Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för landskapsplanering,
trädgårds– och jordbruksvetenskap

Towards a function for an Arabidopsis protein involved in sucrose dynamics

Jonas Skytte af Sätra

"Macavity's a Mystery Cat: he's called the Hidden Paw—
For he's the master criminal who can defy the Law.
He's the bafflement of Scotland Yard, the Flying Squad's despair:
For when they reach the scene of crime—Macavity's not there!"

-Old Possum's practical cats by T.S. Elliot

Självständigt arbete vid LTJ-fakulteten, SLU
Examensarbete 30 hp, Hortonomprogrammet, Alnarp 2013

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Towards a function for an Arabidopsis protein involved in sucrose dynamics
Mot en funktion för ett Arabidopsis-protein involverat i sackarosdynamik

Jonas Skytte af Sätra

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Abstract

System based models of plants rely on descriptions and assigned functions of genes, and currently 50% of the genes in *Arabidopsis thaliana* has either no assigned function or a function based on homology only. Mitogen activated protein kinases (MAPK's) are key players in cell signalling and are conserved among eukaryotes, though their targets are highly diverse. A recent study has described a small Arabidopsis protein (80), without homology to any described protein, as a phosphorylation target of the MAPK's MPK3/6. Further work established α-glucan phosphorylase (PHS) as interacting with 80 and described a delayed senescence phenotype for 80 knock-out plants. This work has examined the effect of flagellin and sucrose on the stability of the 80 protein in seedling culture. In whole plants the effect of 80 on glucose, sucrose and the glucose residue content of the soluble heteroglycan (SHG) pool, as well as 80's stability during senescence has been investigated.

- 80 was found to be degraded under induced leaf senescence, in a way similar to that of induced darkness in whole plant systems.
- At day-time leaves from 80knock-out plants exhibited an increase in sucrose and no changes in glucose content.
- At night-time 80knock-out plants exhibited an increased sucrose:glucose ratio.
- 80knock-out leaves did not exhibit significant changes in sucrose and glucose content during senescence.
- In seedlings, 80 was found to be stabilised by sucrose and flagellin, though the sucrose dependent stabilisation was partly inhibited by flagellin.

Sammanfattning

Systembaserade modeller för växter baseras på dokumenterade beskrivningar av olika geners funktioner, och 50 % av generna i *Arabidopsis thaliana* har antingen ingen känd funktion eller enbart en presumtiv funktion baserad på homologi baserade med kända proteiner. Mitogen aktiverade protein kinaser (MAPK's) är viktiga delar av cellsignalleringen och ser väldigt lika ut mellan eukaryoter. De proteiner som fosforyleras av MAP kinaserna är däremot väldigt skiftande. Nyligen beskrevs a litet protein utan homologi till något känt protein, 80, som ett MPK3/6 fosforyleringsmål. Vidare studier har identifierat ett protein som interagerar med 80, ett α-glukan fosforylas (PHS). En försenad senescens och har också hittats hos blad av 80knock-outplantor som saknar en fungerande 80 gen (80 knockout). Det här arbetet har studerat hur 80 regleras av flagellin och sackaros i sterilkultur. I hela plantor har 80's effekt på glukos, sackaros och den lösliga heteroglukan (SHG) bundna glukospoolen studerats, liksom dess stabilitet under senescens.

- 80 degraderades vid inducerad senescens på samma sätt som under inducerat mörker i hela plantor.
- Under dagen hade blad från 80knockout plantor högre sackaros innehåll men oförändrade glukos nivåer, jämfört med vildtyps plantor.
- Under natten hade 80knockout en signifikant högre sackaros:glukos ratio.
- 80 hade ingen signifikant inverkan på sockerhalten under senescens.
- I sterilkultur stabiliserades 80 av sackaros och flagellin, men den sackaros beroende stabiliseringen delvis motverkades av flagellin.

Index

1 Background

1.1. MAPK module signalling

One of the key mechanisms for converting short lived cellular signals into longer lasting down-stream signals is the mitogen activated protein (MAP) kinase module. This module is highly conserved and is made up of three components, MAP kinase kinase kinase (MAPKKK, upstream), MAP kinase kinase (MAPKK, middle) and MAP kinase (MAPK, downstream). The module relays signals by phosphorylation of various proteins, including gene regulatory proteins as well as other protein kinases (Alberts et al, 2008).

In mammalian cells MAP kinase cascades functions down-stream of for example G-Protein Coupled Receptors (GPCR's), Receptor Tyrosine Kinases (RTK's) or two-component histidine kinases. The plant system is, however, not as well understood and is complicated by extensive cross-talk between modules (Mishra et al, 2006).

In plants the MAP kinase modules are associated with responses to several stresses including pathogens, heavy metals, salt, cold and drought but are also involved in development. Flagellin, containing the 22 amino acid peptide flg22, is the main structural component of the bacterial flagellum and acts as a PAMP (Pathogen Associated Molecular Pattern) in several plant species. In Arabidopsis the MEKK1-MKK4/5-MPK3/MPK6 module is down-streams of the flagellin receptor (FLS2) and up-streams of the WRKY22 and WRKY29 transcription factors, see figure 1. The flg22 induced activation of MPK3/6 occurs within 5min and triggers and stabilizes a large number genes and proteins, including *ASC6* involved in ethylene synthesis (Nakagami, Pitzschke & Hirt, 2005 and Colcombet, J. & Hirt, H., 2008)

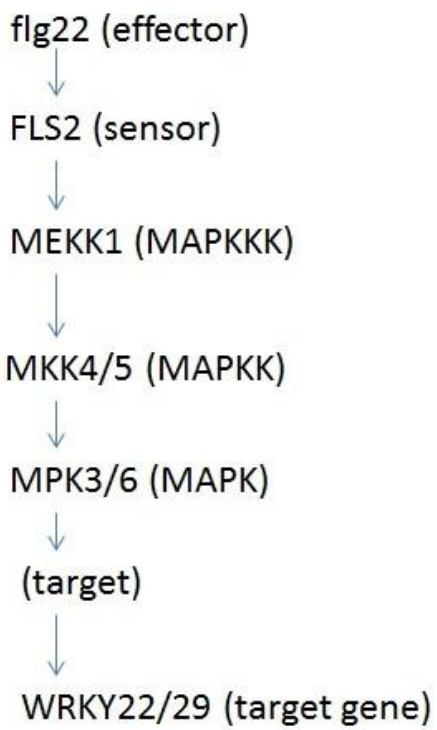

Figure 1. The flagellin induced MAPK signalling cascade in Arabidopsis, modified from Nakagami, Pitzschke & Hirt (2005).

1.2. A novel MPK3/6 substrate

As approximately 50% of all genes in *Arabidopsis thaliana* has no described function or a function based on homology only (Lida et al, 2010) it is important to characterise these genes in order to improve future systems based predictions in plants.

In a recent work by Sörensson et al. (2012) five substrates of MPK3/6 were identified. One of them, a small protein denoted 80, is phosphorylated by MPK3/6 at one out of five phosphorylation sites (serine 105) and lacks assigned function as well as homology to any described Arabidopsis protein. Interestingly, a constitutively expressed phospho-mimetic mutant of 80, 35S:80(S105D)::HA, having the MPK3/6 phosphosite serine-105 replaced by aspartic acid, exhibited a high seedling mortality and increased stomatal density. Further work (Andreasson, unpublished) used a yeast-two-hybrid system (Y2H) screen in the search for proteins interacting with 80, and identified the two α-glucan phosphorylases PHS1 and PHS2 in Arabidopsis with high confidence. The results were confirmed by pull-down and a co-immuno-precipitation assays.

These assays do not, however, distinguish between the two forms of PHS. But as PHS1 is plastidic and PHS2 is cytosolic, and there are no indications that either 80 or MPK3/6 is plastidic, this work will focus on PHS2.

Notably, PHS2 protein levels are not influenced by light-dark transitions, whereas 80 is present only during the day and absent during the night (Sörensson, 2010). 80 is degraded upon induced darkness during daytime. Furthermore, 80 protein stability is sucrose and phosphorylation dependent and 80 is degraded upon de-phosphorylation in a proteasome dependent manner (Lenman, unpublished).

1.3. PHS2 and SHG

Triose phosphates were previously believed to be the main carbohydrate exported from the chloroplast into the cytosol (Bowsher, Steer & Tobin, 2008), however, recent work has revealed a more complex pathway involving PHS2.

As reviewed by Fettke et al (2009), during night-time transitory starch is degraded to, and mainly exported to the cytoplasm as, maltose. Disproportionating enzyme 2 (DPE2) uses the maltose as substrate in a hydrolysis reaction with soluble heteroglycan (SHG) as glucosyl acceptor. A similar, reaction is carried out by the cytosolic phosphorylase isozyme PHS2. PHS2 catalyses the reversible reaction between SHGs and inorganic phosphate releasing glucose-1-phosphate, see figure 2. *dpe2* mutants exhibit minor increases (~20%) in sucrose levels during day-time and 2-fold increases during nigh-time as well as 10-fold increases in glucose during the day but with wild-type levels during the night. *phs2* mutants exhibit minor increases in sucrose during the day, wild-type levels during the night and a 2-fold increase in glucose during the day and minor increase during the night.

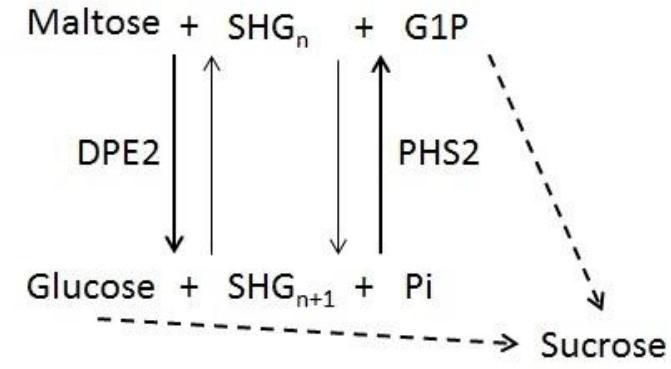

Figure 2. A simplified view of the metabolic interconnection between different saccharides and SHG, modified from Lu et al (2006).

Soluble heteroglycans (SHG), or the water soluble - ethanol/KCl insoluble polysaccharides, were recently reviewed by Fettke et al (2009). The isolation and fractionating of SHG's consists of several steps. Following extraction, SHG's are purified and small sugar compounds are removed, either by dialysis as in Fettke et al (2004) or by precipitation in ethanol/KCl as described by Yang and Steup (1990). The purified SHG's are then divided into a small (SHG_S) and a large (SHG_L) fraction by ultra-filtration with a cut-off at 10kDa. SHG_L can be further divided into sub-fraction I and II, either by field flow fractionation (FFF) coupled to multi-angle laser light scattering (MALLS) as done by e.g. Fettke et al (2004), or by treatment with the (β-glucosyl)3-Yariv reagent. The Yariv reagent reacts with, and precipitates, subfraction II whereas subfraction I is unaffected and remains in solution, see figure 3.

Further more, the different SHG constituents differ in their cellular localisation. Studies using non-aqueous fractionating indicates that SHG_S and subfraction I resides in the cytosol, whereas subfraction II resides in the apoplastic space. Further studies of isolated organella has confirmed that neither SHG_S, nor SHG_L reside in the chloroplast.

Interestingly, the (β-glucosyl)3-Yariv reagent was developed to recognize glycosides, but it also binds to arabinogalactans as well as arabinogalactan-proteins. The fact that the Yariv reagent binds only to subfraction II points to structural differences between the two subfractions. Furthermore, PHS2 interacts with subfraction I and SHG_S, but not subfraction II. With respect to the DPE2/PHS2 mediated reactions, subfraction I seems to be functionally homogenous (Fettke et al, 2009).

SHG's mainly consists of galactose and arabinose residues, and to a lesser degree glucose, xylose and mannose. Glucose makes up less than 3% of the SHG_L fractions (Fettke et al 2005), but is the only constituent of SHG that has been extensively examined.

Reversibly Glycosylated Proteins (RGPs) are involved in, and important for, the incorporation of L-Ara into the plant cell wall. It has been speculated that RGPs might interact with SHGs (Rautengartena et al, 2011). There is however little information on the role of arabinose in plants, though it has been proposed to act as an energy storage.

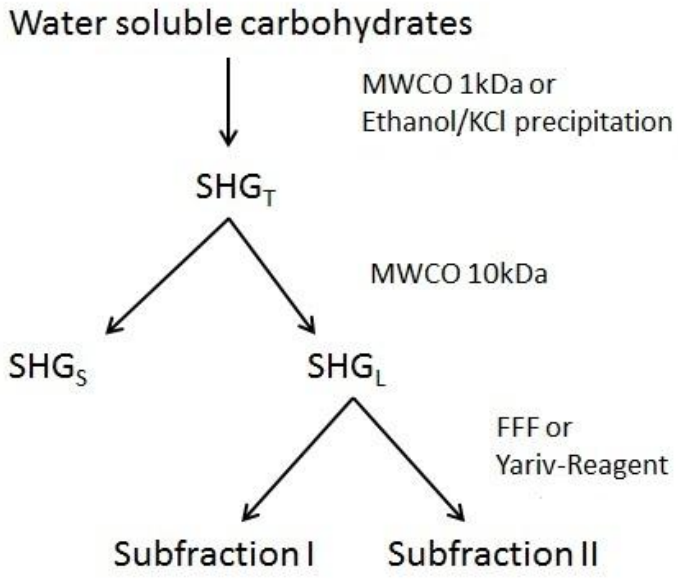

Figure 3. Fractioning of SHG's as described by Fettke et al (2009).

1.4. Senescence

The interaction between 80 and PHS suggests that 80 would have a function in SHG metabolism. Interestingly, knock-outs in 80 exhibit a delayed senescence in detached leaf assays under darkness (Muhlenbock, unpublished). The phenotype is weaker under light conditions and absent in whole plant systems.

The complete progress of leaf senescence has been recently reviewed elsewhere (Brouwer, 2012 and Lim et al, 2007) and will not be discussed in detail. In short, leaf senescence is characterised by degradation of the photosynthetic machinery and allocation of nutrient resources, and a well known enhancer thereof is ethylene. Other phytohormones, including salicylic acid (SA), have also been shown to be involved in leaf senescence. Several proteins involved in senescence are regulated by a SA dependent pathway, and the transcriptomic changes caused by SA are highly similar to that of age-dependent senescence. The role of SA seems to be in age-dependent senescence, and not to as high a degree in dark-induced senescence.

Furthermore, WRKY22 has recently been shown to mediate dark-induced leaf senescence in Arabidopsis, in detached leaf assays (Zhou et al 2011). MPK6 has also been shown to be involved in senescence signalling, downstream of MKK9, with *mpk6* mutants showed delayed leaf senescence in detached leaf assays under light conditions (Zhou et al, 2009).

As 80 exhibit sucrose dependent stabilisation, an interaction with PHS2 and a senescence phenotype of the knock-out it is tempting to speculate that 80, as a MPK target, might be involved in sugar signalling. As recently reviewed by Bolouri-Moghaddam and Van den Ende (2012) sucrose is emerging as an important signalling compound in plant innate immunity. Application of exogenous sucrose activates *PR1* and *PR2* genes, and specifically sucrose seems to trigger anthocyanin accumulation. According to Joeng et al (2010) ethylene seems to inhibit this accumulation.

1.5. Goals

Phenotypic analysis of a confirmed gene knock-out can help in deciphering the function of the corresponding protein. A reversed phenotype of a knock-out complemented with the wild type gene would provide support for the phenotype of the gene.

Based on diurnal changes in 80 and PHS2 protein stability, one might hypothesize that 80 regulates the diurnal shift in PHS2 activity from SHG degrading to SHG synthesizing. In that case a 80knock-out might exhibit changes in sucrose and glucose metabolism, as well as the SHG bound glucose residues, similar to those of *phs2* and *dpe2* mutants. An effect of 80knock-out on SHG would also provide further support for the idea that the interaction is specific towards PHS2.

As the 80knock-outs exhibits a delayed senescence phenotype, though 80 is degraded upon induced darkness in whole plant systems, it would be of interest to investigate if 80 is also degraded during senescence. Thereby providing further clues to 80s function during senescence.

As 80 is phosphorylated by the flagellin induced MPK3/6, it is of interest to investigate whether flagellin treatment influences protein stability, and if so, if it is affected by sucrose.

In short, the project will focus on:

- Confirm 80 insertion line
- Genomic confirmation of a 80 insertion line complemented with a genomic 80 fragment
- Analyse 80's effect on glucose and sucrose levels in Arabidopsis leafs.
- Establish and utilise a method for measurements of SHG's in Arabidopsis leafs.
- Investigate 80 protein stability during induced senescence.
- Investigate the effect of flagellin on the 80 protein stability.

2 Material & Methods

2.1 Material

For experiments analysed by western blot Columbia (col-0) lines carrying 35S:80::HA (haemagglutinin) and 35S:80(S105A)::HA constructs were used. For experiments on carbohydrate metabolism a insertion line in the Nossen ecotype (pst10468 from RIKEN) carrying a transposon insert in the exon region of 80, as well as corresponding wild-type, were used.

Plants for confirmation of rescue were 3.2knock-out (pst10468) that had been transformed with a construct containing a genomic fragment from Nossen, including the 80 gene, its native promoter and down-stream region. The construct also contained a BAR-gene and, accordingly, the plants were sprayed twice with (0,1-0,5%) BASTA prior to sampling of leaves for PCR-confirmation.
Whole plants were grown at 8h darkness and 16h light (150-180µmols^{-1}m^{-2}) in a commercial peat based substrate (*Kronmull Krukväxtjord med Leca*, Weibull Trädgård AB) fertilised with PG Mix NPK 14-7-15 to a conductivity of 2,5-4,5mS/cm and pH 5,5-6,5. Leaf samples were collected from plants being 3-5 weeks of age. Senescence was attained by a detached leaf assay in darkness. The phenotypic growth stage was estimated roughly according to the scale described by Boyes et al (2001).

Seedlings were grown in liquid culture containing ½ Murishage Skoog (MS) basal salt and 30mM sucrose, at 50µmols^{-1}m^{-2} at a density of 5-7 seedlings per 2ml media. After one week, seedlings were transferred to 20ml of either ½ MS with 6mM sucrose or ½ MS without sucrose and incubated for another 20h. Subsequently, flg22 was added to the media to varying concentrations followed by 1h incubation. Afterwards seedling were sampled and snap-frozen in N_2 and stored at -80ºC .

2.2 DNA extraction

Extraction of DNA was done as described by Edwards et al (1991). Frozen tissue was homogenised in a buffer containing 200mM tris pH 7,5, 250mM NaCl, 25mM EDTA and 0,5% SDS. Debris was removed by centrifugation at 13300rpm for 1min followed by collection of the supernatant. Subsequently DNA was precipitated by addition of isopropanol and, after removal of liquid phase, re-suspended in water.

2.3 PCR and gel-electrophoresis

DNA content in samples was measured on a Nanodrop ND-1000 Spectrophotometer and diluted to 15ng/µl. PCR was performed using 11,8µl water, 2µl 10x buffer, 2µl dNTP mix, 1µl 10µM primers, 0,2µl Taq and 2µl DNA sample. PCR-cycling was done according to standard protocol: [94ºC, 3min]x1; [94ºC 30s; 57ºC, 30s; 72ºC, 1min]x32; [72ºC, 3min]x1; [4ºC, infinite]x1. F43 (GGCTCTTCGGGTATAGTCTGG) and R347 (GCAGAGCAGCACCCAAAC) primers were used.
Products were visualised on 1% agarose, 1x TAE, gel and stained with ethidiumbromide. For reference a GeneRuler 1kb DNA Ladder (Fermentas) was included.

2.4 RNA extraction and cDNA synthesis

In short, RNA was extracted using a QIAGEN RNeasy mini kit. RNA was then purified from DNA and cDNA was synthesised using a Superscript Reverse Transcriptase and RNA degraded using RNAse H. Samples were diluted and stored at -20ºC.

2.5 RT-PCR

In short, SYBR green was used for staining of amplified DNA. PCR cycling standard protocol was used: [94ºC, 5min]x1; [95ºC, 15s; 60ºC, 25s; plate read]x35; [95ºC, 3min, 60ºC, 25s, increase from 50ºC to 95ºC, 0,5ºC/5s + plate read; 4ºC, infinite]x1

For expression confirmation in Nossen wild-type and knock-out, the 80F43/80R347 primer pair was used. Primers against Actin were used for normalisation.

2.6 Protein extraction.

For seedling experiments, 6 seedlings were used, and for senescence approximately 0,3g of leaf tissue. Tissue was snap-frozen in N_2 at sampling, then homogenised on ice in a buffer containing 50 mM MOPS pH 7,5, 5% glycerol, 5 mM EDTA, 5 mM ascorbic acid, 0,5%TRITON, 5 mM DTT, 5 µM Na_3OV_4, 1 mM NaMo, 25 mM NaF, 10 nM calyculin, protease inhibitor cocktail P9599, 1 mM PMSF and 0,55% PvPP at approximately 6x ratio. Samples were subsequently ultrasound treated in ice and centrifuged 5min at 13300rpm and +4C. The supernatant was collected and centrifuged for 10min under the same conditions. Supernatants were collected and mixed with an equal volume 2xSDS with 1 mM DTT were-after they were heated to 85ºC for 10min. Samples were stored at -80ºC until use, the following day.

2.7 Western blot

Extract corresponding to approximately 40µg protein was loaded on a 12% SDS-PAGE gel and separated at 110V for 2h. Proteins were then transferred to Amersham Hybond-ECl membranes (GE Healthcare, n. D107765) at 20V for 1h in 39mM glycine, 48nMTris, 0,037% SDS, 20% methanol. To ensure equal protein concentrations membranes were stained with PonseueS. Membranes were then washed in phosphate buffer saline (PBS) with 1% TWEEN 20, blocked in PBST+KCl and 4% dry milk and subsequently incubated with monoclonal rat anti-HA (haemagglutinin, Roche) at 1:1000 at 4ºC over night. Subsequently, membranes were washed with PBST + KCl, were-after it was incubated with monoclonal Horseradish peroxidase -anti rat (Roche) at 1:160000 in PBST + KCl and 4% dry milk for 2h at rt. The membranes were washed with PBST + KCl and finally with PBS. 380µl SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) was added where-after the membranes were exposed to CL – X posure film (Thermo Scientific) for 2-40min. Subsequently, the films were developed and fixed for 3min each using G150 and G354 (AGFA), respectively.

The HA-tagged constructs were used as it has not been possible to develop an antibody that binds specifically to 80.

2.8 Extraction of glucose and sucrose

Tissue was homogenized in a mortar with N_2 or using stainless steel beads (Qiagen) and a FastPrep-24 (MP Biomedicals), incubated with 500µl 80% methanol at 80ºC for 20min and left to cool on ice. After centrifugation for 5min at 13300rpm, 400µl supernatant was removed, distributed into 125µl aliquots and dried in using a SpeedVac SVC1000 (Savant). The pellet was redisolved in 500µl 80% ethanol and the incubation and centrifugation step was repeated. 400µl supernatant was collected, 125µl aliquoted to the methanol pellet and left to dry in completely. The pellet was dissolved in 1,5ml millipore water followed by another incubation and centrifugation step. The supernatant was collected and put on ice until the methanol/ethanol phases had evaporated. Then the dried in pellets were dissolved in the 400µl millipore extract. Samples were deproteinised by the addition of 25µl 36mg/ml potassium hexacyanoferrate (II) (Sigma cat. No P-9287), 25µl 72mg/ml zinc sulphate (Sigma cat. No Z-4750) and 50µl 100mM NaOH followed by centrifugation at 13300rpm for 10min. The supernatant was then collected and kept at -20ºC until analysis.

2.9 Measurement of carbohydrates

Samples were analysed for glucose and sucrose using the K-MASUG 02/11 kit (Megazyme International Ireland). The assay was performed at 1:10th volume in microtiter-plates, and water, buffer and ATP/NAD(H) solution was added as a master-mix. Otherwise the assay was performed according to the K- MASUG protocol. For analysis of SHG glucose residues the protocol was modified to facilitate 200µl sample, due to very low concentrations. Measurements were performed on a Multiskan GO (Thermo Scientific) in UV Flat Bottom Microtiterplates (Thermo, #8404). Essentially hexokinase (HK), glucose-6-phosphate dehydrogenase (G-6-P DH), ATP and $NADP^+$ is added to the samples to convert D-glucose and ATP into glucose-6-phosphate (G-6-P) and ADP followed by a conversion of G-6-P and $NADP^+$ into gluconate-6-phosphate, hydrogen and NADPH. The amount of NADPH produced is measured spectrophotometrically at 340nm. To measure sucrose content, the samples are first treated with α-glucosidase which hydrolyses sucrose into D-glucose and D-fructose. The assay is intended for measurements of maltose content as well, though this was not performed.

2.10 Extraction of Soluble Heteroglycans

SHG's were extracted according to a protocol modified from Lu et al (2006). Tissue was snap-frozen in N_2 and homogenized in falcon tubes with 5 stainless steel beads (Qiagen) two times 30s at 5M/s in a FastPrep-24 (MP Biomedicals),. The homogenate was dissolved in 2ml/g ice-cold 20% ethanol, vortexed and centrifuged 5min at 5000g, subsequently the supernatant was collected and re-centrifuged for 13min at 16000g. Then the supernatant was filtered through a 70µm sieve and incubated at 95ºC for 10min. Following 13min centrifugation at 16000g, SHG's were precipitated in 70% ethanol and 1% KCl, incubated 5min at rt and centrifuged 6min at 16000g. The pelleted SHG's were washed three times in 75% ethanol and 1% KCl to remove free glucose. The SHG's were resuspended in 500µl MQ by 30min incubation at 45°C. SHG's were then separated into large and small fraction by centrifugation at 14000g for 10min using Amicon Ultra 10K Spinn Columns (Millipore). SHG_S were precipitated in 70% ethanol and 1% KCl and redisolved in 100ul water. The extracts were then hydrolysed by 90min incubation at 95°C in 1M HCl and neutralised with NaOH.

2.11 Statistics

For pair-wise comparisons students t-test was performed in Microsoft Excel. General Linear Model (GLM) analysis was performed in Minitab 16 (Minitab) using Dunnet's test.

3 Results

3.1 Confirmation of an 80 insertion line

Two offspring's (3.1 and 3.2) from a heterozygous 80 insertion line (Line 3 of pst10468, RIKEN) were analysed for presence of an intact 80 gene. Three lines of another insertion line (pst05114, RIKEN) were also analyzed.

Genomic DNA was amplified using the 80F43/80R347 primer pair, proper amplification should result in a 304bp product. As seen in figure 4, line 3.1 seems to carry intact copies of 80, whereas line 3.2 does not amplify 80. Line pst05114 was discarded as it was not ready for further analysis. Separate analysis using other primer-pairs (RikenDs5/80R347, F43/R288 & 468LRP) confirmed 3.1 as 80wt and 3.2 as 80ko (data not shown).

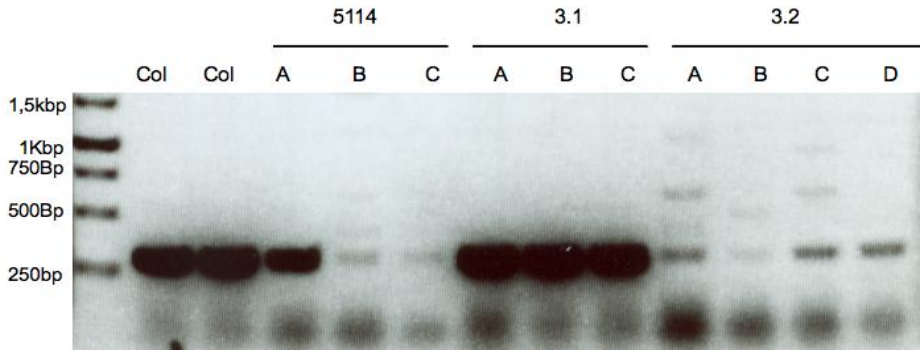

Figure 4. DNA confirmation of 80 knock-out and wild-type. DNA fragments amplified using the 80-directed F43/R347 primer pair, separated on 1% agarose gel and visualised using ethidium bromide. GeneRuler 1kb DNA ladder (Fermentas) for reference. Left to right: two columbia samples, three samples from the 5114 line, three samples from line 3.1 and four samples from line 3.2. Amplification should result in a 304bp segment.

To verify the genomic DNA results, samples for RNA extraction were taken 5h into the light period, when 80 is expected to be expressed (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Analysis of expression confirms that line 3.1 transcribes intact 80 RNA, as opposed to 3.2. Both lines transcribe the house-keeping gene Actin at approximately the same level, table 1.

Table 1. RNA confirmation of 80 knock-out and wild-type. RT-PCR of three plants from line 3.1 and 3.2, respectively. 80 is analysed using the F43/R347 primer pair and Actin as control, samples are taken 5h into the light period. N/A designates that the fluorescence signal did not reach above the threshold level for 35 PCR cycles. 3.1 is confirmed to transcribe 80wt and 3.2 is a 80ko.

Sample	80 C(t)	Actin C(t)
80wt 3.1 A	21,7	20
80wt 3.1 B	22,3	20
80wt 3.1 C	22,8	19,9
80ko 3.2 A	N/A	20,3
80ko 3.2 B	N/A	20,2
80ko 3.2 C	N/A	20

The 80 knockout plants (line 3.2) exhibit a delayed senescence in detached leaf assays under darkness (Muhlenbock, unpublished). The phenotype is weaker under light conditions and absent in whole plant systems.

3.2 Confirmation of genomic 80 fragment insertion

Line 3.2 80ko (pst10468) plants were transformed with a genomic fragment from Nossen, including the 80 gene, its native promoter and downstream region (Mogren, unpublished). Following two treatments with BASTA, leaf tissue was sampled from 30 plants. Analysis of DNA using 80-primers confirmed that all plants contained an 80 fragment, see figure 5 & 6.

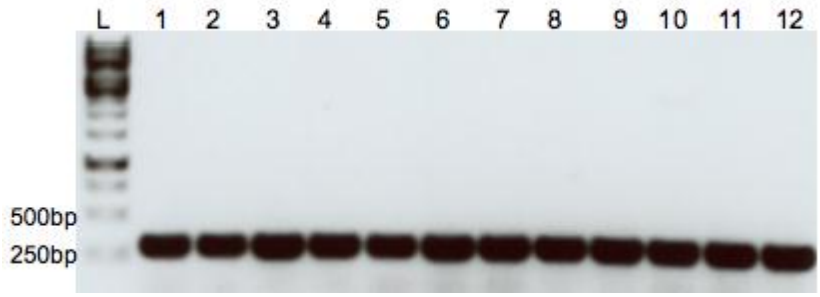

Figure 5. Confirmation of genomic 80 fragment insertion. Gel-electrophoresis of DNA samples following PCR with 80F43/80R347 primers: (1) 1kb ladder , (2-13) sample 1 – 12. Amplification product should be 304bp, all samples produced a band slightly larger than the 250bp band from the ladder.

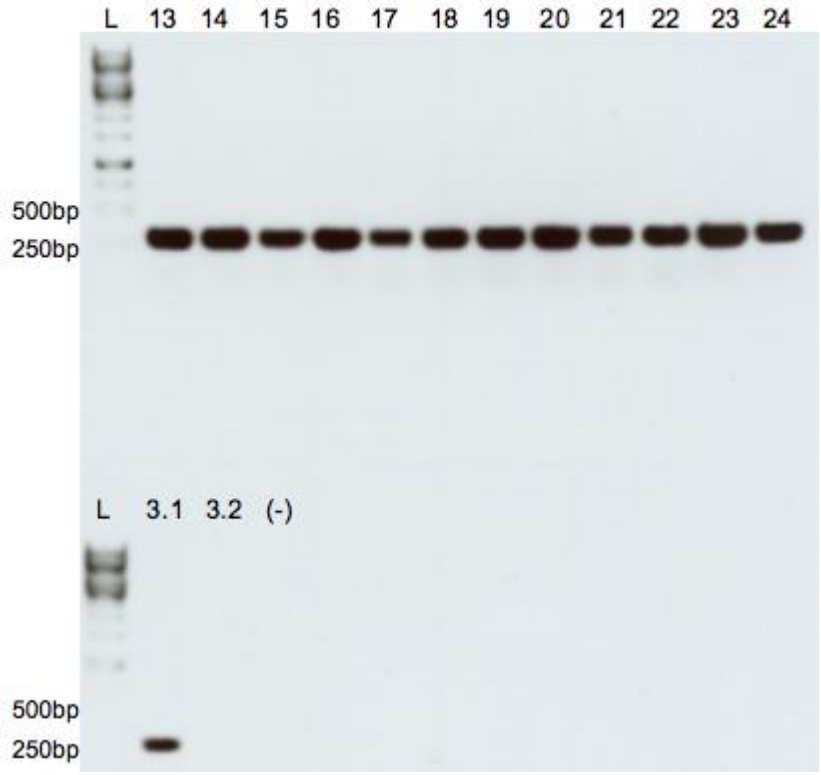

Figure 6. Confirmation of genomic 80 fragment insertion. Gel-electrophoresis of DNA samples following PCR with 80F43/80R347 primers, top row: (1) 1kb ladder , (2-13) sample 13 – 24. Bottom row: (1) 1kb ladder, (2) Nossen 3.1wt(+), (3) Nossen 3.280KO(-), (4) NTC(-). Amplification product should be 304bp, all samples and Nossen 3.1wt produced a band slightly larger than the 250bp band from the ladder. NTC and Nossen 3.2KO produced no bands.

3.3 80 stability during senescence

As the 80 knock-out exhibit a senescence phenotype the stability of the 80 protein during dark detached leaf senescence assay were investigated, using a 35S:80::HA construct in Col. As seen in figure 7, 80 is partially degraded within 30min and completely degraded after 2h of senescence, and remains degraded for up to 96h (data not shown). Similar results have been found in two independent experiment. PonseauS staining confirms that band signal-strength was not due to any major differences in protein concentration between samples. The “ghost-bands” above the 80 band is most likely due to unspecific binding of the secondary antibody.

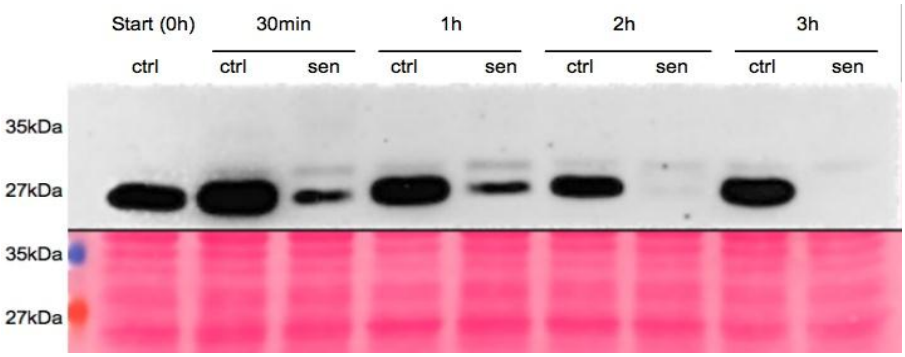

Figure 7. Stability of 80 during senescence in darkness. Top lane shows the immunoblot visualisation after incubation with HRP-substrate, bottom lane is the corresponding region on the membrane visualised by PonseauS-staining. Each lane consists of protein extract from a single leaf. 80 had started to degrade after 30min and was completely absent after 2h of senescence. Results were confirmed in an independent experiment.

3.4 Influence of flagellin on 80 stability

Previous work has shown that, in seedling culture, 80 is stabilised by sucrose containing media and de-stabilised by transfer to media without sucrose (Lenman, unpublished). As 80 is phosphorylated by the flagellin induced MPK3/6, it is of interest to investigate whether flagellin treatment influences 80 protein stability, and if so, if it is affected by sucrose. We also wanted to investigate if the effect of flagellin treatment differed between the 80 wild type and 80(S105A) mutant since the MPK3/6 phosphorylation occurs at serine-105. To investigate the effect of flagellin on 80 protein stability Col-0 seedling transformed with 35S:80::HA and 35S:80(S105A)::HA were grown one week in liquid culture with ½ MS and 30mM sucrose. Subsequently seedlings were transferred to fresh media, either with 6mM sucrose or lacking sucrose, and incubated for 24h where-after they were treated with varying concentrations of flagellin.

The sucrose dependent 80 protein stabilisation was confirmed using 80 wild-type (figure 8, lane -0 and +0). Interestingly, in starved seedling 80 wild-type protein was increasingly stabilised with increasing levels of flagellin. On the other hand, in seedlings still provided with sucrose flagellin treatment resulted in a partial, though not completely, de-stabilisation of the 80 protein. The interaction between sucrose and flagellin has been confirmed in independent experiments with two different lines (not shown).

To investigate if the stabilisation was due exclusively to the MPK3/6 phospho-site, similar experiments were conducted using 80(S105A). The 80(S105A) protein (figure 9) showed the same over-all pattern, though the relative changes in band strength were not as strong.

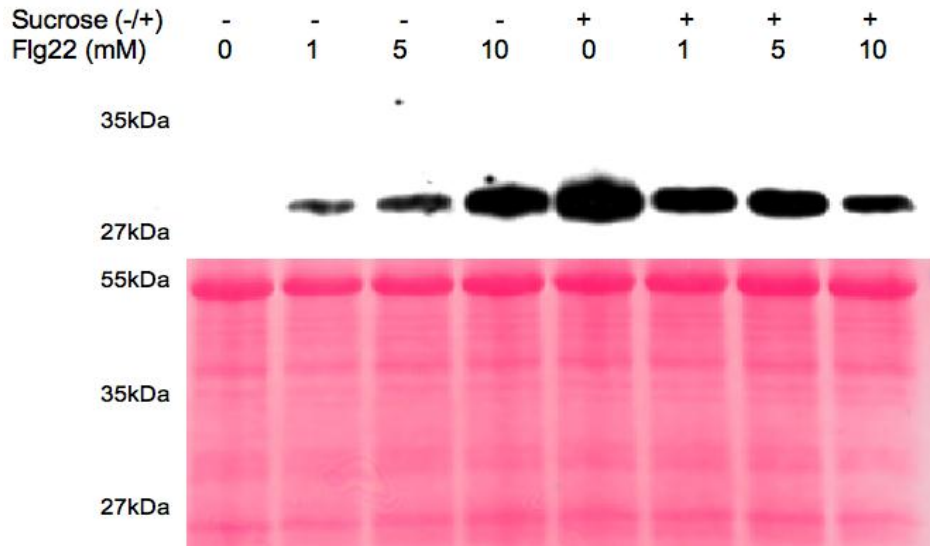

Figure 8. Stability of 80 with and without sucrose and treatment with varying concentrations of flg22, using a 35S:80::HA construct in Col. Top, immunoblot visualisation of 80. Bottom, PonseauS staining of membrane prior to blocking. When sucrose is absent flg22 stabilises 80, when sucrose is present 80 is stabilised and is slightly de-stabilised by flg22.

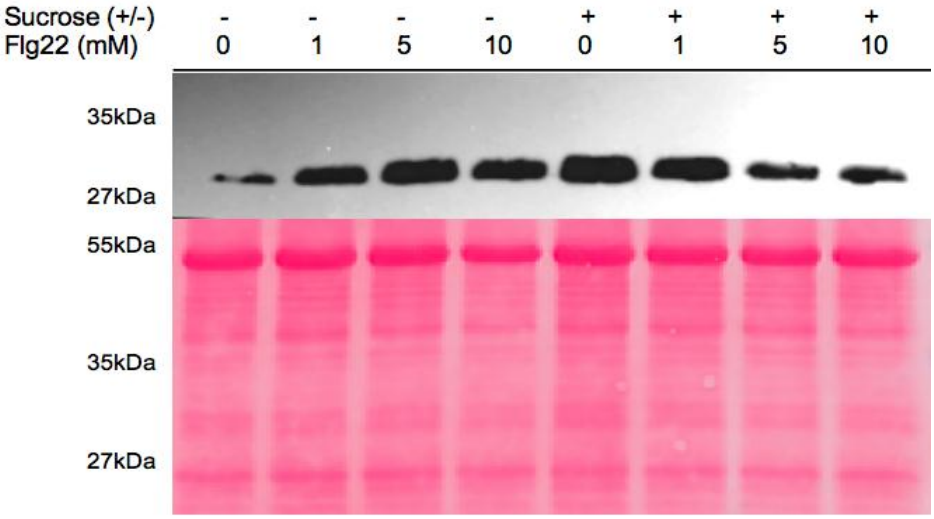

Figure 9. Stability of 80 with and without sucrose and treatment with varying concentrations of flg22, using a 35S:80(S105A)::HA construct in Col. Top, immunoblot visualisation of 80. Bottom, PonseauS staining of membrane prior to blocking. When sucrose is absent flg22 stabilises 80, when sucrose is present 80 is stabilised and is de-stabilised by flg22.

3.5 Influence of 80 on glucose and sucrose

Glucose and sucrose content was measured 3-6h into the light period during three principal growth stages (GS), rosette growth (3), inflorescence emergence (5) and flower production (6). In addition plants were analysed that had reached GS 6 unexpectedly early, possibly due to water stress, and had had their inflorescences removed approximately 24h prior to sampling (6*). Data from all GS analysed in a GLM revealed a significant increase in sucrose content in the 80knock-out(p=0.003, n=24). In growth stage 3 to 6 the sucrose content was 5-11% higher in the 80knock-out than in wild-type though not significant for these GS's analysed separately (p= 0.23; 0.39 & 0.56). For 6*, however, the difference was significant with a 50% increase in 80knock-out over wild-type (p=0.002, n=5), see figure 10.

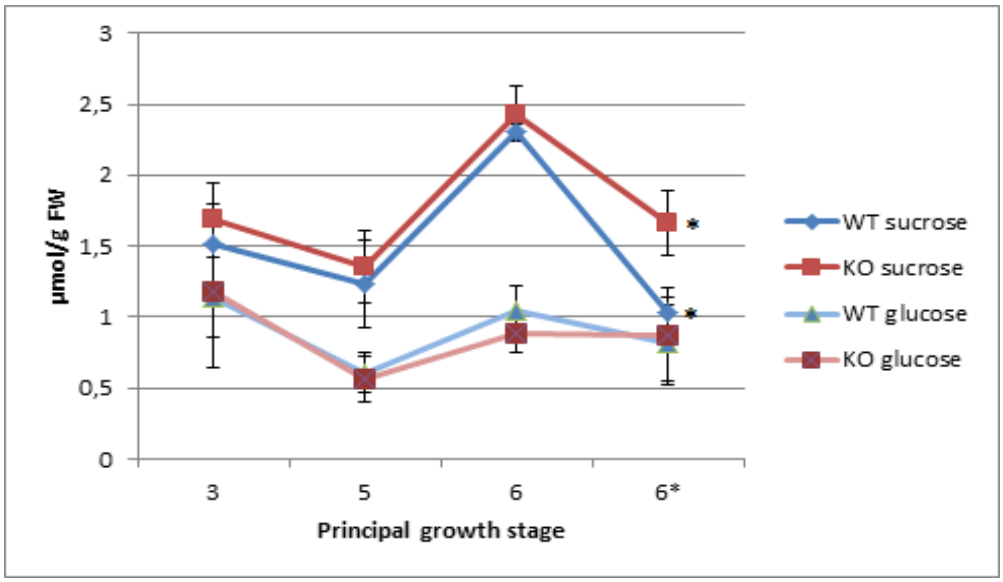

Figure 10. Glucose and sucrose content in leafs of 80knock-out and wild-type, during different growth stages. Differences in sucrose content are significant for GS 6 (p=0,002). Each GS stems from a separate experiment. Error bars denote standard deviation. (n=8, 8, 3 & 5, respectively)*

In samples taken in the middle of the night period, 80knock-out seemed to have a slightly higher sucrose content, though not significant (p=0.14). 80knock-out did, however, exhibit a higher Sucrose:Glucose ratio (p=0.04), see table 1. The interaction between 80 and sucrose during day-time and night-time is illustrated in figure 11. Notably, the glucose content did not exhibit any significant changes.

Table 2. Content of glucose and sucrose in leaf samples collected in the middle of the night period. Differences in sugar content are not significant whereas the difference in sucrose:glucose ratio is significant (p=0.044, n=4(wt)/3(ko)).

(µmol/g FW)	Glucose	stdev	Sucrose	stdev	Sucrose:Glucose	stdev
3.1WT	0,78	0,13	1,73	0,27	2,26	0,56
3.2KO	0,67	0,08	2,36	0,48	3,5	0,57

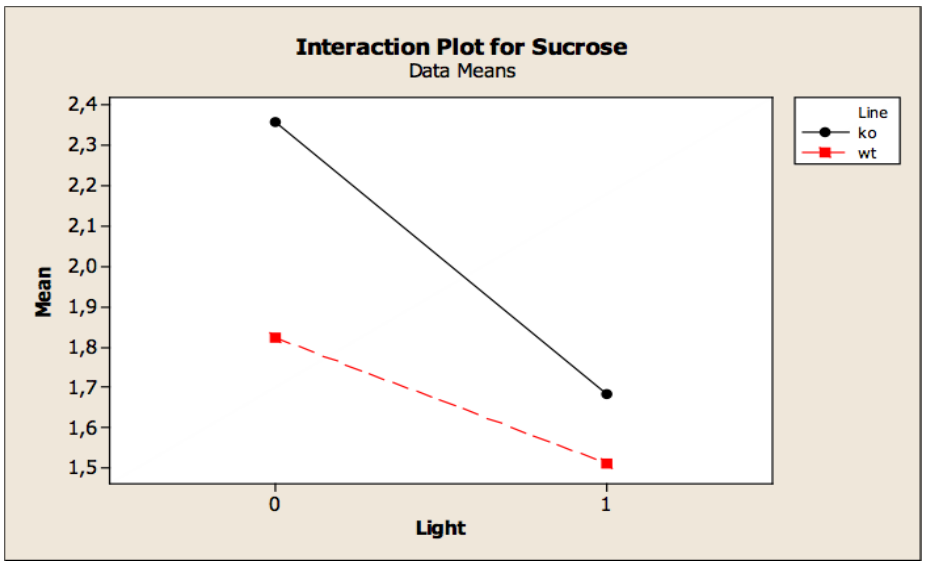

Figure 11. Interaction plot for sucrose content (µmol/g FW). Day-time (Light 1), Night-time (Light 0), 80knock-out (ko) and 80 wild-type (wt). Difference between 80knock-out and wild-type is not significant (p=0.0504)

As the 80ko exhibit a delayed senescence during dark detached leaf assays, sugar contents were measured under senescence conditions as well. Results from a 24h dark detached leaf assay, a 24h light detached leaf assay and a 96h dark detached leaf assay are summarised into main effect plots in figure 12 & 13. The 80knock-out plants did not differ significantly from wild-type plants in any of the experiments, though the over-all trend was towards a higher content of both sugar species.

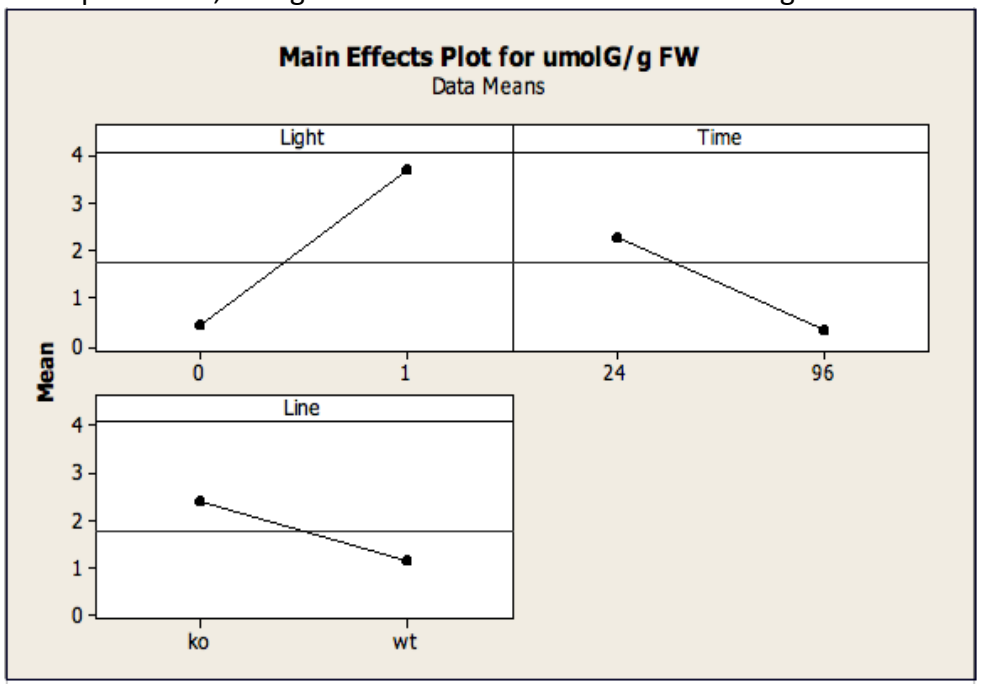

Figure 12. Main Effects Plot for glucose content in three senescence trials. The difference between light and dark senescence is significant (p=0.008) whereas the differences between times and lines are not (p=0.87 & 0.20 resp.).

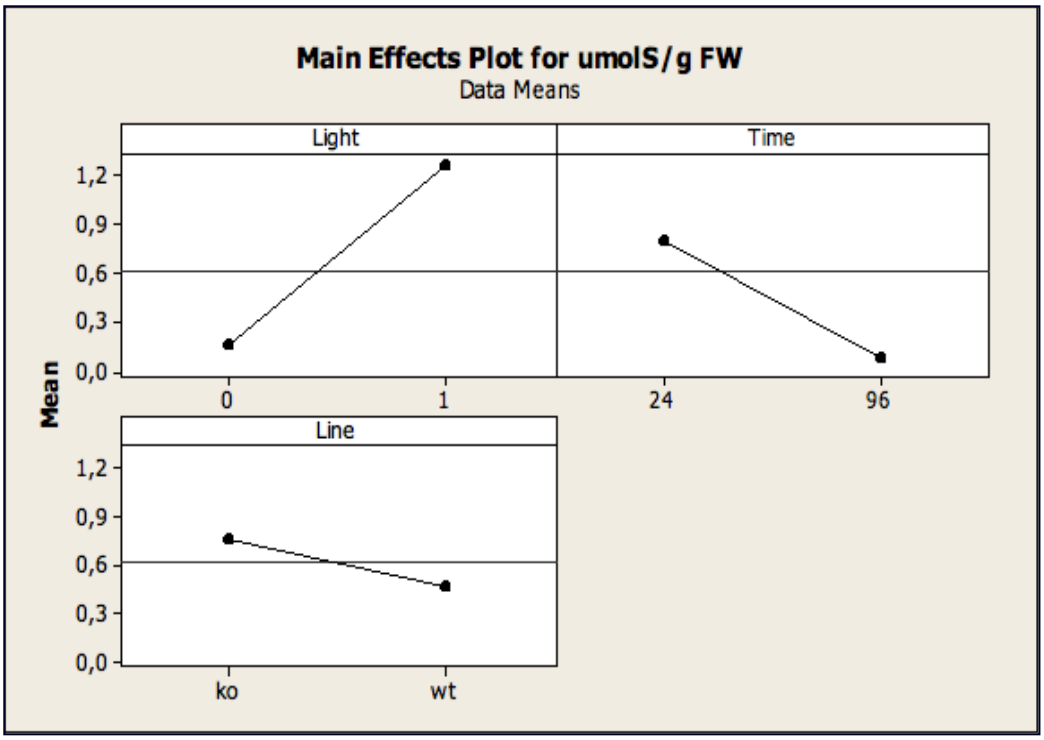

Figure 13. Main Effects Plot for sucrose content in three senescence trials. The difference between light and dark senescence is significant (p=0.028) whereas the differences between times and lines are not (p=0,78 & 0.45 respectively).

3.6 SHG

A method for SHG extraction was established based on Lu et al (2006). Leaf tissue from plants at GS 3, sampled in the middle of the light period, did not exhibit any significant differences in glucosyl residue content, neither in SHG_S nor in SHG_L or SHG_T, see figure 14.

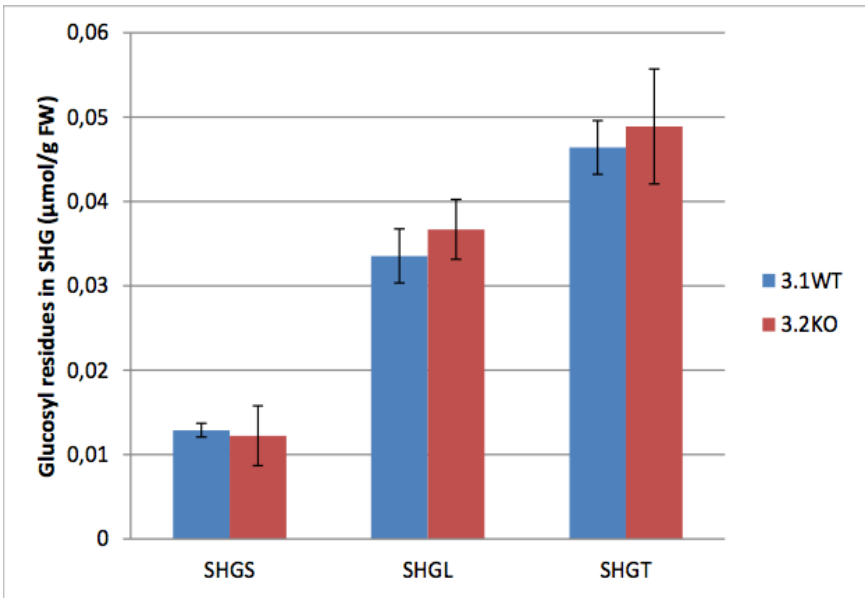

Figure 14. Glucosyl content in 3.2knock-out and 3.1wild-type Arabidopsis leafs in the middle of the light period. Tissue from 2-3 plants were pooled together, differences are not significant (n=3). Error bars denote standard deviation.

4 Discussion

4.1 80 stability during senescence

The results from the senescence experiments indicate that 80 is fully degraded within 2 hours of senescence. Thus, the delayed senescence phenotype observed is most likely due to the effect of a process taking place prior to the leaf detachment (for example build up of a SHG-bound glucose pool) or early on in the experiment (for example wound signalling). As the temporal degradation pattern of 80 is similar to that observed upon induced darkness in whole plant systems (Sörensson, 2010), it is likely to be the induction of darkness rather than the senescence that causes the degradation. It is, however, unclear whether the down-regulation is due to a loss of stabilisation signals (an active kinase), an induced destabilisation (an active phosphatase) or both.

4.2 Influence of flagellin on 80 stability

In seedlings kept in media lacking sucrose for 24h increasing levels of flagellin stabilises wildtype 80. In seedlings kept in sucrose containing media flagellin treatment did, on the other hand, destabilise 80 wild type protein. As a similar pattern was observed for 80(S105A) the flagellin induced stabilisation/destabilisation is most likely under partial control of some of the other sites than the MPK3/6 serine-105 phosphorylation site.

In liquid grown seedlings 80 protein is present when the media contains sucrose and absent in media lacking sucrose both in light and darkness (Marit Lenman, personal communication). Thus, the sucrose dependent stabilisation is likely to be under the control of a pathway separate from that of light sensing (Zhang et al, 2010).

In a study by Thibaud et al (2012) seedlings kept in 6mM sucrose for 7 days exhibited an increased pathogenesis related *PR2* gene expression, through a pathway dependent on SA but down-streams or independent of NPR1. Increasing levels of sucrose resulted in increasing transcription-levels of PR-2, which is generally thought to be a stress response protein. Similarly, increasing levels of sucrose results in increasing levels of 80 (Lenman, unpublished).

The data presented here indicate that 80, as a MAPK-target and potentially involved in sugar signalling, is at the crossroads of abiotic and biotic stress signalling. It would also indicate that these signalling pathways activate 80 partially through different phosphorylation sites. Interestingly a similar interaction between sucrose and flagellin has been discussed by Bolouri-Moghaddam and Van den Ende (2012) in relation to anthocyanin accumulation. Sucrose promotes anthocyanin accumulation and, similar to the effect on 80 protein stability, flagellin inhibits this accumulation.

4.3 Influence of 80 on glucose and sucrose

Over all, the results obtained so far might indicate that 80 is a negative regulator of sucrose. Interestingly the glucose content did not seem to exhibit any differences which, taken together with the sucrose dependent stabilisation discussed above, points to a specific interaction between 80 and sucrose.

In previous work by Lu & Sharkey (2004) *dpe2* mutants in the Wassilewskija (WS) ecotype had approximately 10-fold levels of glucose and approximately 30% higher levels of sucrose, 4h into the light period. Lu et al (2006) investigated *phs2* mutants in Landsberg *erecta* (Ler) ecotype and found a 4-fold increase in glucose and a similar change in sucrose content at the same time of the day.

As 80 might function as a regulatory protein, and thus only having an indirect effect on the PHS2 enzyme activity, a less significant effect on sugar levels would be expected. Interestingly, as both *dpe2* and *phs2* exhibited major differences in glucose content, an effect on glucose would have been expected in 80knock-out as well. The fact that the trend is towards disturbed sucrose levels could indicate that there is a specific interaction between sucrose and 80, or possibly that 80 is interacting with the less well characterised PHS1, though the later is unlikely. Another possibility is the presence of a homologous protein with partial functional over-lap. A candidate for such a protein is the homologous protein 15, however, 80 and 15 exhibit different expression patterns with 80 having a stronger expression in senescing and cauline leafs as well as in mature pollen, whereas 15 has a stronger expression in the shoot apex and the floral bud (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Furthermore both *dpe2* and *phs2* showed significant increases (100 and 10 fold, respectively) in maltose content during the dark period. Thus it would have been interesting to measure the maltose content in 80ko plants as well. Unfortunately the assay used in this work failed to give meaningful results for maltose content, for unknown reasons. It should also be noted that, as discussed by Fettke et al (2009), there is some variation between ecotypes in the expression of starch-related enzymes.

Sucrose has recently been shown to be a global regulator of plant responses to nutrition deficiency (Lei & Liu, 2011; Lei et al, 2011). Sugars have also gained attention as signalling compounds involved in plant innate immunity. As reviewed by Bolouri Moghaddam & Van den Ende (2012), high sucrose:hexose ratios appears to be an important factor in sugar signalling. This relationship is partly regulated by invertases and thought to influence defence responses, specifically inducing anthocyanin production and accumulation of reserve compounds (Bolouri-Moghaddam et al, 2012).

As plants seems to react to the light-dark transition in similar way as to carbon depletion, it's intriguing that 80knock-out night-time samples did exhibit a significant increase in the sucrose:glucose ratio. Even though fructose was not measured, from Lu at al (2006) and Lu and Sharkey (2003), fructose levels seems to follow the same pattern as glucose in wild-type Ler and WS, as well as in *dpe2* and *phs2*, but at much lower concentrations. Thus, this might point towards negative regulation of 80 over the sucrose:hexose ratio. This is a bit surprising as 80 is degraded in darkness (Sörensson, 2010), though the difference might be due to mechanisms at play prior to the degradation of 80.

The differences in sucrose content might be due to small differences in GS development, however, 80knock-out and wild-type doesn't seem to exhibit any significant differences in the time it takes to initiate bolting. These are, on the other hand, rather stochastic observations and a detailed analysis of a large (n>50) number of plants, as described by Boyes et al (2001), might reveal some differences.

One might also question the efficiency of the K-MASUG assay, as the maltose part never yielded meaningful results. For sucrose and glucose, on the other hand, the assay seems to work very well with the standard curves usually having R^2 values exceeding 0,99 within the recommended range (not shown). In general, the extraction method used clearly benefited from bead-beater homogenisation and pooled tissue from multiple plants (GS6, figure 10) as this resulted in much smaller standard deviations compared to when mortars were used (other GS, figure 10). Further studies would also benefit from high-performance chromatography analysis (for example HPAEC-PAD) to get at complete picture of the different metabolites, especially sugars.

4.4 SHG

No significant interaction between 80 and the SHG pool can be distinguished from the results. However, the glucose residue content of the SHG_L pool seems to be 2-3 times larger than that of the SHG_S pool, in contrast with previous work by Lu et al (2006) were the SHG_L pool was smaller. This difference might stem from the different ecotypes used or the diurnal changes in carbohydrate metabolism. Furthermore, the same authors described a 25% increase in the glucosyl residue content in *phs2* mutants. As previously discussed, the small increase observed in the 80knock-out would be reasonable given that it doesn't interact directly with the SHG-pool, though it is hard to prove statistically.

Further division of SHG_L into subfraction I & II might reveal detailed information, since subfraction II is not supposed to be present in the cytosol, and thus will not interact directly with PHS2. Interestingly, a novel protein, HIP1.3, with high affinity for SHG's was recently identified by Fettke et al (2011) adding further complexity to the SHG metabolism.

4.5 Further studies

In vitro studies establishing the mode of interaction between 80 and PHS2 would be of great interest. Also, in general, further phenotype studies on levels of sucrose, glucose and SHG bound glucose should focus on stress conditions, for example pathogen infection and drought.

2D-PAGE analysis of 80 protein dynamics would be interesting. 2D gels can differentiate between different phosphorylation sites, which would be especially valid in relation to the apparent antagonistic interaction between sucrose and flagellin. Studies of double mutants in 80 and different sugar signalling proteins (for example the sucrose transporter SUC2), or 15 would also be of interest. Furthermore, interactions with ethylene and anthocyanin accumulation might also yield interesting results. Also, high-performance chromatography analysis of total SHG residues would be interesting, as glucose makes up a small proportion of the total SHG content.

As RGPs have been speculated to interact with SHG's, and have an important role in pollen maturation, one might further speculate that the high expression of 80 in mature pollen could be of interest.

The 80 complementation lines should be selfed to yield homozygous lines and analyzed for expression of 80 prior to confirmation of an 80 phenotype.

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