGN Functions in Defense against Necrotrophic Fungi and Abiotic Stress Tolerance. Plant Physiol **156**, 2053-2068.

Lee, B.H., Lee, H., Xiong, L., and Zhu, J.K. (2002*)* A mitochondrial complex I defect impairs cold-regulated nuclear gene expression. Plant Cell **14**, 1235-1251.

Lee, S.C., and Luan, S. (2012*)* ABA signal transduction at the crossroad of biotic and abiotic stress responses. Plant, Cell & Environment **35**, 53-60.

Lichtenthaler, H.K. (1987*)* Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In: Methods in Enzymology--Lester Packer, R.D., ed.: Academic Press. 350-382.

Lin, P.C., Pomeranz, M.C., Jikumaru, Y., Kang, S.G., Hah, C., Fujioka, S., Kamiya, Y., and Jang, J.C. (2011*)* The *Arabidopsis* tandem zinc finger protein AtTZF1 affects ABA- and GA-mediated growth, stress and gene expression responses. The Plant journal **65**, 253-268.

Liu, Y., He, J., Chen, Z., Ren, X., Hong, X., and Gong, Z. (2010*)* ABA overly-sensitive 5 (ABO5), encoding a pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3, is involved in the abscisic acid response in *Arabidopsis*. Plant J **63**, 749-765.

Lorrain, S., Lin, B., Auriac, M.C., Kroj, T., Saindrenan, P., Nicole, M., Balague, C., and Roby, D. (2004) Vascular associated death1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. Plant Cell **16**, 2217-2232.

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., *et al.* (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16**, 2089-2103.

Mauch-Mani, B., and Mauch, F. (2005) The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol **8**, 409-414.

Meyer, E.H., Tomaz, T., Carroll, A.J., Estavillo, G., Delannoy, E., Tanz, S.K., Small, I.D., Pogson, B.J., and Millar, A.H. (2009) Remodeled respiration in ndufs4 with low phosphorylation efficiency suppresses *Arabidopsis* germination and growth and alters control of metabolism at night. Plant Physiol **151**, 603-619.

Michalecka, A.M., Svensson, A.S., Johansson, F.I., Agius, S.C., Johanson, U., Brennicke, A., Binder, S., and Rasmusson, A.G. (2003) *Arabidopsis* genes encoding mitochondrial type II NAD(P)H dehydrogenases have different evolutionary origin and show distinct responses to light. Plant Physiol **133**, 642-652.

Millar, A.H., Sweetlove, L.J., Giegé, P., and Leaver, C.J. (2001) Analysis of the *Arabidopsis* mitochondrial proteome. Plant Physiol **127**, 1711-1727.

Millar, A.H., Whelan, J., Soole, K.L., and Day, D.A. (2011) Organization and regulation of mitochondrial respiration in plants. Annu Rev Plant Biol **62**, 79-104.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9**, 490-498.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V., and Van Breusegem, F. (2011) ROS signaling: the new wave? Trends Plant Sci **16,** 300-309.

Moller, I.M. (2001) Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu Rev Plant Physiol Plant Mol Biol **52**, 561-591.

Murayama, M., Hayashi, S., Nishimura, N., Ishide, M., Kobayashi, K., Yagi, Y., Asami, T., Nakamura, T., Shinozaki, K., and Hirayama, T. (2012) Isolation of *Arabidopsis* ahg11, a weak ABA hypersensitive mutant defective in nad4 RNA editing. J Exp Bot **21**, 21.

Nakagawa, N., and Sakurai, N. (2006) A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II intron maturase, causes impaired splicing of mitochondrial NAD4 transcript and altered carbon metabolism in *Arabidopsis thaliana*. Plant Cell Physiol **47**, 772-783.

Noctor, G., De Paepe, R., and Foyer, C.H. (2007) Mitochondrial redox biology and homeostasis in plants. Trends Plant Sci **12**, 125-134.

Okuda, K., and Shikanai, T. (2012) A pentatricopeptide repeat protein acts as a site-specificity factor at multiple RNA editing sites with unrelated cis-acting elements in plastids. Nucleic Acids Research.

Pandey, G.K., Grant, J.J., Cheong, Y.H., Kim, B.G., Li, L., and Luan, S. (2005) ABR1, an APETALA2-domain transcription factor that functions as a repressor of ABA response in *Arabidopsis*. Plant Physiol **139**, 1185-1193.

Parsons, R., Weyers, J.D.B., Lawson, T., and Godber, I.M. (1997) Rapid and straightforward estimates of photosynthetic characteristics using a portable gas exchange system Photosynthetica **34**, 265-279.

Prinsen, E., Redig, P., Strnad, M., Galis, I., Van Dongen, W., and Van Onckelen, H. (1995) Quantifying phytohormones in transformed plants. Methods Mol Biol **44**, 245-262.

Ramel, F., Sulmon, C., Bogard, M., Couee, I., and Gouesbet, G. (2009) Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. BMC Plant Biol **9**, 28.

Rasmusson, A.G., Heiser, V.V., Zabaleta, E., Brennicke, A., and Grohmann, L. (1998) Physiological, biochemical and molecular aspects of mitochondrial complex I in plants. Biochim Biophys Acta **1364**, 101-111.

Rasmusson, A.G., Soole, K.L., and Elthon, T.E. (2004) Alternative NAD(P)H dehydrogenases of plant mitochondria. Annu Rev Plant Biol **55**, 23-39.

Rosenwasser, S., Rot, I., Sollner, E., Meyer, A.J., Smith, Y., Leviatan, N., Fluhr, R., and Friedman, H. (2011) Organelles Contribute Differentially to ROS-Related Events during Extended Darkness. Plant Physiol **156**, 185-201.

Saeed, A.I., Bhagabati, N.K., Braisted, J.C., Liang, W., Sharov, V., Howe, E.A., Li, J., Thiagarajan, M., White, J.A., and Quackenbush, J. (2006) TM4 microarray software suite. Methods Enzymol **411**, 134-193.

Saha, D., Prasad, A.M., and Srinivasan, R. (2007) Pentatricopeptide repeat proteins and their emerging roles in plants. Plant Physiol Biochem **45**, 521-534.

Schmitz-Linneweber, C., and Small, I. (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci **13**, 663-670.

Shikanai, T. (2006) RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol Life Sci **63**, 698-708.

Somerville, S.C., and Somerville, C.R. (1983) Effect of Oxygen and Carbon Dioxide on Photorespiratory Flux Determined from Glycine Accumulation in a Mutant of *Arabidopsis thaliana*. Journal of Experimental Botany **34**, 415-424.

S**tern, D.B., Goldschmidt-Clermont, M., and Hanson, M.R.** (2010) Chloroplast RNA metabolism. Annu Rev Plant Biol **61**, 125-155.

Sung, T.Y., Tseng, C.C., and Hsieh, M.H. (2010) The SLO1 PPR protein is required for RNA editing at multiple sites with similar upstream sequences in *Arabidopsis* mitochondria. Plant J **63**, 499-511.

Szabados, L., and Savoure, A. (2010) Proline: a multifunctional amino acid. Trends Plant Sci **15**, 89-97.

Takenaka, M. (2010) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the nad7 transcript in mitochondria of *Arabidopsis*. Plant Physiol **152**, 939-947.

Takenaka, M., Verbitskiy, D., Zehrmann, A., and Brennicke, A. (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. J Biol Chem **285**, 27122-27129.

Tang, J., Kobayashi, K., Suzuki, M., Matsumoto, S., and Muranaka, T. (2010) The mitochondrial PPR protein LOVASTATIN INSENSITIVE 1 plays regulatory roles in cytosolic and plastidial isoprenoid biosynthesis through RNA editing. Plant J **61**, 456-466.

Timmis, J.N., Ayliffe, M.A., Huang, C.Y., and Martin, W. (2004) Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. Nat Rev Genet **5**, 123-135.

Verbitskiy, D., Hartel, B., Zehrmann, A., Brennicke, A., and Takenaka, M. (2011) The DYW-E-PPR protein MEF14 is required for RNA editing at site matR-1895 in mitochondria of *Arabidopsis thaliana*. FEBS Lett **585**, 700-704.

Verbitskiy, D., Zehrmann, A., van der Merwe, J.A., Brennicke, A., and Takenaka, M. (2010) The PPR protein encoded by the LOVASTATIN INSENSITIVE 1 gene is involved in RNA editing at three sites in mitochondria of *Arabidopsis thaliana*. Plant J **61**, 446-455.

Verbruggen, N., and Hermans, C. (2008) Proline accumulation in plants: a review. Amino Acids **35**, 753-759.

Xiong, L., Gong, Z., Rock, C.D., Subramanian, S., Guo, Y., Xu, W., Galbraith, D., and Zhu, J.K. (2001a) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in *Arabidopsis*. Dev Cell **1**, 771-781.

Xiong, L., Lee, B., Ishitani, M., Lee, H., Zhang, C., and Zhu, J.K. (2001b) FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. Genes & development **15**, 1971-1984.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci **10**, 88-94.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57**, 781-803.

Yuan, H., and Liu, D. (2012) Functional disruption of the PPR protein SLG1 affects mitochondrial RNA editing, plant development, and responses to abiotic stresses in *Arabidopsis*. Plant J **70**, 432-444.

Zehrmann, A., van der Merwe, J., Verbitskiy, D., Hartel, B., Brennicke, A., and Takenaka, M. (2012) The DYW-class PPR protein MEF7 is required for RNA editing at four sites in mitochondria of *Arabidopsis thaliana*. RNA Biol **9**, 156 - 162.

Zehrmann, A., Verbitskiy, D., Hartel, B., Brennicke, A., and Takenaka, M. (2010) RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. FEBS Lett **584**, 4181-4186.

Zehrmann, A., Verbitskiy, D., van der Merwe, J.A., Brennicke, A., and Takenaka, M. (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell **21**, 558-567.

Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53**, 247-273.

Zhu, Q., Dugardeyn, J., Zhang, C., Takenaka, M., Kuhn, K., Craddock, C., Smalle, J., Karampelias, M., Denecke, J., Peters, J., *et al.* (2012a) SLO2, a mitochondrial PPR protein affecting several RNA editing sites, is required for energy metabolism. The Plant journal **71**, 836-849.

Zhu, Q., E, H.M., and Van Der Straeten, D. (2012b) Functional analysis of SLO2 provides new insight into the role of plant PPR proteins. Plant Signal Behav **7**, 1209-1211.

Zhu, Q., Dugardeyn, J. Zhang, C., Mühlenbock, P., Valcke, R., De Coninck, B., Oden, S., Karampelias, M., Cammue, B., Prinsen, E., and Van Der Straeten, D. (2013) The *Arabidopsis* PPR RNA editing factor SLO2 participates in multiple stress and hormone responses. Molecular Plant, **accepted for publication**.

Zsigmond, L., Rigo, G., Szarka, A., Szekely, G., Otvos, K., Darula, Z., Medzihradszky, K.F., Koncz, C., Koncz, Z., and Szabados, L. (2008) *Arabidopsis* PPR40 connects abiotic stress responses to mitochondrial electron transport. Plant Physiol **146**, 1721-1737.

Chapter 7:

Conclusions and Perspectives

7.1 Summary of the *slo2* phenotypes

slo2 mutants display a myriad of phenotypes, which are summarized in Table 1.

Phenotypes of *slo2*			
General Phenotype	Germination	*slo2* germinates later than WT (average of 2-3 days delay)	5.2.1
	Cotyledon Opening	after germination, in mutants, cotyledon opening is delayed by one additional day	5.2.1
	Rosette Leaves at Bolting	bolting of slo2 is delayed compared to WT resulting in a bigger number of rosette leaves at bolting	5.2.1
		followed by a delayed senescence of the rosette leaves	
	Rosette Size	rosette size of mutants are smaller than those of WT	5.2.2
Response to Sugar	Germination	in the absence of sucrose, slo2 shows a severe growth arrest	5.2.6
		in the presence of 1% sucrose germination of slo2 is enhanced more strongly than that of WT	
	Post-embryonic growth	in the presence of 7% sucrose, germination of slo2 is more strongly inhibited than that of WT	5.2.6
	SLO2 Expression	SLO2 expression is enhanced by 1% sucrose	5.2.6
	Gene Expression	Expression of *RBCS* and *PC* is more strongly reduced by 7% sucrose than WT (compared to 1% sucrose)	5.2.6
		Sugar-induced expression of *CHS* is abolished in the *slo2*	
importance of Carbon Status	CO_2-Fertilization	CO_2-fertilization (partially) reduces the general phenotype of *slo2*	5.2.7
	External Carbon Source	*slo2* mutants can not survive in the absence of sucrose in MS/2 medium	5.2.7
	high light dose	an increase of light dose or light period reduces the general phenotype of *slo2*	5.2.7
Response to Ethylene	DLE	reduced DLE on Low Nutrient Medium	5.2.1
	Triple Respons	root and hypocotyl growth inhibition less pronounced than WT when treated with ACC	6.2.3
		normal exaggeration of the hook	
	Leaf Expansion	Cotyledon and leaf expansion is less reduced by ACC or ethylene than WT	6.2.3
	Root Growth	root growth inhibtion in light conditions by ACC is less pronounced in the mutant	6.2.3
	Shoot Growth	shoot growth inhibtion in light conditions by ACC is less pronounced in the mutant	6.2.3
Response to ABA	Germination	germination on ABA is further delayed in *slo2* than in WT	6.2.4
	Seedling Development	ABA induced delay of seedling development is stronger in the mutant	6.2.4
	Root Growth	root elongation is more reduced in ABA treated mutants than in WT	6.2.4
	ABA levels	*slo2* mutants contain higher endogenous ABA levels	6.2.4
	Gene Expression	*slo2* has an enhanced expression of ABA and stress related genes in response to ABA treamtent	6.2.8
Abiotic Stress	Root Growth	inhibition of root elongation is stronger in NaCl or sorbitol treated mutants than in WT	6.2.5
	Shoot Growth	inhibition of shoot elongation is stronger in NaCl or sorbitol treated mutants than in WT	6.2.5
	Germination	germination of mutants is much more inhibited on medium containing NaCl or sorbitol than WT	6.2.5
	Drought Tolerance	adult *slo2* mutants are more tolerant to drought stress (and salt stress)	6.2.5
	Stomata	in normal growing conditions, adult *slo2* mutants have more stomata, but with a smaller aperture	6.2.5

Phenotypes of *slo2*			
Biotic Stress	*Botrytis cinerea*	*slo2* mutants show a higher % infected leaves and an increased necrosis and leaf decomposition	6.2.7
Respiration Pathway	Editing Defects	editing of nad4L-110, mttB-144, mttB-145, mttB-666, nad1-2, nad1-40 and nad7-739 is disturbed in *slo2*	5.2.8
	Protein Levels	levels of complex I, complex III and complex IV of the mETC are reduced in *slo2*	5.2.9
	Gene Expression	alternative respiratory pathway transcripts are up-regulated in slo2 (in response to ABA)	6.2.9
	ATP/ADP	levels of ATP are reduced in *slo2,* as are levels of adp (but to a smaller extent)	5.2.10
	NADH/NAD	levels of NADH are higher in *slo2,* levels of NAD reduced	5.2.10
Oxydative Stress	Reactive Oxygen Species	mutants contain higher levels of H_2O_2 and O_2^-	6.2.10
Amino Acids	AA Homeostasis	*slo2* contains higher levels of alanine, glycine, valine, isoleucine, serine, proline, asparagine, glutamine, ornithine, histidine, and tyrosine	6.2.11
		slo2 contains lower levels of aspartic acid	
Global Gene Expression	Gene Regulation	in mutants, 562 genes are up-regulated and 216 genes are down-regulated compared to WT	6.2.12

Table 1: Phenotypes of *slo2*

7.2 SLO2, a mitochondrial PPR protein, plays a vital role in energy metabolism

The aim of this thesis was to characterize the function of a novel mitochondrial PPR protein, SLO2 which is a mitochondrial PLS-E+ type PPR protein (Zhu *et al.*, 2012). *slo2* loss-of-function mutants are characterized by retarded leaf emergence, restricted root growth, and late flowering. This phenotype is strongly enhanced in the absence of sucrose, with inability of *slo2* mutants to complete their life cycle, suggesting a defect in energy metabolism. The *slo2* growth retardation phenotypes are largely suppressed by supplying sugars, but also by increasing light dosage, or the concentration of CO_2. A first contribution to understanding SLO2 function was the identification of RNA editing defects in *slo2* mutants, some of which result in amino acid changes in subunits of complex I of the mitochondrial electron transport chain (mETC). Both complex I abundance and activity are highly reduced in the *slo2* mutants, as well as the abundance of complexes III and IV. Moreover, ATP, NAD+, and sugar contents are much lower in *slo2* as compared to the WT. We propose that SLO2 (directly or indirectly) modulates carbon and energy balance, by maintaining the abundance and/or activity of complexes I, III and IV of the mETC, thereby playing an essential role in a plant's life cycle under certain growth conditions, possibly in interaction with stress factors.

7.3 A mutation in SLO2 has an effect in multiple (stress) responses

The *slo2* mutant was identified in a screening for ethylene-insensitivity of *Arabidopsis* seedlings grown in the light (Zhu *et al.*, 2012). Since ethylene has effects which are

antagonistic to ABA, a well-known stress related hormone, SLO2 has the potential to participate in plant stress response. Our investigations support this hypothesis. We showed that mutation in *SLO2* causes hypersensitivity to ABA, salt, osmotic stress, *Botrytis cinerea* infection and insensitivity to ethylene. In addition, adult plants showed an increased drought and salt tolerance. SLO2 is necessary for the expression of nuclear encoded stress-responsive genes, as well as mitochondrial encoded *NAD* genes of complex I and genes of the alternative respiratory pathway both under normal conditions and upon ABA treatment. In addition, H_2O_2 accumulation and higher photorespiration levels were recorded in *slo2* mutants. A transcriptome analysis of *slo2* provided evidence for gene expression alterations that correlated with its putative function in mitochondria, development and in stress. We conclude that SLO2 is required for plant sensitivity to ABA, ethylene, biotic and abiotic stress. Overall, this study supports a putative link between mitochondrial RNA editing events and stress response.

7.4 SLO2 could be part of a protein complex

Although much progress has been made, a number of questions need to be further addressed to reveal the functional mechanism of SLO2. The following considerations can be made to elucidate this mechanism. First and foremost, several lines of evidence suggest that SLO2 needs a partner to fulfill its molecular function. SLO2 is involved in RNA editing in mitochondria, regulating editing of components of the mETC (Zhu *et al.*, 2012). SLO2 however does not carry a catalytic deaminating region since it lacks a DYW domain. It was proposed that exactly this DYW domain of PPR proteins, containing a conserved Zn-binding domain which confers cytidine deaminase activity, is responsible for the C-to-U RNA editing events (Salone *et al.*, 2007). Hence, SLO2 is expected to form a complex(es) with a protein(s) which carries (carry) the catalytic activity. Hence, we hypothesize that the actual editing activity is conferred by another PPR protein, binding to SLO2. This has already been shown for DYW1 This interactor of CCR4 containins a DYW motif, but no PPR motifs and has been shown to be necessary for CCR4 induced editing of ndhD-1 site (Boussardon *et al.*, 2012). Secondly, we showed that SLO2 is involved in plant stress response; however, whether its involvement is direct or indirect remains unknown. Analysis of its putative binding-partner (s) will provide more information of the role of SLO2 in this process. Thirdly, SLO2 is a key regulator in plant growth and development, probably through controlling mitochondrial energy metabolism of plants. The study of SLO2 interactors could be instrumental in future unraveling the inter-organellar energy network of mitochondria, plastids and peroxisomes. Furthermore, using our SLO2-GFP lines, the hypothesis whether stress conditions may alter the subcellular localization of SLO2, can be challenged, which could provide a first indication for an interaction between mitochondria and other energy engines in the cell. Fourthly, regulation of mitochondrial energy metabolism by SLO2 may not be limited to RNA editing, but may also include the post-translational regulation of proteins/enzymes that are involved in energy metabolism. Again, analysis of binding partners of SLO2 can shed light on this aspect of its working mechanism. Moreover, we found that SLO2 mutation leads to the defects of mitochondrial complex I, III and IV (Zhu *et al.*, 2012). This is the first example of a single gene mutation that results in

changes in 3 mitochondrial complexes. Further investigation of the functional mechanism of SLO2 in the protein assembly of mitochondrial complexes will be very interesting.

Based on our observations, *slo2* mutants show a reduced sensitivity to ethylene and hypersensitivity to ABA (Zhu *et al.*, unpublished), while also being hypersensitive to sucrose (Zhu *et al.*, 2012). An intricate signaling network links sugar responses to ABA and ethylene, both involved in stress responses (Leon and Sheen, 2003). We hypothesize that stresses may affect RNA editing, and that SLO2 is part of this network by interacting with proteins that are specific for these signaling cascades.

7.5 Isolation of interaction partners

As discussed in the previous paragraph, SLO2 most likely functions by interaction with other (PPR) proteins. Identification of the binding partners of SLO2 will help to unveil its mode of action in the fundamental processes it is involved in. As a first step towards identification of interacting proteins, immunoprecipitation (IP) could be performed. Since no SLO specific antibodies are available yet, anti-GFP antibodies could be used, on an extract from SLO2-GFP lines (both under control of the *SLO2* promoter, *pSLO2::SLO2-GFP*, and under control of the *CaMV-35S* promoter, *p35S::SLO2-GFP*), with the WT as a negative control. Candidate binding proteins can then be identified in the SLO2-GFP-complex by peptide mass fingerprinting (PMF) by mass spectrometry after which their interaction should be confirmed (for example by the BiFC (bimolecular fluoresence complementation technique). or by co-immunoprecipitation. For this purpose, *myc* tagged fusions of the possible interactors should be created. As soon as an anti SLO2 antibody is available, a co-immunoprecipitation experiment with WT plants can be executed in parallel (by tagging possible interactors with GFP or myc). An alternative for this approach may be the tandem affinity purification (TAP) method. Both techniques however may show an aberrant result when GFP or myc antibodies are used. As SLO2 may be present in the plants, it could possibly interact better with its natural interacting partners, since no GFP or myc tags are added. Problems with transient expression will be reduced by using a construct under control of the *SLO2* promotor.

A second approach may be the analysis of the subcellular localization of all the proteins containing a DYW motif. Around a fifth of the PPR proteins contain a DYW domain. At first this analysis may occur in silico, selecting all the DYW containing proteins that are predicted to show a mitochondrial localization or (like SLO2) lacking a clear localisation signal. Confirmation of true interaction *in vivo* may be provided by BiFC experiments.

7.6 Molecular genetic analysis of mutants from interactors

Once in vivo interaction of SLO2 with (DYW) candidates has been established, the biological relevance of these interactions should be determined by molecular-genetic analysis. Therefore, loss-of-function mutants in the genes encoding interactors, should be created and purified. Phenotypic similarities with *slo2* mutants can be expected if SLO2 serves to functionally support the interactor; conversely, phenotypic opposites can occur in case SLO2

acts as an inhibitor. Mutants in the (DYW) interactors as well as the double mutant combinations with *slo2* should then be tested for altered RNA editing of the complex I components which are affected in *slo2* mutants, to prove that the editing targets are indeed the same.

Double mutant combinations are likely to be very severely affected in growth. Since the *slo2* phenotype is dependent on sucrose, this feature in single/double mutants of binding partners should also be tested. NAD+, NADH, ATP, ADP and sugar measurements could be done in the double mutants as previously described (Zhu *et al.*, 2012) to assess the influence of the loss of DYW function.

In summary, the analysis of putative SLO2-binding partners should be performed in order to unravel the mechanism of SLO2. Once we can confirm their binding ability with SLO2, genetic experiments (single and double mutant analysis) and biochemical experiments (eg. RNA binding, cytidine deaminase activity of SLO2 and binding partners) should follow.

7.7 SLO2 and RNA editing in stress responses

Our data indicate hypersensitivity of *slo2* to ABA combined with partial insensitivity to ethylene. Moreover, we found that SLO2 is necessary for plant tolerance to salt and drought stress (Chapter 6). Until now, only two reports showed the RNA editing factors may involve in plant stress response, *ABA hypersensitive germination 11* (*ahg11)* and *slow growth 1* (*slg1)* (Murayama *et al.*, 2012; Yuan and Liu, 2012). Since our data showed that *SLO2* participates in plant stress response, several possibilities need further investigation:

- Is SLO2 directly or indirectly involved in stress response?
- Since SLO2 is necessary for RNA editing events, are those RNA editing target genes involved in stress response?

These questions can be answered by studying the effect on RNA editing of various types of stresses, drought and temperature stress.

Last but not least, using our SLO2-GFP lines, the hypothesis whether stress conditions may alter the subcellular localization of SLO2 could be investigated. Several lines of evidence exist for dual localization of proteins, including PPR proteins (Ding *et al.*, 2006; Carrie *et al.*, 2009; Hammani *et al.*, 2011). Our observations during the determination of the subcellular localization of SLO2 indicate that SLO2 is possibly not uniquely localized in mitochondria, but may also show a peroxisomal localization (data not shown). Therefore, the SLO2-GFP lines should be exposed to different abiotic stresses and consecutively a change in the subcellular localization of the SLO2 protein should be determined. This may yield an important contribution towards unraveling the inter-organellar energy network of mitochondria, plastids and peroxisomes.

Moreover, experiments could be performed, checking whether the editing status of SLO2-target sites correlates to the physiological phenotype under different stress conditions.

7.8 The role of SLO2 in photorespiration and photosynthesis

Our results showed that mutation of SLO2 leads to an enhanced photorespiration rate. However, the functional mechanism is largely unknown. We were able to prove that the mitochondrial electron transfer chain is defective because of the *SLO2* mutation, and that a high concentration of CO_2 could partially complement the *slo2* mutant phenotype (Zhu *et al.*, 2012). We also observed increased amino acids and lower ATP, NADH in *slo2* mutants (Zhu *et al.*, 2012). The exact role of SLO2 in photosynthesis and photorespiration pathways should therefore be examined into more detail. Firstly, measurements of photosynthesis and photorespiration related-parameters under various conditions, such as, high/low CO_2, high/low nitrogen, high/low light conditions, day/night difference could be performed. Secondly, parameters which reflect the carbon and energy status in plant, such as ATP, ADP, NAD^+, NADH, sugar, amino acids should be determined under the conditions above, in order to provide new insight in the effect of *slo2* mutation in global energy metabolism.

7.9 Functional analysis of interactions among mitochondrial complexes

Our results showed that a mutation in *SLO2* leads to RNA editing changes in *mttB*, *nad4L*, *nad1* and *nad7*. To check whether *slo2* mutant phenotypes resulted from the changes or defects in those genes, complementary experiments should be performed by transforming WT *MTTb*, *NAD4L*, *NAD1* and *NAD7* genes into *slo2* mutant background. By genetic and biochemical analysis of the transformants (eg. western blotting, complex activity measurements), it could be shown whether any of these genes play a key role in the complex assembly in plants. By testing these lines under different stress conditions, the role of mitochondria in stress response can further be determined.

SLO2 mutation results in the dysfunction of mitochondrial complex I, complex III and complex IV, affecting the mitochondrial electron transport (Zhu *et al.*, 2012). Except *mttB*, all belong to complex I. We hypothesize that *mttB*, which functions in membrane targeting and secretion of cofactor-containing proteins, such as iron-sulphur clusters (Weiner *et al.*, 1998), may play a central roles in the dysfunction of mitochondria complex III and complex IV.

7.10 References

Andrés, C., Lurin, C. and Small, I. (2007) The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiologia Plantarum **129,** 14-22.

Boussardon, C., Salone, V., Avon, A., Berthomé, R., Hammani, K., Okuda, K., Shikanai, T., Small, I., and Lurin, C. (2012) Two interacting proteins are necessary for the editing of the NdhD-1 site in *Arabidopsis* plastids. Plant Cell **24**, 3684-3694

Carrie, C., Kühn, K., Murcha, M.W., Duncan, O., Small, I., O'Toole, N. and Whelan, J. (2009) Approaches to defining dual-targeted proteins in *Arabidopsis*. The Plant Journal **57**, 1128-1139.

Chateigner-Boutin, A.L. and Small, I. (2010) Plant RNA editing. RNA Biology 7**,** 213-219.

Ding, Y.H., Liu, N.Y., Tang, Z.S., Liu, J. and Yang, W.C. (2006) *Arabidopsis* GLUTAMINE-RICH PROTEIN23 is essential for early embryogenesis and encodes a novel nuclear PPR motif protein that interacts with RNA polymerase II subunit III. Plant Cell **18,** 815-830.

Hammani, K., Gobert, A., Hleibieh, K., Choulier, L., Small, I. and Giegé, P. (2011) An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation. Plant Cell **23,** 730-740.

Leon, P. and Sheen, J. (2003) Sugar and hormone connections. Trends in Plant Science **8,** 110-116.

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., Lecharny, A., Le Ret, M., Martin-Magniette, M.L., Mireau, H., Peeters, N., Renou, J.P., Szurek, B., Taconnat, L. and Small, I. (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16**, 2089-2103.

Murayama, M., Hayashi, S., Nishimura, N., Ishide, M., Kobayashi, K., Yagi, Y., Asami, T., Nakamura, T., Shinozaki, K. and Hirayama, T. (2012) Isolation of *Arabidopsis* ahg11, a weak ABA hypersensitive mutant defective in nad4 RNA editing. The Journal of Experimental Botany **21**, 21.

Salone, V., Rudinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B., Small, I., Knoop, V. and Lurin, C. (2007) A hypothesis on the identification of the editing enzyme in plant organelles. FEBS Letters **581**, 4132-4138.

Takenaka, M., Verbitskiy, D., Zehrmann, A. and Brennicke, A. (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. The Journal of Biological Chemistry **285,** 27122-27129.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A. and Turner, R.J. (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell **93,** 93-101.

Yuan, H. and Liu, D. (2012) Functional disruption of the PPR protein SLG1 affects mitochondrial RNA editing, plant development, and responses to abiotic stresses in *Arabidopsis*. The Plant Journal **70,** 432-444.

Zehrmann, A., Verbitskiy, D., van der Merwe, J.A., Brennicke, A. and Takenaka, M. (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. Plant Cell **21,** 558-567.

Zhu, Q., Dugardeyn, J., Zhang, C., Takenaka, M., Kuhn, K., Craddock, C., Smalle, J., Karampelias, M., Denecke, J., Peters, J., Gerats, T., Brennicke, A., Eastmond, P., Meyer, E.H. and Van Der Straeten, D. (2012) SLO2, a mitochondrial PPR protein affecting several RNA editing sites, is required for energy metabolism. The Plant Journal **71**, 836-849.

Zhu, Q., Dugardeyn, J. Zhang, C., Mühlenbock, P., Valcke, R., De Coninck, B., Oden, S., Karampelias, M., Cammue, B., Prinsen, E., and Van Der Straeten, D. (2013) The *Arabidopsis* PPR RNA editing factor SLO2 participates in multiple stress and hormone responses. Molecular Plant, **accepted for publication**.

Chapter 8: Summary/Samenvatting

8.1 Summary

The goal of this research was the functional characterization of the *SLO2* gene of *Arabidopsis thaliana*. SLO2 is a PPR protein with an E+ motif at its C-terminus. It does not contain a DYW motif. s*lo2* was isolated as an ethylene mutant based on a slower emergence of the first leaf pair. This process normally is accelerated by adding ACC to the growth medium, but the emergence of the leaves is less stimulated by ACC in the mutants than that of WT seedlings. Besides this, *slo2* shows a myriad of aberrant responses to ethylene treatments.

Based on these first data, the research initially focused on the unraveling of the role of SLO2 in ethylene signaling. As a first step a literature study on the interaction of ethylene with other hormones was performed and an in silico study on the interaction between ethylene and gibberellins in roots was executed.

Research on the subcellular localization of SLO2 pointed towards a mitochondrial localization; although based on its sequence, this was not predicted. Moreover *slo2* mutants are hypersensitive to sucrose and their phenotype is dependent on the presence or absence of an external carbon source. Therefore the focus of the research shifted towards discovering the function of SLO2 in the mitochondria.

Based on their structure, PPR proteins are predicted to execute a function in RNA editing, stability, splicing… For a couple of proteins this was already proven. Our research demonstrated a role for SLO2 in the editing of important components of the complex I of the mitochondrial electron transport chain (NAD4L; NAD7, NAD1, mttB). In mutants the level of editing is much reduced or absent, leading towards a lower abundance of complex I, but also to a lower abundance of complex III and complex IV. Because of this, the mitochondrial electron transport chain is no longer functional, reflected in lower amounts of ATP, NAD+ and sugars. Either directly or indirectly caused by this lack of energy, *slo2* mutant displays a myriad of different aberrant responses to several stress and hormone treatments. The mutants are less sensitive to ethylene, hypersensitive to ABA and sucrose, and to drought. In addition, the infection rate by *Botrytis cinerea* is higher in the mutants as compared to WT.

By investigating the general gene expression levels in the mutant, we were able to show that a vast number of phenotypes is also reflected in a molecular response. 778 genes show an obvious difference in expression levels compared to WT. These genes are (as expected) involved in stress responses, response to different hormones, plant development and electron transport.

Further research on the working mechanism of SLO2 is required. It can be hypothesized that the subcellular localization of SLO2 changes under the influence of stress. Further research of the localization of SLO2 under these conditions could explain the vast myriad of responses in which SLO2 is involved.

In order to unravel the working mechanism of SLO2, research to isolate its possible interaction partners is required. SLO2 does not contain a DYW domain and it is exactly that sequence that has been shown to be able to function as the catalytic part required for RNA editing. The determination of interaction partners carrying this domain could explain how SLO2 is able to play its role in RNA editing of some of the key components of the electron transport chain. Further analysis of (double) mutants or overexpressors of these interaction

factors will further help to clarify the exact working mechanism of SLO2, as will be the study of mutants in the RNA targets of SLO2.

Summarized we can state that SLO2 facilitates the editing of key components of the electron transport chain, leading to a broad phenotype in plant development and their response to stress.

8.2 Samenvatting

Het doel van dit onderzoek was het karakteriseren van de *slo2* mutant van *Arabidopsis thaliana*. SLO2 is een PPR-eiwit met een E+ motief aan de C-terminus. Het bezit geen DYW motief. *slo2* werd geïsoleerd als een ethyleenmutant op basis van een vertraagd verschijnen van het eerste bladpaar. Dit proces wordt versneld in de aanwezigheid van ACC, maar de mutant reageert minder sterk op ACC als WT planten. Daarnaast vertoont *slo2* nog een groot aantal afwijkende responsen op verschillende (hormoon)behandelingen.

Op basis van deze eerste data focuste dit onderzoek oorspronkelijk op het nagaan van de rol van SLO in de ethyleensignalisatie. Daarom werd de interactie van ethyleen met andere hormonen onderzocht en werd een *in-silico* studie uitgevoerd over de interactie tussen ethyleen en gibberellines.

Onderzoek van de subcellulaire localisatie van SLO2 wees echter op een mitochondriale localisatie, alhoewel dit op basis van de sequentie niet voorspeld werd. Bovendien zijn de mutanten overgevoelig aan sucrose en is het vertoonde fenotype duidelijk afhankelijk van de aan- of afwezigheid van een externe koolstofbron. Daarom werd de focus verlegd naar het onderzoeken van de functie van SLO2 in de mitochondria.

Op basis van hun structuur wordt voorspeld dat PPR-eiwitten een functie uitoefenen in RNA editing, stabiliteit, splicing… Dit werd voor een aantal eiwitten reeds aangetoond. Ons onderzoek heeft aangetoond dat dit ook voor SLO2 het geval is. SLO2 staat in voor het editeren van belangrijke componenten van complex I van de mitochondriale elektronentransportketen (NAD4L, NAD7, NAD1 en mttB). In mutanten gebeurt deze editing minder, wat leidt tot een verlaagde concentratie van complex I, maar ook tot lagere aanwezigheid van complex III en complex IV.

Hierdoor is de mitochondriale elektronentransportketen niet langer volledig functioneel, wat zich ook reflecteert in lagere hoeveelheden ATP, NAD+ en suikers. De plant vertoont dus een continu energietekort.

Al dan niet rechtstreeks verzoorzaakt door dit energietekort vertoont *slo2* nog een wijd gamma aan afwijkende responsen. De mutanten zijn minder gevoelig aan ethyleen, hypergevoelig aan ABA en sucrose en als gevolg ook aan droogte. De besmettingsgraad van de mutanten door *Botrytis cinerea* ligt ook gevoelig hoger dan bij WT.

Door het onderzoeken van de algemene gen expressie in de mutant werd aangetoond dat het groot aantal aan fenotypes zich ook reflecteert in een duidelijk moleculaire respons. Zo vertonen 778 genen een duidelijk verschillend expressieniveau t.o.v. WT. Deze genen zijn zoals verwacht betrokken in stressresponsen, respons op verschillende hormonen, plant ontwikkeling en elektronentransport.

Verder onderzoek over het werkingsmechanisme van SLO2 is nodig. Zo is het waarschijlijk dat SLO onder invloed van stress een verschillende lokalisatie vertoont. Verder onderzoek van de SLO-lokalisaties onder verschillende condities kan bijdragen tot het verklaren van het grote gamma aan fenotypes vertoond in *slo2.*

Om het actieve werkingsmechanisme van SLO2 te verklaren, is onderzoek naar mogelijke interactiepartners noodzakelijk. SLO2 bevat immers zelf geen DYW sequentie. Net deze sequentie wordt verwacht dat ze een katalytische werking kan uitoefenen. Het bepalen van mogelijke interactiepartners van SLO2 en het analyseren van dubbelmutanten,

overexpressoren van deze partners… kan het mechanisme van SLO2 verder helpen ontrafelen.
Samengevat kan gesteld worden dat SLO2 door het faciliteren van editing instaat voor een groot aantal fenotypes in de ontwikkeling van planten en hun respons op stress.

Chapter 9: Publications Jasper Dugardeyn

A1 articles (ISI Web of Science) (IF= impact factor; R= ranking)

Zhu, Q., Dugardeyn, J. Zhang, C., Mühlenbock, P., Valcke, R., De Coninck, B., Oden, S., Karampelias, M., Cammue, B., Prinsen, E. and Van Der Straeten, D. (2013). The *Arabidopsis* PPR RNA editing factor SLO2 participates in multiple stress and hormone responses. Molecular Plant, **accepted for publication**.

Zhu, Q., Dugardeyn, J., Zhang, C., Takenaka, M., Kuhn, K., Craddock, C., Smalle, J., Karampelias, M., Denecke, J., Peters, J., Gerats, T., Brennicke, A., Eastmond, P., Meyer, E.H. and Van Der Straeten, D. (2012). SLO2, a mitochondrial PPR protein affecting several RNA editing sites, is required for energy metabolism. The Plant Journal **71**, 836-849. IF=6.946; R= 6/188 (Plant Sciences). *Shared first author.

Gu, Q., Lynen, D.F., Rumpel, K., Dugardeyn, J., Van Der Straeten, D., Xu, G. and Sandra, P. (2011) Evaluation of automated sample preparation, retention time locked GC-MS and data analysis methods for the metabolomic study of *Arabidopsis* species. Journal of Chromatography A **1218**:3247-3254. IF=4.101; R= 7/73 (Analytical Chemistry)

McDonnell, L., Plett, J.M., Andersson-Gunneras, S., Kozela, C., Dugardeyn, J., Van Der Straeten, D., Glick, B.R., Sundberg, B. and Regan, S. (2009) Ethylene levels are regulated by a plant encoded 1-aminocyclopropane-1-carboxylic acid deaminase. Physiologia Plantarum, **136**: 94-109 IF = 2.334; R= 26/188 (Plant Sciences)

De Grauwe, L., Chaerle, L., Dugardeyn, J., Decat, J., Rieu, I., Vriezen, W., Moritz, T., Beemster, G., Phillips, A., Harberd, N., Hedden, P. and Van Der Straeten, D. (2008) Reduced gibberellin response affects ethylene biosynthesis and responsiveness in the *Arabidopsis* gai eto2-1 double mutant. New Phytologist **177**:128-141 IF = 5.249; R= 7/188 (Plant Sciences)

Dugardeyn, J., Vandenbussche, F. and Van Der Straeten, D. (2008)To grow or not to grow: what can we learn on ethylene-gibberellin cross-talk by in silico gene expression analysis? Journal of Experimental Botany **59**:1-16 IF = 3.917; R= 12/188 (Plant Sciences)

Dugardeyn, J. and Van Der Straeten, D. (2008) Ethylene: fine tuning plant growth and development by stimulation and inhibition of elongation. Plant Science **175**: 59-70. IF = 1.795; R= 36/188 (Plant Sciences)

A2 articles (international & peer-reviewed, not in ISI Web of Science)

De Grauwe, L., Dugardeyn, J. and Van Der Straeten, D. (2008). Novel mechanisms of ethylene-gibberellin crosstalk revealed by the *gai eto2-1* double mutant. Plant Signaling & Behavior **3**:1113-1115.

P1 articles (articles in Proceedings included in ISI Web of Science)

Van Der Straeten, D., Vandenbussche, F., De Grauwe, L., Dugardeyn, J. and Hagenbeek, D. (2007) Interactions with the ethylene pathway: a puzzle yet to be completed. In Advances in Plant Ethylene Research, A. Ramina *et al.*, eds., Springer, 61-68 [ISBN 978-1-4020-6013-7]

Dugardeyn, J., Hagenbeek, D., Zhang, C. and Van Der Straeten, D. (2007) A novel growth modulator interconnects ethylene, ABA, and sugar signaling. In Advances in Plant Ethylene Research, A. Ramina *et al.*, eds., Springer, 111-112 [ISBN 978-1-4020-6013-7]

Hagenbeek, D., Dugardeyn, J., Zhang, C. and Van Der Straeten, D. (2007) A PPR protein, required for normal plant development, may be involved in control of the ethylene pathway at the post transcriptional level. In Advances in Plant Ethylene Research, A. Ramina *et al.*, eds., Springer, 119-120 [ISBN 978-1-4020-6013-7]

B2 Chapters in books

Dugardeyn, J. and Van Der Straeten, D. (2008) Ethylene: inhibitor and stimulator of plant growth. In Plant Cell Monographs, Volume 10, Plant Growth Signaling, L. Bogre & G.T.S. Beemster, eds., Springer-Verlag Berlin Heidelberg, 199-221 [ISBN 978- 3-540-77589-8].