Plastid-to-nucleus communication

Paulina Sowinska

Umeå Plant Science Centre
Department of Forest Genetics and Plant Physiology, SLU, Umeå

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Faculty of Forest Sciences, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Umeå

Author: Paulina Sowinska

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Supervisors: Dr Åsa Strand, Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-901 87 Sweden and Dr Karin Ljung, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, SE-901 83 Sweden

Examiner: Prof Thomas Moritz, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, SE-901 83 Sweden

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Introduction.

The plant cell contains two distinct organelles, which have their own genomes where genetic information is stored. Such division was due to the endosymbiotic theory, which claims that mitochondria and plastids entered the ancestor cell as prokaryotic organisms. During evolution the vast majority of genetic information from organelles has been exported to the nucleus. That is why mitochondrial and chloroplast genomes do not contain a full set of housekeeping genes. Around 95% of the 3000 proteins which are detected in the chloroplast are encoded in the nucleus. Although the majority of genes are nuclear encoded, a significant number of genes are encoded by the chloroplastic and mitochondrial genomes. Some of the protein complexes have subunits which are products of nuclear and organellar genes, meaning that it must be a tight coordination between those compartments. They must communicate to have proper expression from their genomes. It was discovered that information flows from nucleus to plastid, called anterograde signalling and in opposite direction, from plastid to nucleus, known as, retrograde signalling. Anterograde mechanisms coordinate gene expression in the organelle with cellular and environmental cues that are perceived and choreographed by genes in the nucleus. This type of traffic includes nuclear-encoded proteins that regulate the transcription and translation of organellar genes. Retrograde signalling, coordinates the expression of nuclear genes encoding plastid proteins with metabolic and developmental state of the plastid and mitochondria (Pinas Fernandez and Strand, 2008, in press). Communication between plastids and the nucleus is necessary for the initiation of chloroplast development in the light, and also for the ability of the plant cell to respond correctly to fluctuations in the environment (Strand et al., 2003). The developmental and metabolic status of plastids affects the expression of nuclear genes that encode plastid proteins (Larkin et al., 2003). It has been shown that plastid signals are also important for efficient metabolism and leaf development (Surpin et al., 2002). Genetic, physiological and biochemical studies have identified several retrograde signalling pathways as demonstrated in Figure 1: the redox state of plastoquinone, the reactive oxygen species, and intermediates of the tetrapyrrole pathway.

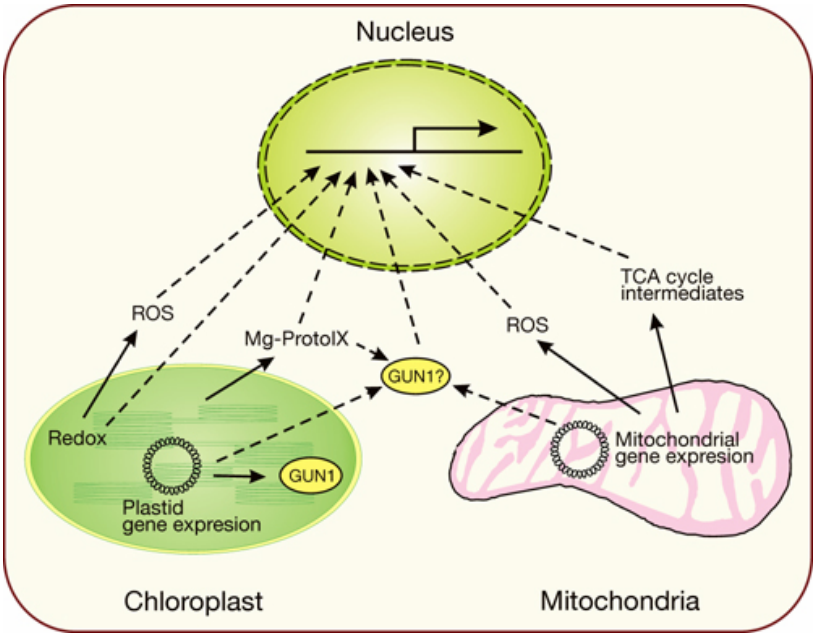

Figure1 *Model of retrograde signalling between the organelles and the nucleus. The organelles produce multiple signals at different times of their development, and in response to changes in the environment, that orchestrate major changes in nuclear gene expression (Pinas Fernandez, Strand, 2008 in press).*

The changes in the redox poise of the electron transport system between photosystem II (PSII) and the cytochrome b6/f complex will trigger expression of nuclear encoded genes. The redox state of the connecting electron carrier, the plastoquinone pool, may be viewed as a plastidic photon-sensing system used to adjust gene expression to the intensity of irradiation (Beck, 2001). There is convincing evidence that the redox state of the plastoquinone pool is also instrumental in controlling the expression of nuclear genes; i.e., it is the start site of a plastid-nucleus connecting signalling pathway that enters cytosol/nucleus and there, by modifying factor(s), alters gene expression (Beck et al., 2001).

Another example of retrograde signalling is phosphorylation of specific proteins in thylakoids membrane, which in turn influence expression of nuclear genes. Also by using different kinds of chloroplast-specific inhibitors of translation the expression level of nuclear-encoded proteins related to photosynthesis will decrease (Beck et al., 2005).

It was shown that reactive oxygen species generated in chloroplasts regulate the expression of nuclear genes. Under stress conditions such as high light chloroplast produce singlet oxygen in PSII and hydrogen peroxide in water-water cycle. Singlet oxygen thus qualifies as a plastid generated signal that could play a specific role in the activation of a genetically determined stress response program (op den Camp et al., 2003). Hydrogen peroxide is thought to diffuse as freely as water across biological membranes and therefore could directly interact with extraplastidic

signalling components (Beck et al., 2005). Not only production of reactive oxygen species plays role in signal transduction but also changes in concentration. Increases in foliar H_2O_2 concentrations are important for the induction of the ascorbate peroxidase gene *APX2* (Strand, 2004).

The last type of retrograde signal transduction, and the mainly investigated in this project is mediated by tetrapyrroles, which accumulate under stress conditions affecting the structure and function of the thylakoid membrane and serves as plastid signal to regulate the expression of nuclear genes for photosynthetic proteins (Strand, 2003). Such type of communication was discovered using *genome uncoupled (gun)* mutants. Five mutants have been identified (*gun1-5*) that express nuclear-encoded photosynthetic genes in the absence of proper chloroplast development (Mochizuki et al., 2001). Characterization of the five *gun* mutants revealed that two different signalling pathway are affected, the GUN1 and GUN2-5 pathway, (fig.2).

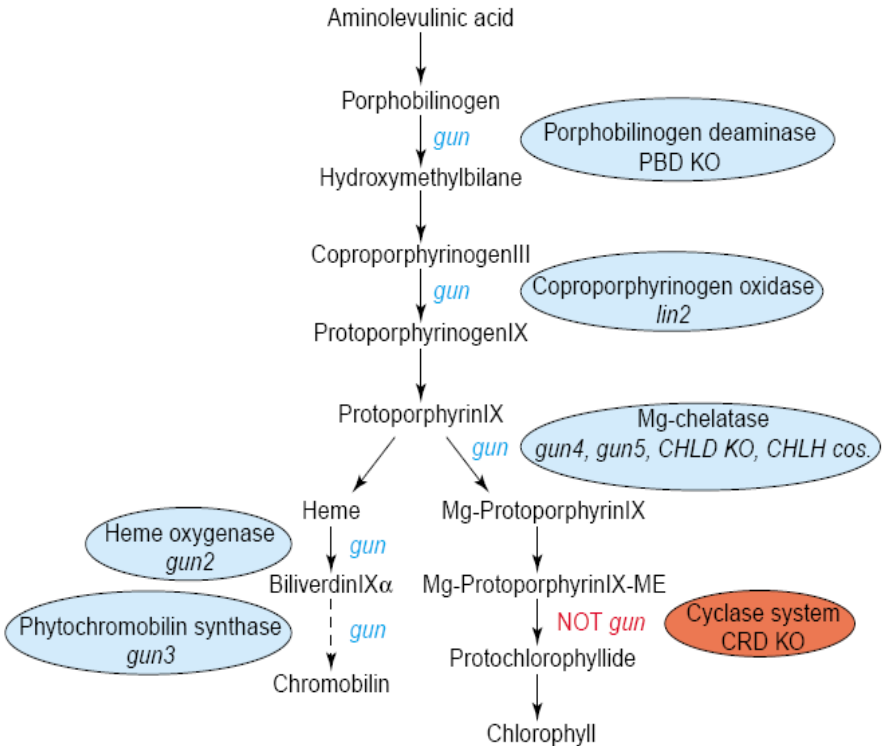

Figure2 *The tetrapyrrole biosynthetic pathway. Steps of the pathway defined by mutants are indicated. All mutants tested with different lesions in the pathway upstream of Mg-ProtoIX demonstrated a gun phenotype (shown in blue). By contrast, a T-DNA knockout (KO) mutant downstream od Mg-ProtoIX did not exhibit a gun phenotype when grown on norflurazon (shown in red) (Strand, 2004).*

In all living organisms from bacteria to mammals, which use oxygen, heme plays key role in molecular and cellular processes. Intracellular heme concentration tightly depends on oxygen tension, so in anaerobic growth conditions heme amount is decreased and in aerobic growth conditions heme concentration goes up. It is also known as a secondary signal for oxygen. It has been found in yeast *Saccharomyces cerevisiae* that heme influence expression of genes which encode function, required for respiration and for controlling oxidative damage, by the heme activator protein Hap1. Hap1 is a DNA binding transcriptional activator (Zhang et al., 1999), which contains of three domains: the heme domain, the DNA binding domain, and the activation domain. Hap1 activity is highly responsive to heme concentration; increasing heme concentration increases Hap1 transcriptional activity, whereas decreasing heme concentration decreases Hap1 transcriptional activity (Zhang et al., 1999). Hsp90 and Hsp70 are critical for the precise regulation of Hap1 activity by heme. When heme concentration increases, heme enhances the interaction of Hsp90 with Hap1 and binds to Hap1, causing conformational changes in the multichaperone-Hap1 complex and leading to Hap1 activation (Lan et al., 2004). It was shown that heme synthesized in mitochondria regulate transcription of nuclear genes encoding mitochondrial proteins. The analogical situation was found in higher plants. Mg-protoporphyrinIX is known as a plastid signal emitting while treating plants with different stress stimuli. It accumulates under stress condition in cytosol and was found to trigger expression of nuclear and plastid genes. The Mg-ProtoIX released by chloroplasts must be bound by regulatory protein to further transmit the stress signal to nucleus. It was demonstrated *in vitro* that two of the HSP90 proteins, HSP81-2 and CR88 interacted with Mg-ProtoIX and that the binding is direct. These identified interactions strongly suggest that HSP90 proteins may be components of a regulatory complex similar to the yeast system responding to the accumulation of the plastid signal and controlling nuclear gene expression in response to stress conditions (Kindgren et al., 2008 submitted Plant Cell). In *Chlamydomonas*, magnesium protoporphyrin IX and magnesium protoporphyrin IX methyl ester were shown to substitute for light in the induction of nuclear gene HSP70 (Kropat et al., 2000). It was discovered that the cis-acting sequence element is employed for induction of HSP70a by both MgProto and light, lending support to the model that light induction of this gene is mediated via MgProto (von Gromoff et al., 2006).

The goal of this project was to identify downstream components communicating the Mg-ProtoIX signal to the nucleus. Two approaches were investigated to identify additional components in the Mg-ProtoIX-mediated pathway: the suppressor mutant, sog2, and the interaction between Mg-ProtoIX and HSP81-2. The purpose of the suppressor screen was to identify mutants where the *gun5* phenotype is lost, which indeed can reveal downstream components targeting the Mg-ProtoIX mediated signal to the nucleus. The *gun5* mutation was crossed into a transgenic line containing *LHCB1::Luciferase* promoter reporter construct. The advantage of luciferase activity was used to detect expression of LHCB1. The *gun5xLHCB1::LUC* line, in Columbia background, was mutagenized using EMS. The *gun5* mutation together with *LHCB1::LUC* transgene was integrated to Landsberg to make mapping faster. Map based cloning method was used to narrow down *sog2* mutation, using SSLP and CAPS markers.

The interaction between plastid signal Mg-ProtoIX and heat shock protein HSP81-2 was investigated *in vivo*. Mg-ProtoIX accumulates in stress conditions and influence expression of nuclear encoded genes. In this project expression level of *LHCB* and *COR15* nuclear encoded gene was investigated to answer the question if Mg-ProtoIX and HSP81-2 form a regulatory complex controlling gene expression. Various mutants were used, which have lesions in tetrapyrrole pathway, the Mg-ProtoIX over- and under- accumulating mutants, *crd* and *gun5*, respectively, also in addition to and in combination with a *hsp81-2* mutant. The expression levels were determined using Real Time PCR technique and the conditions that were used trigger Mg-ProtoIX accumulation: low temperature exposure, norflurazon treatment and treatment with far red light.

Materials and methods:

Plant material: Seeds from *Arabidopsis thaliana* wt (ecotype Columbia), *gun5*, *hsp81-2, gun5/hsp81-2, crd, crd/hsp81-2*, and *sog2* were sterilized and plated on the 1xMS medium (Duchefa Biochemie), containing 2% sucrose (for Low temperature and Norflurazon experiment). In Far Red experiment 1xMS medium without sucrose was used. All seeds were taken to 4^0C over night for vernalization. Then seeds were grown in the growth chamber with continuous white light (22^0C) for 7 days. Afterwards seedlings were shifted to 5^0C for 12 h (such treatment was done for Low temperature experiment). For the Far Red experiment seeds were taken to continuous white light (22^0C) for 24 h, after to dark (22^0C) for 24 h, then for 72 h in Far Red light (controls stayed in dark conditions), and all the seeds were taken to continuous white light (22^0C) for 24 h. For the Norflurazon experiment seeds were grown in 12-h-ligth /12-h-dark cycle (22^0C) for 7 days. All plant material was harvested, frozen in liquid nitrogen and stored at -80^0C.

RNA extraction: RNA was isolated using the RNeasy Plant RNA Kit (E.Z.N.A, OMEGA, bio-tek) according to the manufacturer's instructions. Total RNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Saveen-Werner, Limhamn, Sweden).

cDNA synthesis and Real time PCR: The iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) was used for cDNA synthesis using 1ug of total RNA according to the manufacturer's instructions. cDNA was diluted 10-fold (for the Far Red and Norflurazon experiment) and 20-fold (for the Low temperature experiment). 2 uL and 4 uL of the diluted cDNA was used in a 20 ul iQ SYBR GreenSupermix reaction (Bio-Rad, Hercules, USA). All reactions were performed in triplicates. Thermal cycling consisted of an initial step at 95^0C for 3 min, followed by 40 cycles of 10 s at 95^0C, 30 s at 55^0C, and 10 s at 72^0C.

Primers used in Real Time PCR:

In RT PCR, which is known as a very sensitive method, for every experiment specific housekeeping genes should be used to have comparison between expression of your target gene and a reference gene. This method is mainly about relative quantification of a target gene in comparison to a reference gene (Pfaffl et al., 2001). Housekeeping genes are present in all nucleated cell types since they are necessary for basic cell survival. The mRNA synthesis of these genes is considered to be stable and secure in various tissues (Pfaffl et al., 2001). In Far Red experiment Yellow-leaf-specific gene 8 (YLS8) was used as a reference gene: YLS8 (At5g08290): forward primer: 5′-CATGACTGGGATGAGACCTG -3′; reverse primer 5′- TCCTGAAGAACATGAGGC-3′. For the Low temperature experiment Ubiquitin-like protein was used: Ubiquitin (At4g36800): forward primer: 5′-CTGTTCACGGAACCCAATTC-3′, reverse primer: 5′- GGAAAAAGGTCTGACCGACA -3′. As a target gene, which expression was investigated in the Far red and Norflurazon experiment was: LHCB2.4 (At3g27690): forward primer: 5′- GCCATCCAACGATCTCCTC -3′, reverse primer: 5′- TGGTCCGTACCAGATGCTC -3′, and LHCB1.1 (At1g29920): forward primer 5′- GGAACGGAGTCAAGTTTGGA-3′, reverse primer 5′-CAAAATGCTCTGAGCGTGAA-3′. In the Low temperature experiment as a target gene COR15a (At2g42540) forward primer: 5′-AACGAGGCCACAAAGAAAGC-3′, and reverse primer: 5′- CAGCTTCTTTACCCAATGTATCTGC-3′ was used. SYBR Green dye, which binds to minor groove double-stranded DNA, is used in RT PCR. The fluorescence, which emits is recorded by My IQ (Bio-Rad Laboratories Inc.). For the mathematical model it is necessary to determine the crossing points (CP) for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background (Pfaffl et al., 2001). The output data were converted into Microsoft Excel. Then data were transferred and analysed in linear regression software (LinRegPCR). The samples which didn't have PCR efficiency between 1, 8 -2, and CP values were various between each triplicates, were removed from further calculations. The relative expression ratio (R) of a target gene is calculated based on E and the CP deviation of an unknown sample versus a control, and expressed in comparison to a reference gene. Formula which was used is given below:

$$Ratio = \frac{(E_{\text{Target}})^{\Delta \text{CP}_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control - sample)}}$$

Plant material and methods for mapping: The *gun5* mutation was crossed into a transgenic line containing *LHCB1::Luciferase* promoter reporter construct. The advantage of luciferase activity was used, and the expression of LHCB1 can be monitored in the quick way using Typhoon scanner (Amersham Biosciences). Luciferase reacts with luciferin and converts it to oxyluciferin with light emission (Fig.4).

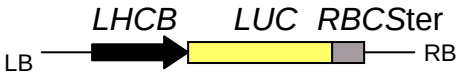

Figure3. Promoter reporter construct.

http://www.bioart.co.uk/lux/image5IO.JPG

Figure4. The luciferase reaction.

The *gun5xLHCB1::LUC* line, in Columbia background, was mutagenized using EMS. The *gun5* mutation together with *LHCB1::LUC* transgene was integrated to Landsberg to make mapping faster. The M_2 seeds were sown one by one and grown on 1xMS (Duchefa Biochemie) medium containing 5uM Norflurazon. Then seeds were screened for luciferase activity. Sog2 is a dominant mutation, with a strong suppression of the *gun5* phenotype, so it should be mapped on wt. In screening only samples with strong signals were transferred to 1xMS with 2% sucrose for regeneration. Afterwards seedlings, which restored photosynthesis, were transferred to the soil, and one leaf was taken from each plant for DNA extraction.

DNA extraction: DNA was isolated using Extraction buffer (200 mM Tris HCl, ph=7,5, 250 mM NaCl, 25mM EDTA, 0,5% SDS). Extracted DNA was stored in 100ul TE.

PCR reaction: PCR reaction was performed using MJ Mini Thermal Cycler (Bio-RAD).PCR program was as follow: 5 min at 95^0C, followed by 40 cycles of 30 s at 95^0C, 30 s at 55^0C, 30 s at 72^0C , and 10 min. at 72^0C , 4^0C forever. For some of the primers annealing and extension temperature was different according to various product lengths.

Tab.1.Primers tested for mapping:

Primer	**Primer sequence**
LFY3	**F**: 5′- GACGGCGTCTAGAAGATTC-3′, **R**: 5′-TAACTTATCGGGCTTCTGC-3′,
DFR.1	**F**: 5′-TGTTACATGGCTTCATACCA-3′, **R**: 5′-AGATCCTGAGGTGAGTTTTTC-3′,
RBCS-B	**F**: 5′- TGAATGGAGCGACCATGGTGGC-3′, **R**: 5′- TATCGACAATTTATTGTGG-3′,
SO191	**F**: 5′- CTCCACCAATCATGCAAATG-3′, **R**: 5′-TGATGTTGATGGAGATGGTCA-3′,
PHYC	**F**: 5′-AAACTCGAGAGTTTTGTCTAGATC-3′, **R**: 5′CTCAGAGAATTCCCAGAAAAATCT-3′,
T2L5.3	**F**: 5′GCTGCGAAGGCTGAATGAAG-3′, **R**: 5′-TCGCCGGGAAAAACAGTAAC-3′,
CUE1	**F**: 5′-TCTCGTTCTGATGGCTCCTGTG-3′, **R**: 5′GTGTAACCGGTGATACTCTCGCC-3′,
T18F2-SP6	**F**: 5′AGCTTCGATAACAAACTCACC-3′, **R**: 5′-AGAAGATAAATCAACTAAACAAAATG-3′,
F13K20-T7	**F**: 5′-TTTGTGCAATTTATTAGGGTAG-3′, **R**: 5′ATTTGCAGAAGTTGAAGTTGGTC-3′,
NGA76	**F**: 5′-AGGCATGGGAGACATTTACG-3′, **R**: 5′-GGAGAAAATGTCACTCTCCACC-3′,
NGA139	**F**: 5′-GGTTTCGTTTCACTATCCAGG-3′, **R**: 5′-AGAGCTACCAGATCCGATGG-3′,

Results:

The Mg-ProtoIX triggers repression of *LHCB* expression under norflurazon treatment.

A large number of nuclear encoded genes are regulated by accumulation of Mg-ProtoIX. This plastid signal accumulates under different stress conditions. The *LHCB* gene was used as a marker gene to see whether the expression of nuclear encoded photosynthesis genes is repressed in response to the accumulation of the plastid signal Mg-ProtoIX in cytosol. The *Lhcb* gene family in green plants encodes several light-harvesting Chl *a/b* binding (*LHC*) proteins that collect and transfer light energy to the reaction centres of PSII (Teramoto et al., 2001). In this project different stress conditions were used, which triggered Mg-ProtoIX accumulation such as low temperature exposure, norflurazon treatment and treatment with far red light followed by white light exposure. Two different mutants, with lesions in defined step in tetrapyrrole biosynthesis were used, the Mg-ProtoIX over- and under- accumulating mutants, *crd*, and *gun5* respectively. I also included the *hsp81-2* mutant to test for gun phenotype according to model presented in fig9, the crd/hsp81-2 and gun5/hsp81-2 double mutants. Double mutants were chosen to check if particular genes act in the same signalling pathway or in parallel pathways. The mRNA levels of the mutants were determined by real time PCR. *LHCB1.1, LHCB2.4 and COR15a* mRNA levels were compared with reference genes. I used *Ubiquitin* and, *YLS8*, genes which show the same expression in controls and treated samples.

Norflurazon is a herbicide, which inhibits phytoene desaturase in carotenoid biosynthesis. The photooxidation caused by noflurazon treatment is limited to the plastid and results in complete destruction of the thylakoid membrane (Puente et al., 1996). The *crd* and *gun5* mutants show different phenotype after norflurazon treatment (Fig.1). The *crd* mutant display expression of *LHCB2* similar to wild type, due to the same reaction under stress condition, Mg-ProtoIX will be accumulated in cytosol in both genotypes, so it will suppress the *LHCB2* expression. The *gun5* mutant did not show wild type phenotype. This mutant under-accumulate Mg-ProtoIX under stress condition, and Mg-ProtoIX is not emitted to cytosol, which causes de-repression of nuclear encoded gene. The *crd* has the wild type phenotype and *crd/hsp8-2* also shares wild type phenotype. So repression of *LHCB2* in mentioned genotypes is similar. The *gun5/hsp81-2* double mutant shows gun5 phenotype. The expression level of *LHCB2* in those phenotypes is comparable. The *hsp81-2* single mutant did not show gun5 phenotype, so expression of *LHCB2* is more repressed compared to *gun5* mutant. The ubiquitin is used here as a reference gene, which expression should be the same in all samples (Fig.2).

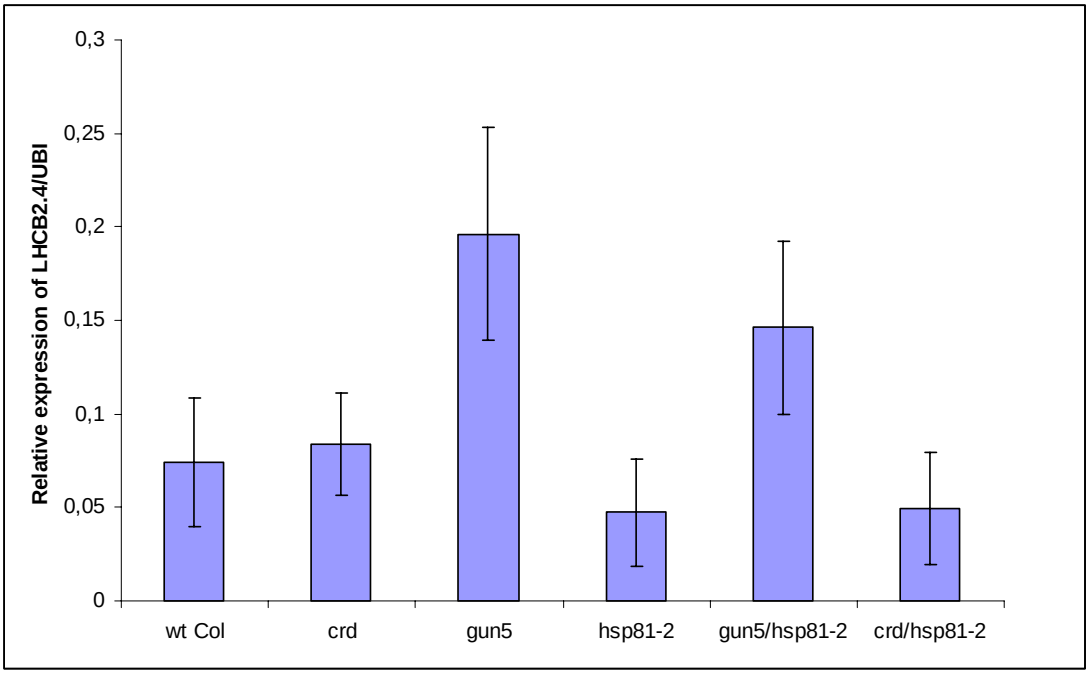

Figure1. The relative expression of LHCB 2.4 gene in wild type (Colombia ecotype) and mutants following norflurazon treatment. The expression was related to the amount present in the respective non-norflurazon grown sample. Transcription of the Ubiquitin like protein, RCE1 (At4g36800) was used as internal standard in the different cDNA samples. Each bar represents the mean of expression of triplicates with standard deviation and error bars.

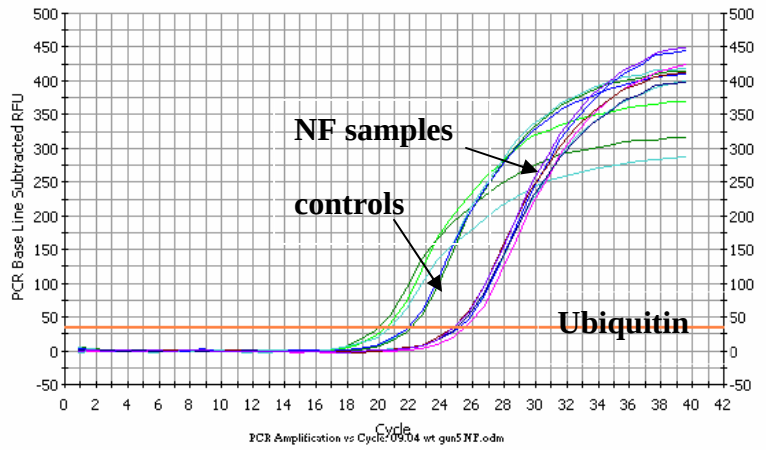

Figure2. Data from real time PCR. Curves illustrate DNA amplification, which is emitted as fluorescence from Sybr green. Red line is so called Cp (crossing point) it shows number of cycles where amplification rise above the background fluorescence. In gun5 mutant the Cp of LHCB2 is greater in the controls than in the norflurazon treated sample. Ubiquitin is used here as a reference gene, which expression should be the same in all samples (controls and NF treated).

The Mg-ProtoIX acts as a negative regulator of photosynthetic genes under FR block-of-greening treatment.

The far red light treatment was the next stress condition used to investigate *LHCB1* expression level in the different mutants. The FR block-of-greening treatment leads to an enhanced accumulation of Mg-ProtoIX in cytosol, which will in turn repress expression of *LHCB1* in wild type. It is due to the fact that Mg-ProtoIX is known to be a negative regulator of photosynthetic genes. The expression level of *LHBC1* is strongly suppressed in wild type and in *crd* mutant. The *gun5* mutant did not show wt phenotype. The *gun5* mutant demonstrates the de-repression of *LHCB1* expression. In contrast, *hsp81-2* mutant showed high repression of marker gene. The *gun5/hsp81-2* double mutant showed *gun5* phenotype with comparable expression level of *LHCB1*. The level of repression in *crd/hsp81-2* was greatly lower than in *crd* single mutant.

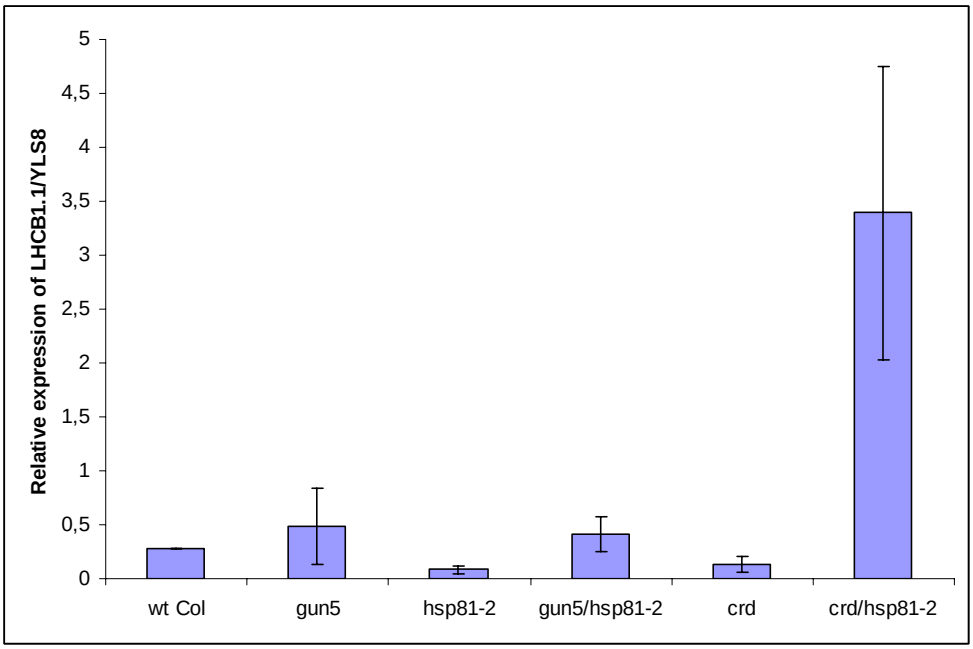

Figure3. The relative expression of LHCB 1.1 gene in wild type (Colombia ecotype) and mutants following far red light treatment. The expression was related to the amount present in the respective dark grown sample. Transcription of the Yellow-leaf-specific gene,YLS8 (At5g08290) was used as internal standard in the different cDNA samples. Each bar represents the mean of expression of triplicates with standard deviation and error bars.

Low temperature causes changes in Mg-ProtoIX accumulation, which influence expression of cold regulated genes.

The low temperature treatment was the last stress condition used to see changes in *COR15a* expression levels in the different mutants (Fig.4), which is known as cold response gene. The *COR15a* gene reacts positively to stress condition, so induction of these genes is visible under low temperature treatment. In contrast, photosynthetic genes such as *LHCB* react negatively to stress. In the *crd* mutant *COR15a* induction was similar to wild type. In these two genotypes Mg-ProtoIX accumulates in cytosol under low temperature treatment, so plastid signal can be targeted to nucleus and influence expression of specific genes. The induction of expression of *COR15a* is lower in *gun5* compared to wild type. The *gun5* and *hsp81-2* mutants did not show wild type phenotype. In *hsp81-2* induction of *COR15a* is slightly higher than in *gun5* mutant. In the *hsp81-2* mutant possibly there is an effect on the induction of the COR15a expression. The Mg-ProtoIX is emitted to cytosol and is bound with HSP81-2, this interaction can be sufficient to trigger induction of COR expression. The *gun5/hsp81-2* double mutant showed *gun5* phenotype with comparable expression level of marker gene. The *crd/hsp81-2* reveals comparable expression of *COR15a* to *crd* single mutant. The induction of both genotypes is similar to wild type.

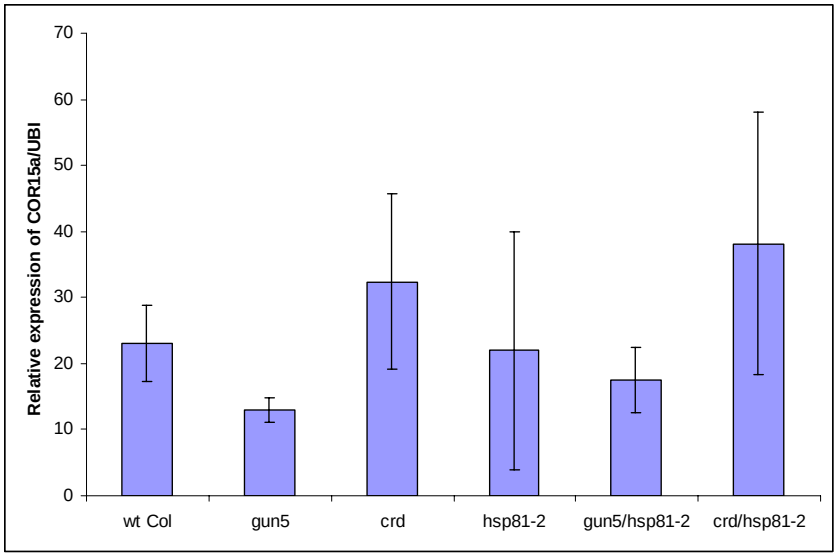

Figure4. The relative expression of COR15a gene in wild type (Colombia ecotype) and mutants following low temperature treatment. The expression was related to the amount present in the respective warm grown sample. Transcription of the Ubiquitin like protein, RCE1 (At4g36800) was used as internal standard in the different cDNA samples. Each bar represents the mean of expression of triplicates with standard deviation and error bars.

Mapping of *sog2* mutation, which is positioned on the lower arm of chromosome V.

The forward genetics was used as a tool to find a gene which is mutated starting from phenotype. Map-based cloning strategies use the fact that, as distances between the gene of interest and the analyzed markers decrease, so does the frequency of recombination (Peters et al., 2003). Next, two markers are found that are about 5% recombination apart and define a genetic interval containing the mutation (Lukowitz et al., 2000). Then mutation must be narrow down by designing new sets of primers, which should be more tightly linked to region of interest. The big advantage to map-based cloning is that it is a process without prior assumptions. Essentially, one is looking at all of the genes in the genome at the same time to find the ones that affect the phenotype of interest (Jander et al., 2002).

The sog2 mutation was isolated in a screen where the gun5 mutation was crossed into a transgenic line containing a *LHCB1::Luciferase* promoter reporter construct. Then the *gun5xLHCB1::LUC* line was mutagenized using EMS. M_2 seeds were used for the suppressor screening. The M_2 seedlings were grown on norflurazon and screened for loss of luciferase activity. The *sog2* mutation was crossed to a Landsberg line where the *gun5* mutation and the *LHCB::LUC* construct had been integrated. Luciferin screening was done on the mapping population and from the population individuals with strong luciferase signal were chosen (Fig5) for further investigation, because *sog2* is a dominant mutation and has to be mapped on wild type.

Then using various markers, such as SSLP and CAPS, parental generation was tested. Those markers, which generate polymorphism (PHYC, RBSC-B, SO191, DFR.1, LFY3) between Colombia and Landsberg, were then tested on mapping population (Fig.6). The next step was to confirm our hypothesis that sog2 is suppressor of gun5. Just to make sure that we did not have any seed contamination the Real Time analyses were done. Different seeds batches of *sog2* were tested (Fig. 8). The *sog2a and sog2org* showed decreased level of *LHCB2.4* expression. *Sog2* and *sog2P2II* revealed similar level of marker gene expression.

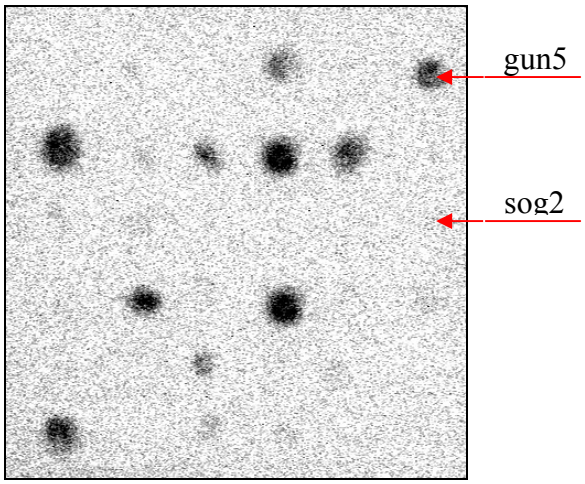

Figure5. Luciferase screening for sog2 (suppressor of gun5) mutation. The sog2 mutation is dominant and that is why it has to be mapped on wild type. The individuals with strong signals were chosen to further investigation.

Primer	Number of individuals	Number of Ler homozygous	Number of Col homozygous	Number of heterozygous	% recombination	Heterozygous in F2
PHYC	61	35	0	26	21,3	1, 5, 6, 7, 8, 15, 16, 23, 26, 28,36, 42, 43, 44, 45, 49, 50, 51, 54, 57, 58, 59, 62, 63, 64, 65
PHYC-2	35	20	0	15	21,4	6, 7, 8, 9, 10, 45, 49, 50, 31, 15, 21, 43, 23, 27, 36,
SO191	54	40	1	13	13,8	6, 7, 8, 23, 31, 45, 46, 54, 57, 58, 59, 63, 64
RBSC-B	51	20	3	28	33,3	2, 5, 7, 6, 8, 10, 11, 12, 23, 31,34, 24, 26, 27,28, 29, 36, 40, 42, 43, 44, 45, 46, 47, 48, 58, 59,65
DFR.1	58	25	1	32	29,3	2, 3, 5, 6, 7, 8, 21, 23, 24, 26, 28, 29, 31, 32, 34, 36, 37, 40, 44, 45, 46, 48, 49, 50, 51, 54, 57, 58, 59,63, 64, 65
LFY.3	58	17	12	29	45,68	42, 2, 3, 6, 12, 13, 18, 20, 23, 26, 27, 28, 30, 31, 32, 36, 37, 39, 40, 44, 46, 48, 49, 53, 54, 56, 57, 61, 64

Figure6. Map based cloning was used as a method to map sog2 mutation. SSLP and CAPS markers were used to detect polymorphism between Columbia and Landsberg ecotypes. All this markers are found on chromosome V.

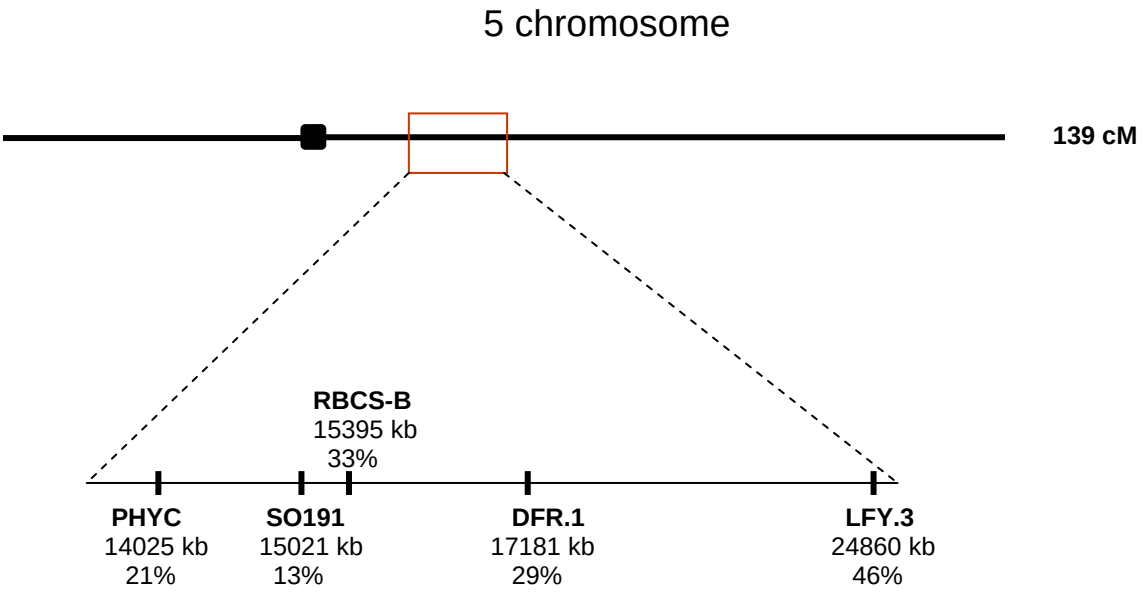

Figure7. SSLP and CAPS markers were used to map sog2 mutation and which are situated on the chromosomeV.

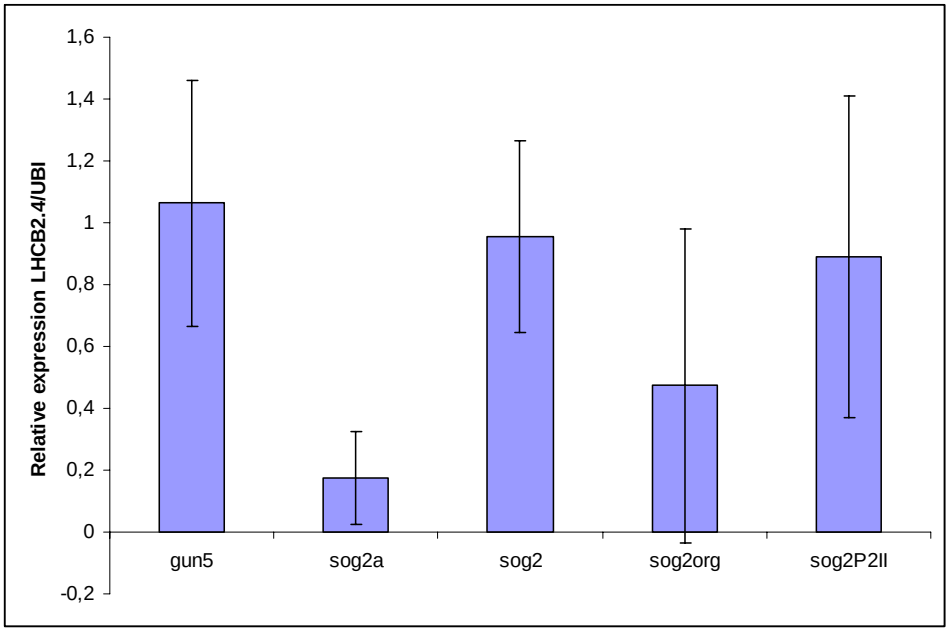

Figure8. The relative expression of LHCB 2.4 gene in gun5 and sog2 mutants following norflurazon treatment. The expression was related to the amount present in the respective gun5 norflurazon grown sample. Transcription of the Ubiquitin like protein, RCE1 (At4g36800) was used as internal standard in the different cDNA samples. Each bar represents the mean of expression of triplicates with standard deviation and error bars. Different seeds batches of sog2 were sown to see if this genotype is indeed the suppressor of gun5.

Taken these results together from two approaches (the gun5 suppressor mutant, sog2, and the interaction between Mg-ProtoIX and HSP81-2) working model was created as shown in Fig.9.

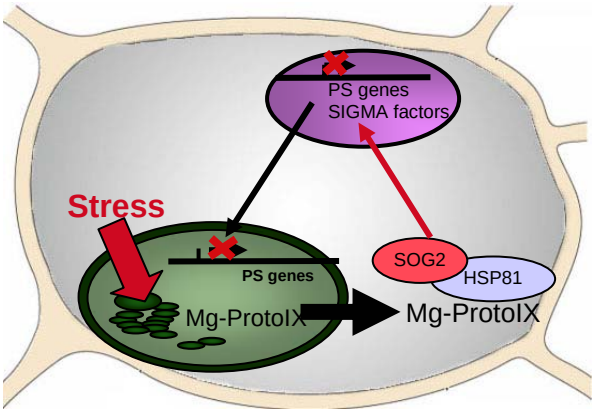

Figure9. Model where the sog2 takes part in retrograde signalling pathway.

Discussion:

In this project I was interested in one of the retrograde communication between the plastid and the nucleus. It is an intermediate in tetrapyrrole biosynthesis, Mg-ProtoIX. The goal of this project was to identify downstream components communicating the Mg-ProtoIX signal to the nucleus. Two approaches were used to identify additional components in the Mg-ProtoIX-mediated pathway: a biochemical approach which revealed the interaction between Mg-ProtoIX and HSP81-2, and mapping of the *gun5* suppressor mutant, *sog2*.

Three experiments were performed, with different stress conditions: norflurazon, far red light, and low temperature treatment. Mg-ProtoIX under such conditions accumulates in cytosol and its accumulation triggers large changes in nuclear gene expression (Kindgren et al., 2008 in preparation). It was shown in *Chlamydomonas reinhardtii* that chlorophyll intermediates (Mg-ProtoIX and Mg-ProtoIX methyl ester) induce the nuclear-encoded heat shock genes *HSP70A* and *HSP70B*, mimicking the normal light induction of these genes (Kropat et al., 2000). In Arabidopsis interaction between the plastid signal and Heat Shock protein 90-type proteins was identified using Mg-ProtoIX affinity column (Kindgren et al., 2008 in preparation). My task was to investigate whether the interaction between the plastid signal Mg-ProtoIX and heat shock protein has any biological relevance by affecting on the expression levels of nuclear encoded genes. As a marker gene the photosynthetic gene *LHCB* was used, which expression is repressed by the accumulation of Mg-ProtoIX. In the low temperature

experiment *COR15a* was used as a marker gene. It is also nuclear encoded and expressed specifically under cold stress. It was shown that Mg-ProtoIX acts as a negative regulator of photosynthetic genes, so I expected to get repression of *LHCB1.1* and *LHCB2.4* expression under norflurazon and far-red light treatment in the wild type. The *crd* mutant over-accumulate Mg-ProtoIX and expression of photosynthetic nuclear encoded gene, LHCB is repressed similarly to wild type. In contrast, the *gun5* mutant under-accumulate Mg-ProtoIX so plastid signal is lost under stress condition and expression of nuclear gene *LHCB* is maintained. In norflurazon and far red light experiment the *hsp81-2* mutant revealed very strong suppression of *LHCB* expression. From these results we can not say that the HSP81-2 protein is required for repression of nuclear encoded photosynthetic genes. We expected that *hsp81-2* mutant will display gun5 phenotype. Only under low temperature *hsp81-2* showed induction of *COR15a* lower than in wild type and similar to the *gun5* mutant. There is possibly an effect on the induction of the *COR15a* expression. The *gun5/hsp81-2* double mutant showed *gun5* phenotype with comparable expression level of marker gene. It suggests that both of these components act in the same signalling pathway. The Mg-ProtoIX is emitted to cytosol and is bound with HSP81-2, this interaction can be sufficient to trigger expression of nuclear encoded genes. The reason why we did not get a clear result can be explained by the fact that HSP81-2 is a member of gene family, which share at least 85% sequence identity along their entire lengths (Krishna et al., 2001). When only one gene was mutated, the rest of genes can take over its function. To obtain expected phenotype all genes from HSP90 family should be down regulated at the same time.

The goal of the second approach was to map the *sog2* mutation. This mutation is situated on the lower arm of V chromosome (Fig7). The lowest % of recombination which we obtained was 13%. It was generated by SO191 marker (Fig6), but it is still too high to say exactly where the mutation is. It is also important to decrease number of Colombia and also number of heterozygosity in mapping population. In PHYC (21% of recombination) marker there was no Colombia but still number of heterozygosity was quite high. Thus, the sog2 mutation is between PHYC and SO191. For future work mutation must be narrow down by designing new sets of primers, which should be more tightly linked to region of interest.

Our working model as presented in Fig9. shows that under different stress conditions Mg-ProtoIX is emitted from chloroplast and accumulates in cytosol. We speculate that it can be bound by heat shock proteins (HSP90) and SOG2 protein. Sog2 might be a transcription

factor, similar to HAP1 in Yeast system. In the wild type situation Mg-ProtoIX can interact and activate SOG2 together with HSP81. Mg-ProtoIX is part of a regulatory complex, which can influence expression of nuclear-encoded genes. When we mutate SOG2, it will be activated all the time, so it will not need Mg-ProtoIX to do its job. Maybe it will enter to nucleus by its own and affect expression of specific genes. For future work it is good to knock out expression all four genes from HSP-81 family. It will be done using RNAi, which will silence all genes HSP81-1, HSP81-2, HSP81-3, and HSP81-4. Then RNAi construct will be transformed to wild type, gun5, sog2, and crd background to see what phenotype we will get.

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