UMEÅ UNIVERSITY

Immunodetection studies to characterize the FtsHi3 protease in chloroplasts of Arabidopsis thaliana

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Abstract

FtsHi3 is a protease which can be found in thylakoid membranes[1]; it plays an essential role in chloroplast and plant development [2]. Members of the FtsH protease family are involved in the recycling of D1 protein, located in the PSII[3]. FtsHi3 is, however, proteolytical inactive; its function remains, therefore, unknown. To determine it, the protein has been removed from the *Arabidopsis thaliana* plant, a model organism [4] for photosynthetic plants. The impact of the FtsHi3 protease would be studied, by comparing the abundance of photosynthetic proteins (CP43, Lhca1, PsaD, Lhcb1) in wild type plants and knock-down mutants of this protease. Because of the different properties between those proteins, I did a screening in order to determine which analytical methods would suit each the best. Henceforth, an adapted protocol would be established for each specific protein. Screening is an essential tool before a new experiment, as use of the wrong technique could lead to unreliable results. This hypothesis has been observed during this project whilst comparing two immunodetection techniques onto four selected proteins; although fluorescence could eventually be suitable for quantitative experiments regarding CP43, its total irrelevance has been probed regarding the other proteins. Without a screening, it would've been suggested that results are caused by the mutation, and not the techniques used. Since analysis on *Arabidopsis thaliana* impacts proteome research on photosynthetic plants[5] in general, getting results that are as relevant and accurate as possible is crucial. Not only does it play a role in the plant life cycle, it also extends to life of most of the living species; plants are primary producers of the food chain and are the main contributors of CO_2 uptake. Their optimum development is a first step toward sustainability regarding fauna and flora.

Introduction

A protease is an enzyme breaking down other proteins by cleaving the peptide bond in the primary sequence of its substrate. Just like any other enzyme, it is not consumed during the reaction it is involved in. Its role is to lower the activation energy and, therefore, trigger the targeted reaction faster. Proteases can cleave a specific or broad range of substrates. The substrates of many proteases within plant cells are not known. Among the proteases found in bacteria, plants and animals is the family of FtsH proteases (Filament temperature sensitive H). These membrane-bound enzymes [6] are located in organelles; in plants, they can

be found in mitochondria and chloroplasts [6]. Plant FtsH proteases are involved in the degradation of the damaged D1 protein, the reaction centre protein of the PS II [7–9].
FtsH proteases are ATP-dependent metalloproteases; they possess a so-called AAA ring involved in ATPase activity and a HEXXH motif, corresponding to the active site, where a Zn^{2+} ion binds to [6,10] and. Interestingly, in the plant chloroplast, not only proteolytically active FtsH members have been detected, some members have mutations in the HEXXH motif; a change of one or more amino acids and/or their order in this sequence. Because of this, Zn^{2+} ion, which is a cofactor to the enzyme[7], can no longer bind to the active site, resulting in proteolytically inactive enzymes, FtsHi. Despite these mutations, they still are essential for chloroplast development in *Arabidopsis* [11]; it's been observed that a mutation of these inactive proteases can lead to abnormalities during plant embryogenesis [6,11] or embryo-lethality (i.e. FtsHi1-2)[6,11]. Their functions in the organelle, in spite of their inactivity regarding the D1 protein, is essential and should be studied. The work is focused on the FtsHi3 protease, located in the envelope membrane of the plant chloroplast, and missing the whole HEXXH motif in its sequence. An homozygous knock-down mutant of this enzyme had been generated, called FtsHi3-1 generating a pale seedling phenotype [6]. The main goal is to investigate the impact of this enzyme on the photosynthetic machinery, specifically Photosystem I and Photosystem II; those systems are interconnected, so are the proteins located in there. The four targeted proteins were CP43, PsaD, Lhc A1 and Lhc B1.
CP43 is a protein of the Photosystem II core complex, its name arises from early electrophoretic studies on photosynthetic proteins and stands for chlorophyll protein (CP). PsaD is a reaction centre protein of the Photosystem I. Lhcb1 and Lhca1 are proteins from the light harvesting complexes surrounding the photosystems. Lhcb1 belongs to the Photosystem II and Lhca1 to the Photosystem I.
The work for my project consisted in screening different immunodetection methods onto each of these proteins, plus the FtsHi3 protease via protein tags. From this, the most suitable analytical technique would be used for further experiments directly related to the FtsHi3 function. Immunoblotting was performed during this screening, using fluorescence and chemiluminescence. I've also investigated relative amount of the photosynthetic proteins cited above in the knock-down mutant compared to wild type. To ensure that the observed phenotype is caused by the knock-down mutation and not by random mutations anywhere

else in the genome, a complemented mutant was generated besides, in which the wild type gene has been restored. This recombinant mutant is expected to behave like the wild type. Biological material differs from each plant under the same growth conditions. To differentiate changes between wild type and knock-down plants from biological variations, leaves from three/four different plants were investigated in each type.

Abbreviations

FtsH	Filament temperature sensitive H
PS	Photosystem
AAA	ATPase Associated with various cellular Activities
WT	Wild Type
Mut	Mutant
Comp.	Complemented
Oxp.	Overexpressed
CP43	Chlorophyll-binding Protein
Lhc	Light-harvesting complex
HA	Human influenza Hemagglutinin
gg	GFP-GUS
GFP	Green Fluorescent Protein
WB	Western Blot
LDS	Lithium Dodecyl Sulphate
TBS-T	Tris-Buffered Saline - Tween
HRP	Horseradish Peroxidase
DTT	Dithiothreitol
PVDF	Polyvinylidene Fluoride
cpDNA	Chloroplast DNA

Materials and methods

Before the start of the project, seeds from a four-months-old *Arabidopsis* were germinated for 10 days on MS plates to select Ftshi3-1-homozygous seedlings for further experiments. Wild Type, knock-down mutant (deletion of FtsHi3 gene), complemented (introduction of the WT FtsHi3 gene into the Ftshi3-1-homozygous mutants) and overexpressed (introduction of the WT FtsHi3 gene into the WT) plants had been grown. Gene introduction, named 'Gene recombination' had been done using Agrobacteria containing FtsHi3 in their t-DNA. The plasmid was introduced in the plants by Agrobacterium transformation method [12]. Two different plasmid types, one containing the gene reporters GFP and Gus (Kanamycin resistant), and one containing HA (Hygromycin resistant) had been used.

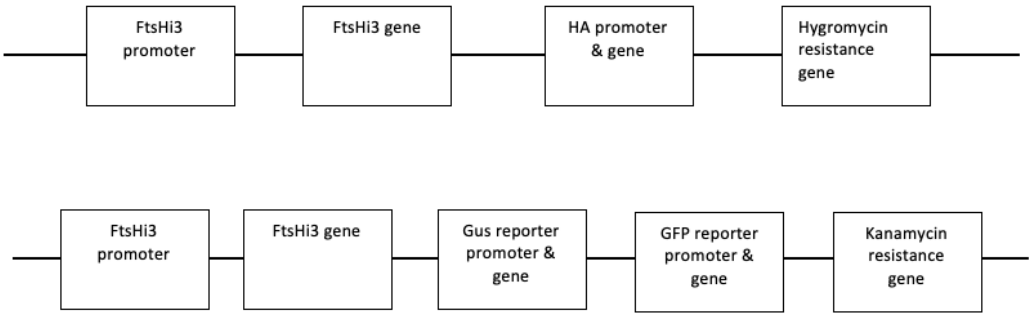


Figure 1: Illustration of HA-containing and gg-containing plasmids

For verifying the similarity in terms of the quantitative pattern (i.e. protein concentration), several complemented and overexpressed lines were grown. The screening has been applied on 4-months-old leaves and 10-days-old seedlings.

Harvesting

My project has started from the harvesting stage; leaves from plants 1, 4 and 7 of the complemented lines containing the GFP & Gus reporters (*i3i3 gg*), plants 9 and 10 of the complemented lines containing the HA-tag reporter (*i3i3 HA*), plants 1, 2 and 3 of the overexpressed lines containing the GFP & Gus reporters (*i3i3 gg WT*) and plants 8, 9 and 10 of the overexpressed lines containing the HA-tag reporter (*i3i3 HA WT*) have been harvested. Seedlings from plants 9 and 10 of complemented lines with HA-tag (*i3i3 HA*) and plants 1 to 10 of the overexpressed line with HA-tag (*i3i3 HA WT*) have been selected.

To make sure that the plasmids have been introduced into the cpDNA, and therefore FtsHi3, the presence of GFP and HA, which tagged FtsHi3 in the samples has been verified. Due to a lack of time, Gus hasn't been studied, hence it won't appear farther in the Results.

Preparation for protein quantification

The total protein concentration for all the plants has been determined via the absorbance of the crude extracts of proteins, using the "Bradford protein assay" procedure. Two leaves, from the *Arabidopsis*' rosettes were selected on each plant and individually frozen. A few seedlings have been frozen as well.

Due to the amount of the leaves and seedlings used for each plant type, the replicates have been named as A1, A2 and B for the leaves for WT and knock-down mutant samples, and from 1 A-C to 10 A-C for the leaves and seedling for the Comp. and Oxp. samples.

I've prepared the crude extracts of my samples by grinding the frozen samples into 100μL of 4xLDS buffer. Because SDS buffer easily precipitates, LDS was preferred.

The grounded samples have been heated (70°C, 10 min) and centrifuged (13 000 RPM, 10 min) for preparing the extract. 5μL of the obtained supernatants have been diluted in 4xLDS (*factor 5*) for the protein quantification preparation. Two blanks of 4XLDS buffer have been prepared besides.

In both the blanks and the diluted samples, 125 μL of DC Reagent I and 125 μL of DC Reagent II have been added, followed by centrifugation (17 000 RPM, 8 min) for isolating the proteins in a precipitated form. After separation from the supernatant (air-dry), I've dissolved the pellets again in 127 μL of Reagent A (mixture of 5 μL DC Reagent S for 250 μL DC Reagent A) before incubation (room temperature, 15 min). 1 000 μL of DC Reagent B has been added to the dissolved solutions for preparing them for absorption measurements at 750 nm.

DC Reagent A contains an "alkaline copper tartrate solution" and DC Reagent B "a diluted Folin reagent" [*] (*Biocompare.com*, 'RC-DC Protein Assay from Bio-Rad'). When DC Reagent A reacts with DC Reagent B, the absorbance of the sample can be measured at 750 nm, mainly due to changes on Tyrosine and Tryptophan. [*]

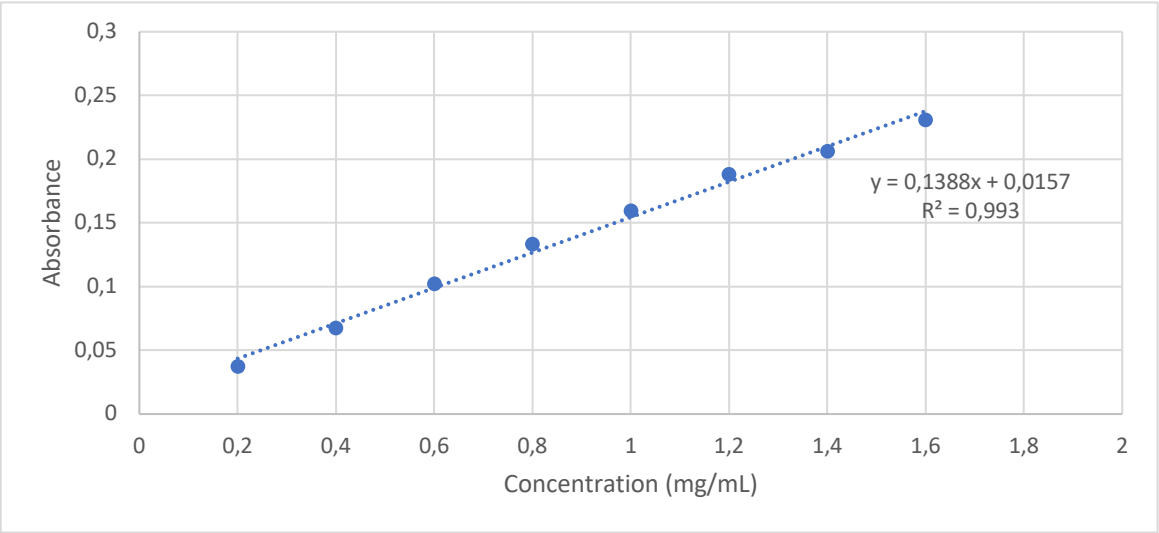
Measurements of absorption have been performed via UV-Spectrophotometer ("*Varian 50 Bio*"), with data displayed via "*Simple Read*" software. The absorbance values of

the total proteins have been converted into concentrations ($A = \varepsilon.l.C$), via the following equation:

y = 0,1388x + 0,0157 *y=Absorbance* *x=Concentration (mg/mL)*

Equation 1: Protein Assay calibration's equation

It corresponds to the linear equation from the following curve. It's been built from samples of Bovine Serum Albumin with known concentrations.



Graph 1: Standard curve, obtained from BSA samples, with known concentrations.

Western blot

From here, two Western Blot methods have been followed; automatic, with the Amersham® WB device, and manual with Bio-Rad's equipment.

The following proteins, with their molecular weights, have been immunodetected.

Proteins	**FtsHi3-1**	**CP43**	**PsaD**	**Lhc A1**	**Lhc B1**	**HA-containing protein in HA+ Ctrl**	**GFP**
Size (kDa)	66	43	18	20,5	20	18-28	27

Table 1: Sizes of the targeted proteins

During this period of time, only automatic method has been used for observing the proteins above. Immunoblots have been prepared for the WT and knock-down mutants.

Detection has been done via secondary antibodies. The membrane containing all the separated proteins was incubated into a solution containing some protein-specific antibodies, to bind onto the targeted proteins. A secondary antibody was then used to bind onto those primary ones and permit their detection. The type chosen (Rabbit/Mouse) was depending on the origin of the primary antibody.

Depending on the visualization technique, the antibody was tagged with a chemical; a fluorescein for visualization via fluorescence, the HRP enzyme for visualization via chemiluminescence.

When excited by light, the fluorescein emits some energy whom the detection indirectly permits that of the proteins. Chemiluminescence relies on the emission of energy via a chemical reaction. Peroxidase catalyses the oxidation of luminol[13] which emits light.

I've prepared samples containing 5μg and 10 μg of total proteins, and a Reducing Loading buffer, which would be mixed with the samples for the loading stage. The buffer contained 29 μL of DTT in 700μL of Loading buffer. Then, a blank containing 100μL of the Reducing Loading Buffer in 100μL of ultrapure water has been prepared. A 1:10 dilution of *Amersham WB Cy5* in ultrapure water has been used to trace our proteins. A 1:20 dilution of MW markers in some blank has been loaded for the gel ladder.

Each sample has been stained in 1μL Cy5 dye and incubated 30 minutes for allowing the dye to bind to the DNA. Then, 20μL of the Reducing Loading Buffer has been added in each tube before heating them (75°C, 10 min). The loading has been executed on an *Amersham WB* gel card for an-hour separation, then the proteins have been transferred onto an *Amersham WB* PVDF card (membrane). The membrane has been blocked, for preventing non-specific binding of the antibodies onto it. To do so, I've incubated it into a 5% skim milk probing block (5g skim milk, 100mL TBS-T). Then, the membranes have been incubated in antibodies, with TBS-T washes in between; the protein-specific primary one for 90 minutes (10μL into 10mL of TBS-T), then the secondary one for 30 minutes (*Goat Anti-rabbit/mouse Cy3*, 5μL into 10mL of TSB-T) for 30 minutes. After drying the membrane, I've visualized the targeted protein via fluorescence, for checking the presence and relative ratio of the targeted protein with the help of its size.

As for determining the presence of the reporter GFP-Gus and HA in the transformed plants, protein separations have been executed on them, via the manual Western Blot method. Samples from the same type have been mixed with 1-3 μL of DTT and heated (75°C, 10 min). The gel has been prepared, with 12/15 % Running gel (40% Acrylamide) depending on the protein's size and run in some 1x Running buffer. A *PageRuler Prestained Protein Ladder* and a blank have been loaded. The transfer has been done to a PVDF membrane with 1x Towbin

buffer. After an-hour blocking, the membrane has been incubated in the primary antibody (4°C, overnight / room temperature, 3h) and eventually a secondary one (room temperature, 30 min), the blot was washed with TBS-T in-between those incubations. The membrane has been analysed under Chemiluminescence by using the *ChemiDoc ™ Touch Imaging System*. Luminol solution, accompanied by a signal enhancer, was spread on the membrane; the presence of Peroxidase catalyses the oxidation of Luminol, generating light.

Results and discussion

Only the WT and mutated samples have been used for immunodetecting the four selected proteins via fluorescence.

Each membrane was incubated first with antibody directed against one protein (CP43, Lhcb1, Lhca1 or PsaD), then with the *Goat Anti-Rabbit* secondary antibody. The images obtained from their immunodetection via fluorescence is shown below. The WB has been done twice (runs 1 & 2), with new diluted samples, for the immunodetection of all the selected proteins except PsaD.

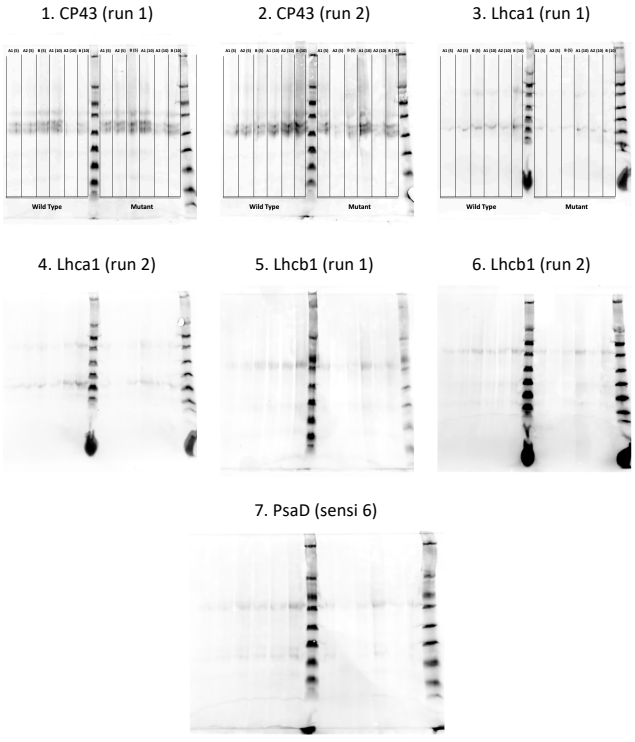


Figure 2: Immunoblot images using antibodies directed the proteins indicated above the gel. The Amersham® WB scan unit was used to perform the procedure.

Figure 3: Left, molecular weight ladder used for the WB. Right, Amersham® electrophoresis & scan unit.

Each membrane picture shown below displays, to the left or right of the proteins' area, the size corresponding to the ladder bands. Replicate names are indicated for each column, with the amount of total proteins in parenthesis. The WT, Mutant, Comp. and Oxp. zones are also displayed. Where a stain/band is displayed so is the secondary antibody and, therefore, the protein it bounded onto; it could be the selected primary antibody as it could be another protein whom the selected secondary antibody can bind onto.

CP43

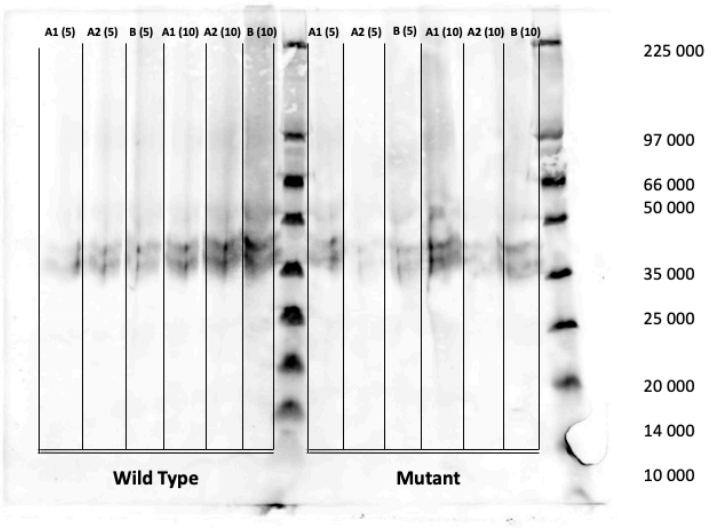


Figure 4: Immunostaining using an antibody directed against the Photosystem II protein CP43 (run 2), visualized via fluorescence.A1, A2 an B correspond to different biological samples (triplicates) of wild type and mutant leaves. In brackets the amount of proteins loaded is given (5 or 10 µg protein).

This blot displays CP43 (43 kDa) very clearly; some bands are located between 35 and 50 kDa. A coherence between the signal strengths and the protein amounts can also be observed for the WT samples; they increase proportionally between each other.
In comparison with the run 1, the signal strengths are similar, although the coherence previously announced doesn't apply for the first run; signal strength increases proportionally with the protein amount for the blot from the first run, whereas it is inversely proportional for the blot from the second run.
Regardless of this incoherence, the screening regarding CP43 detection led to the following result: an automatic run of WB followed by a fluorescence analysis could be applied for further experiments on the CP43 protein. Otherwise, an immunodetection via fluorescence should eventually be tried on again.

Lhca1

The screening regarding Lhca1 detection (20,5 kDa) has started with an immunodetection via fluorescence; the blot displayed too weak signals, because of this irrelevance, chemiluminescence, which is a more sensitive analysis, has been performed using a HRP-tagged secondary antibody.

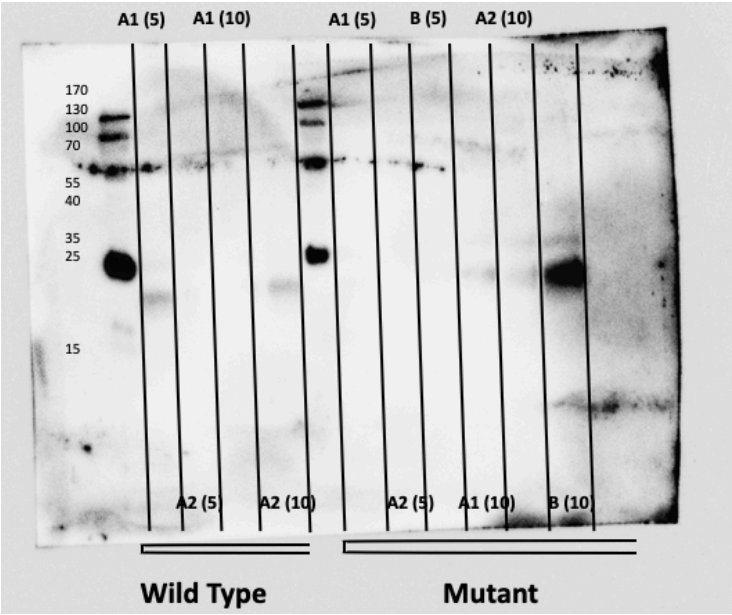


Figure 5: Immunostaining using an antibody directed against Lhca1, visualized via chemiluminescence. A1, A2 an B correspond to different biological samples (triplicates) of wild type and mutant leaves. In brackets the amount of proteins loaded is given (5 or 10 µg protein).

This blot indicates the presence of a strong background; some stains dispersed on the membrane aren't corresponding to the secondary antibody.
The blocking process might have failed here; some marker bands are also stained, indicating that the secondary antibody had the possibility to bind onto the proteins located there.
The proteins have been detected for a few replicates only; A1 (5) and A2 (10) for the WT, A1 (5) and B (10) for the mutants.
This screening led to the following result: neither fluorescence nor chemiluminescence, at that stage, are valuable enough for further experiments regarding Lhca1.
Another try would be needed, with higher protein amounts (for the signals), longer washes (for a weaker the background) and a longer blocking time (for the secondary antibody fixation). If results are still irrelevant, another method should be tried on.

PsaD

The screening regarding PsaD (18 kDa) has also started with an immunodetection via fluorescence; bands obtained via fluorescence analysis were not corresponding to the targeted protein. For the chemiluminescence detection, an HRP-tagged secondary antibody has been used.

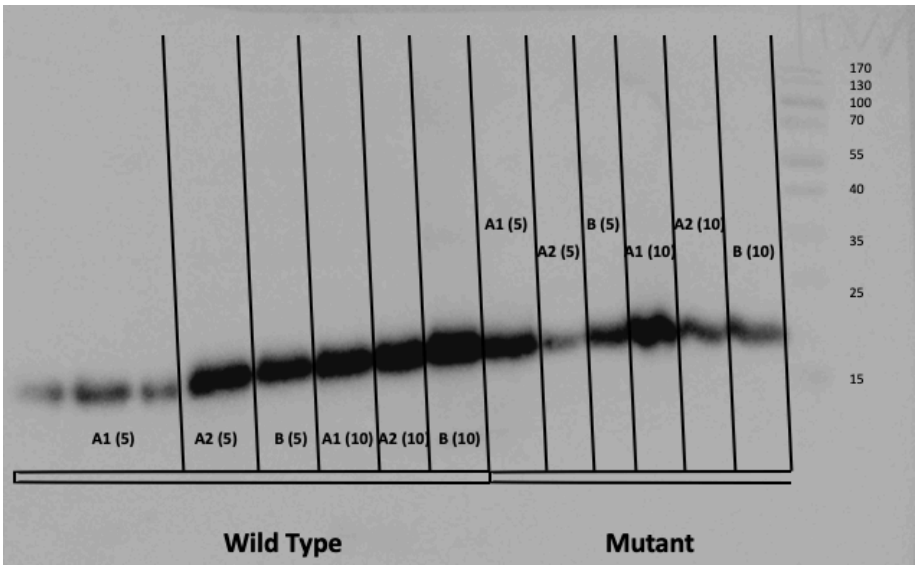


Figure 6: Immunostaining using an antibody directed against PsaD, visualized via chemiluminescence. A1, A2 an B correspond to different biological samples (triplicates) of wild type and mutant leaves. In brackets the amount loaded is given (5 or 10 µg protein).

The protein PsaD (18 kDa) is displayed very clearly; intense bands are located between 15 and 25 kDa on the blot above. The immune-response was beyond the linear detection, as the intense signals show. Regardless of this intensity, screening regarding the immunodetection of PsaD has shown, so far, that visualization via chemiluminescence could be applied for further experiments. Otherwise, this method should be tried again, with more diluted samples.

Lhcb1

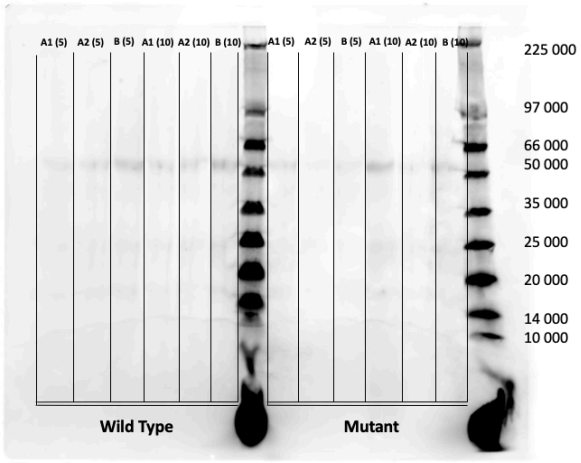


Figure 7: Immunostaining with an antibody directed against Lhcb1, visualized via fluorescence. A1, A2 an B correspond to different biological samples (triplicates) of wild type and mutant leaves. In brackets the amount loaded is given (5 or 10 µg protein).

Lhcb1 (20 kDa) is one of the most common membrane proteins in plant material; the fact that only very weak bands were detected with incorrect molecular weight allows the conclusion that the experiment failed. Manual WB followed by chemiluminescence (not executed) should, therefore, be tried here.

The next part of this section regards the FtsHi3 protein.

As explained above, the plasmids introduced into the overexpressed and complemented lines contain the FtsHi3 gene along with the GFP, HA and Gus reporter genes. For allowing its immunodetection by commercially available antibodies, FtsHi3 protein has been tagged *in vivo* with the HA or GFP synthesized by those lines. By detecting them, we could indirectly detect FtsHi3. The screening was regarding the protein of interest here; the tag giving the best results would be selected for further experiments.

HA-tag

The HA-tag has first been detected via fluorescence. A semi-manual WB has been done here; only the blocking and antibody incubation processes were manual. As the commercial antibody was produced in mouse, a *Goat Anti-Mouse* secondary antibody had to be used. Analysis via fluorescence did not properly display a HA positive control, (18-28 kDa; used to verify the correct functioning of the HA-tag), making the remaining data irrelevant.
To get more relevant results out of this screening, a chemiluminescence analysis has been tried on, using an HRP-tagged *Anti-HA* antibody, and no secondary antibody.

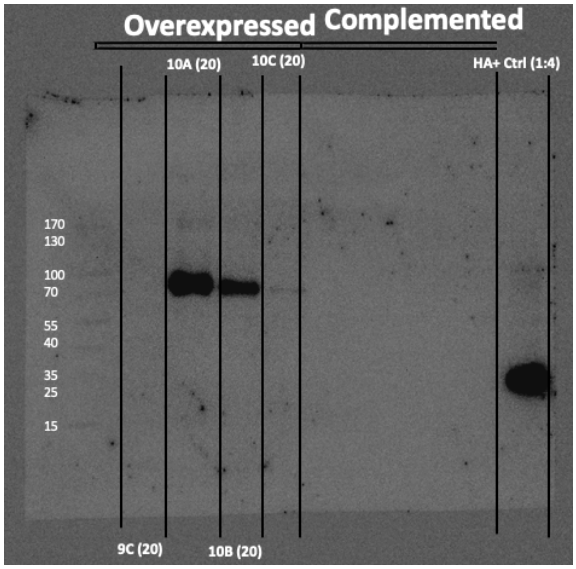


Figure 8: Immunostaining with an antibody directed against the HA-tag via chemiluminescence (20 µg protein loaded). Analysed samples were leaves of overexpressed lines (9C, 10A, 10B and 10C) and complemented lines both with high amounts of total proteins. The last column displays the positive control.

The intense signal observed at the far right and located between 25 and 35 kDa corresponds to the control. The bands observed for the Oxp. samples seem to be related to a protein whom the size is above 66 kDa. However, due to the intense signal, we'd assume that it actually corresponds to FtsHi3. To confirm and this and get results for the other replicates and the Comp. samples, the immunodetection has been repeated, Higher protein amounts have been loaded, specifically for the complemented samples, and only Oxp. 8A has been loaded, as a reference.

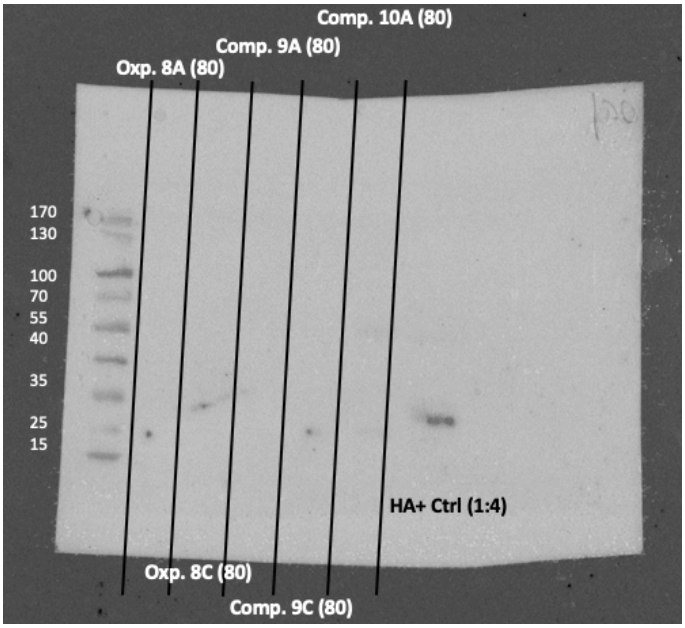


Figure 9: Immunostaining with an antibody directed against the HA-tag of FtsHi3 visualized via chemiluminescence (80 µg protein of Comp.). Beside the positive HA control, only the Oxp. 8A and 8C, and the Comp. 9A, 9C and 10A have been loaded.

While the control is present here, no FtsHi3 is. The amount of control sample has been loaded, yet, a much weaker signal is observed. The transfer of the proteins to the membrane is questionable. Due to the lack of further samples, a higher concentration of the samples could not be loaded. At this stage of the screening regarding the FtsHi3 immunodetection, HA isn't an appropriate tag to use on our protein of interest. To confirm it, another try would be needed, with a new control solution and eventually a higher protein amount.

GFP

Fluorescence hasn't been tested for GFP immunodetection. I've visualized the protein by using chemiluminescence first. The first try has failed; strong background, leaning tendency of the samples during their separation and intense signal of the GFP control.
A new WB, accompanied with a Coomassie blue, has been performed for those samples. A few parameters have changed; longer washes, shorter transfer and longer incubation time of the luminol on the membrane. Because of the high number of samples, four gels were loaded with both some complemented and overexpressed lines loaded into each of them; two were used for the Coomassie blue and two others for immunodetection.

Two gels (noted *Gel 1*) contained, from the left to the right:

Marker Comp. 1A 1B 1C Oxp. 2A 2B 2C Comp. 7A 7B 7C GFP control Marker.

Two other gels (noted *Gel 2)* contained:

Marker Oxp. 1A 1B 1C Comp. 4A 4B 4C Oxp. 3A 3B 3C GFP control Marker.

One of each have been taken for a Coomassie staining, visualized below.

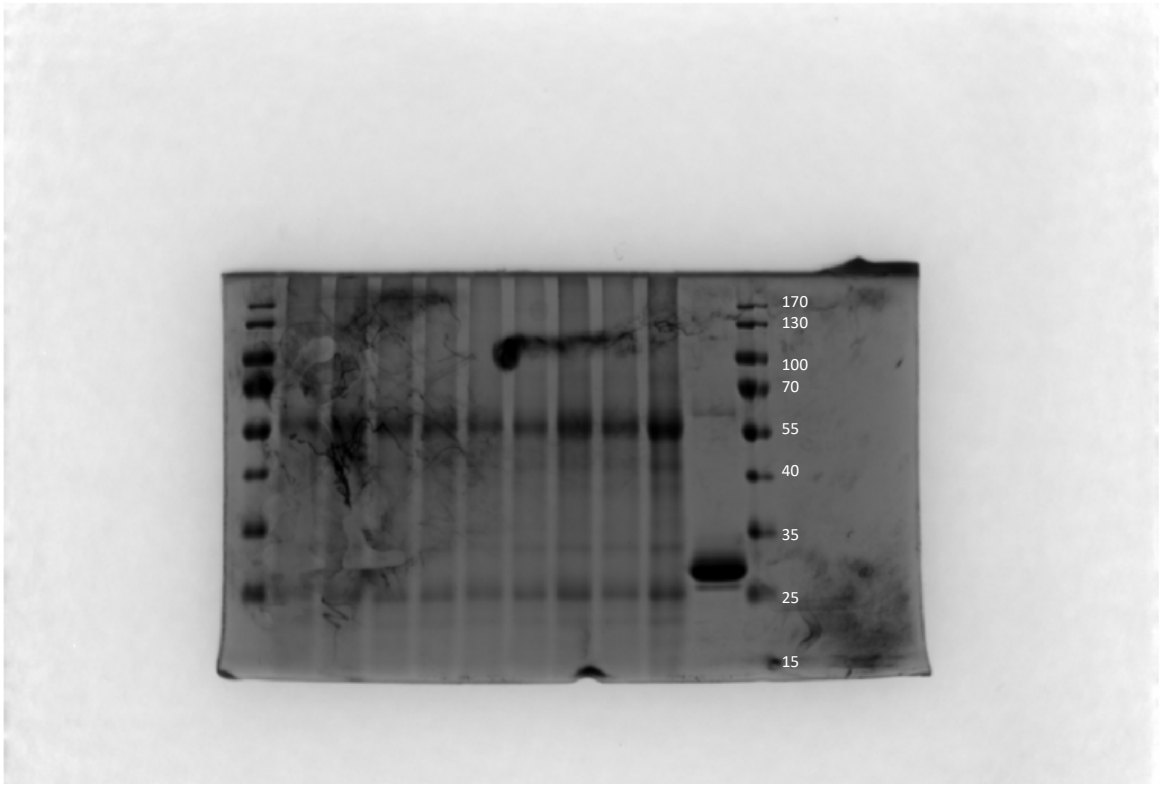


Figure 10: Grey picture of the Coomassie-stained Gel 1 containing the samples listed above.

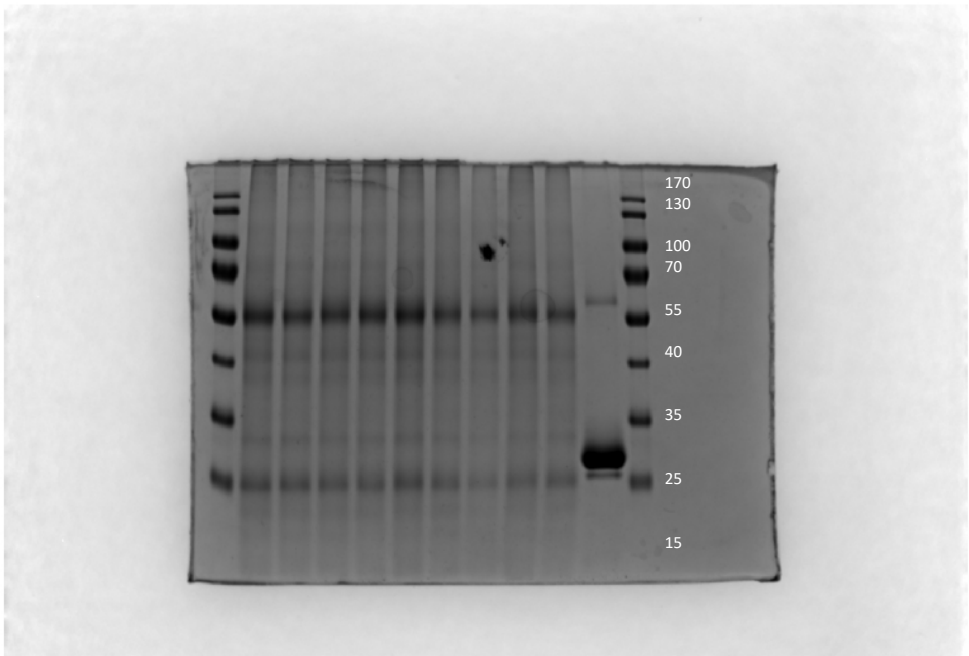


Figure 11: Grey picture of the Coomassie-stained Gel 2 containing the samples listed above.

Both gels display a strong signal for the GFP positive control (27 kDa) with molecular weight between 25 and 35 kDa. Bands, possibly corresponding to GFP-tagged FtsHi3 can observed at molecular weight of 55 and 70kDa. These Coomassie gels are controls showing that the plasmid has successfully been introduced (intense FtsHi3 bands) and GFP control is reliable. The two remaining gels have been taken for a transfer onto a membrane; the post-transfer gels have been stained with Coomassie blue to account for the efficiency of the transfer.

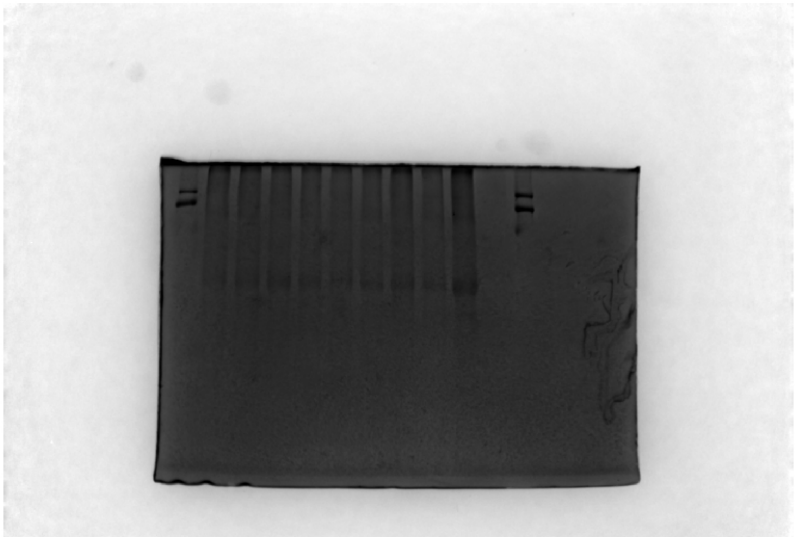


Figure 12: Image of the Gel 1 obtained after transferring the proteins; stained with Coomassie blue solution.

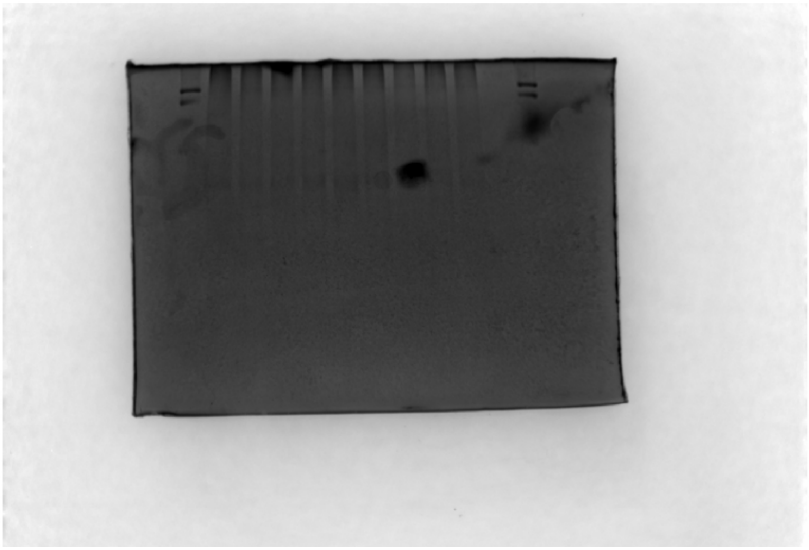


Figure 13: Image of the Gel 2 obtained after blotting the proteins to the membrane; stained by Coomassie.

Obviously, the transfer of proteins to the membrane has not been 100% done. The Gel 1 displays bands in the range of 55 to 70kDa, which might correspond to some FtsHi3 left. The

Gel 2 displays one strong signal near the 70kDa, which might be an artefact or correspond to GFP-tagged FtsHi3. Theoretically, we should get a few proteins of interest left on both membranes, more on the Gel 2. The membranes have been visualized via chemiluminescence; they're displayed below.

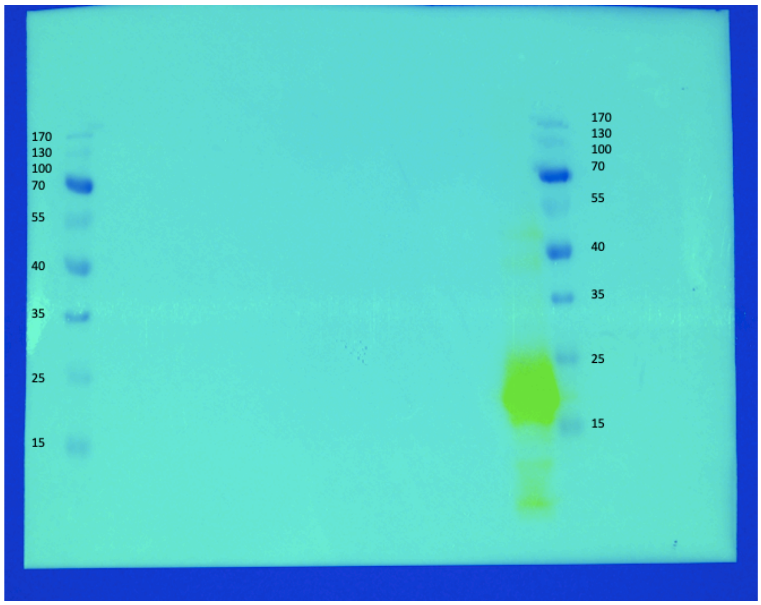


Figure 14: Immunoblot (from Gel 1) using an antibody recognizing GFP visualized via chemiluminescence. Comp. 1 A-C, Oxp. 2 A-C and Comp. 7 A-C samples have been loaded for this WB.

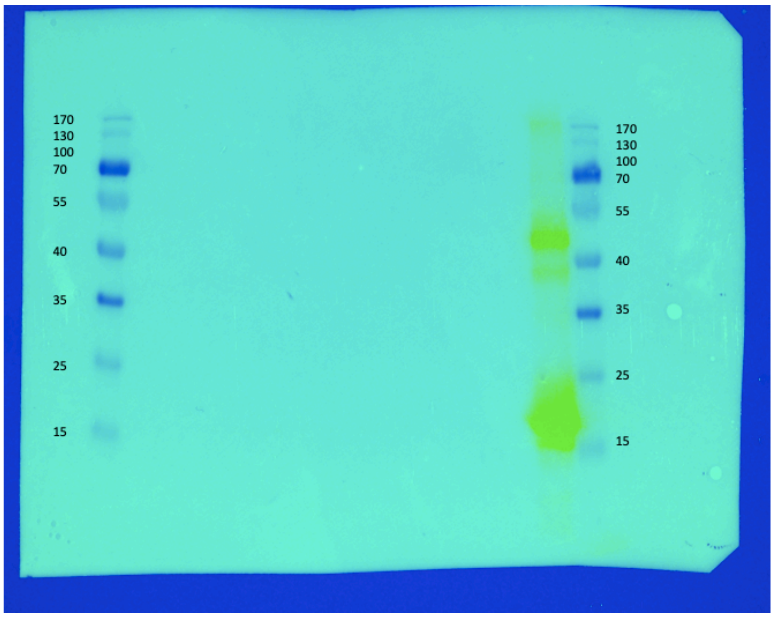


Figure 15: Immunoblot (from Gel 2) using an antibody recognizing GFP visualized via chemiluminescence (second gel). Oxp. 1 A-C, Comp. 4 A-C and Oxp. 3 A-C samples have been loaded.

Both membranes display a strong signal for the GFP control, but no other protein bands. The amount of proteins transferred might have been too low; a much longer transfer time could give more efficient results. Loading a higher protein amount of the samples and a more diluted GFP control could improve the result. At this stage, when it comes to the screening regarding FtsHi3 immunodetection, GFP isn't an appropriate tag either and shouldn't be used for further experiments.

Many factors influence the outcome of screenings; repetitive freezing-thawing processes of the sample, quality of the reagents, settings during gel electrophoresis (voltage, temperature), etc. In order to test the influence of those factors on the screening process, each experiment should have been repeated several times. The protein separation could have been optimized by different types of gels (acrylamide concentrations) or variations in the transfer times. During each immunoblot, various controls have been applied: a good example is the good separation of the molecular size marker. Coomassie staining after transfer is another example.
As previously explained, performing biological replicates is an important part of the screening; learning how similar or different the protein pattern is in different plants not only emphasizes the fact that the chloroplast´s metabolism isn't a fixed activity, it also gives average concentrations for further analyses.

Conclusion

Through the screening, I've been able to exclude a few analytical techniques that couldn't be used for some protein immunodetections. If techniques were selected without being tested, results later obtained, regarding FtsHi3 function, would've probably been interpreted depending on the samples only, when techniques could've impacted them too.
This emphasizes the importance of screening prior to any specific experiment; this helps building an optimized protocol for further experiments. When applied to the FtsHi3 function, the experiment is specific as the strategy used is to focus on selected proteins, FtsHi3 included. Adding a screening step to an experiment is crucial since, as said above, it'll contribute to the interpretation of the results of each of those proteins.

Screening also points the fact that what works for one protein might failed for another one, cause can be biological or technical; the unique properties of the proteins, but also the parameters applied for their detection. Prior tests can positively influence the results.
This screening has been supported by staining with Coomassie Blue, which helped checking the successful introduction of the GFP-containing plasmid into the plant cells. Other supporting tests, such post-transfer membrane analysis have permitted to know whether the lack of results was depending on biological materials or the selected techniques.
Both fluorescence and chemiluminescence have brought interesting results as some point (CP43 and PsaD) but, as said earlier, more tests giving relevant results are needed in order to confirm the suitability of something, here the analytic techniques tested.
This project has given an insight into the complexity of a screening, how important this can be. It also helped eliminating a few techniques which wouldn't be relevant for a specific protein. At this stage of the screening and of the overall experiment, we wouldn't be able to know what FtsHi functions or if, in spite of their presumable proteolytic inactivity, they play an important role in plant cells.
Once the most suitable analytical technique will be determined for WT and mutants, immunodetection of the selected proteins should, later on, be repeated using complemented plants or mutants overexpressing FtsHi3. More research on these enzymes is the starting point that might, in the future, impact the food industry and, thus, our society.

Social aspects

Photosynthetic organisms provide us with oxygen and food. Also, they counteract the greenhouse effect by taking up CO2. Optimization of a healthy plant's growth is crucial for our survival. Knowledge about the way plants grow and adapt can be used, later, as a tool for an optimal growth. Many studies have been, and are still done for this purpose, the characterization of FtsH proteases is one of them. Characterizing the FtsHi enzymes is a first step, which can lead to a better understanding on how their mutations will affect photosynthesis and, in a bigger scale, plant development.

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[*] *Biocompare.com*, 'RC-DC Protein Assay from Bio-Rad'

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The mass-associated columns indicate the volume (in μL) of sample containing the indicated amount (in μg) of total proteins. They've been diluted in LDS, into the volumes indicated on each next column at the right.

Table 2: Absorbance dilution data for WT and Mutants plants' leaves

Samples	**Abs (750nm)**	**[C] (mg/mL)**	**5ug**	**LDS**	**10ug**	**LDS**	**15ug**	**LDS**	**20ug**	**LDS**	**40ug**	**LDS**
Col A1	0,19	6,38	0,8	14,2	1,6	13,4	2,4	12,6	3,1	11,9	6,3	8,7
Col A2	0,27	9,05	0,6	14,4	1,1	13,9	1,7	13,3	2,2	12,8	4,4	10,6
Col B	0,13	4,16	1,2	13,8	2,4	12,6	3,6	11,4	4,8	10,2	9,6	5,4
i3-1 A1	0,21	7,05	0,7	14,3	1,4	13,6	2,1	12,9	2,8	12,2	5,7	9,3
i3-1 A2	0,17	5,58	0,9	14,1	1,8	13,2	2,7	12,3	3,6	11,4	7,2	7,8
i3-1 B	0,15	4,91	1,0	14,0	2,0	13,0	3,1	11,9	4,1	10,9	8,1	6,9

Table 3: Absorbance and dilution data for HA-containing Comp. and Oxp. plants' leaves

Samples	**Abs (750 nm)**	**[C] - mg/mL**	**15ug**	**LDS***	**20ug**	**15uL***	**40ug**	**20uL***	**60ug**	**30uL***	**80ug**	**40uL***
i3i3 HA WT 9C	0,10	2,90	5,2	4,8	6,9	8,1	13,8	6,2	20,7	9,3	27,6	12,4
i3i3 HA WT 10A	0,09	2,60	5,8	4,2	7,7	7,3	15,4	4,6	23,0	7,0	30,7	9,3
i3i3 HA WT 10B	0,18	5,98	2,5	7,5	3,3	11,7	6,7	13,3	10,0	20,0	13,4	26,6
i3i3 HA WT 10C	0,11	3,31	4,5	5,5	6,0	9,0	12,1	7,9	18,1	11,9	24,2	15,8
i3i3 HA 9B	0,12	3,60	4,2	5,8	5,6	9,4	11,1	8,9	16,7	13,3	22,2	17,8
i3i3 HA 9C	0,13	4,06	3,7	6,3	4,9	10,1	9,9	10,1	14,8	15,2	19,7	20,3
i3i3 HA 10B	0,09	2,67	5,6	4,4	7,5	7,5	15,0	5,0	22,4	7,6	29,9	10,1
i3i3 HA 10C	0,08	2,19	6,8	3,2	9,1	5,9	18,2	1,8	27,3	2,7	36,5	3,5

Table 4: Absorbance and dilution data for GFP-containing Comp. and Oxp. plant's leaves

Samples	**Abs (750 nm)**	**[C] - mg/mL**	**50 ug**	**1x LDS**	**75 ug**	**1x LDS**	**100 ug**	**1x LDS**	**125 ug**	**1x LDS**
i3i3 gg 1A	0,661	23,25	2,2	17,8	3,2	11,8	4,3	14,7	5,4	14,6
i3i3 gg 1B	0,5608	19,64	2,5	17,5	3,8	11,2	5,1	13,9	6,4	13,6
i3i3 gg 1C	0,6562	23,07	2,2	17,8	3,3	11,7	4,3	14,7	5,4	14,6
i3i3 gg 4A	0,5888	20,64	2,4	17,6	3,6	11,4	4,8	14,2	6,1	13,9
i3i3 gg 4B	0,4061	14,06	3,6	16,4	5,3	9,7	7,1	11,9	8,9	11,1
i3i3 gg 4C	0,5499	19,24	2,6	17,4	3,9	11,1	5,2	13,8	6,5	13,5
i3i3 gg 7A	0,474	16,51	3,0	17,0	4,5	10,5	6,1	12,9	7,6	12,4

i3i3 gg 7B	0,5785	20,27	2,5	17,5	3,7	11,3	4,9	14,1	6,2	13,8
i3i3 gg 7C	0,1752	5,75	8,7	11,3	13,1	1,9	17,4	1,6	21,8	-1,8
i3i3 gg WT 1A	0,4752	16,55	3,0	17,0	4,5	10,5	6,0	13,0	7,6	12,4
i3i3 gg WT 1B	0,6278	22,05	2,3	17,7	3,4	11,6	4,5	14,5	5,7	14,3
i3i3 gg WT 1C	0,5353	18,72	2,7	17,3	4,0	11,0	5,3	13,7	6,7	13,3
i3i3 gg WT 2A	0,6694	23,55	2,1	17,9	3,2	11,8	4,2	14,8	5,3	14,7
i3i3 gg WT 2B	0,5188	18,12	2,8	17,2	4,1	10,9	5,5	13,5	6,9	13,1
i3i3 gg WT 2C	0,572	20,04	2,5	17,5	3,7	11,3	5,0	14,0	6,2	13,8
i3i3 gg WT 3A	0,6616	23,27	2,1	17,9	3,2	11,8	4,3	14,7	5,4	14,6
i3i3 gg WT 3B	0,5916	20,75	2,4	17,6	3,6	11,4	4,8	14,2	6,0	14,0
i3i3 gg WT 3C	0,5778	20,25	2,5	17,5	3,7	11,3	4,9	14,1	6,2	13,8
GFP solution	0,6471	22,74	1,1	18,9	1,1	22,7	1,1	22,7	5,5	14,5

Table 5: Absorbance and dilution data for HA-containing Comp. and Oxp. plants' seedlings

Samples	**Abs**	**Diluted conc.**	**[C] - mg/mL**	**15 ug**	**1x LDS**	**20 ug**	**1x LDS**	**40 ug**	**1x LDS**
i3i3 HA WT 1A	0,292	1,99	10,0	1,5	18,5	2,0	18,0	4,0	16,0
i3i3 HA WT 1B	0,3568	2,46	12,3	1,2	18,8	1,6	18,4	3,3	16,7
i3i3 HA WT 2A	0,3437	2,36	11,8	1,3	18,7	1,7	18,3	3,4	16,6
i3i3 HA WT 2B	0,5228	3,65	18,3	0,8	19,2	1,1	18,9	2,2	17,8
i3i3 HA WT 3A	0,3312	2,27	11,4	1,3	18,7	1,8	18,2	3,5	16,5
i3i3 HA WT 3B	0,4491	3,12	15,6	1,0	19,0	1,3	18,7	2,6	17,4

i3i3 HA WT 4A	0,2346	1,58	7,9	1,9	18,1	2,5	17,5	5,1	14,9
i3i3 HA WT 4B	0,3263	2,24	11,2	1,3	18,7	1,8	18,2	3,6	16,4
i3i3 HA WT 5	0,3418	2,35	11,7	1,3	18,7	1,7	18,3	3,4	16,6
i3i3 HA WT 6A	0,2718	1,85	9,2	1,6	18,4	2,2	17,8	4,3	15,7
i3i3 HA WT 6B	0,3107	2,13	10,6	1,4	18,6	1,9	18,1	3,8	16,2
i3i3 HA WT 7A	0,3674	2,53	12,7	1,2	18,8	1,6	18,4	3,2	16,8
i3i3 HA WT 7B	0,3198	2,19	11,0	1,4	18,6	1,8	18,2	3,7	16,3
i3i3 HA WT 8A	0,1386	0,89	4,4	3,4	16,6	4,5	15,5	9,0	11,0
i3i3 HA WT 8B	0,3661	2,52	12,6	1,2	18,8	1,6	18,4	3,2	16,8
i3i3 HA WT 9A	0,3806	2,63	13,1	1,1	18,9	1,5	18,5	3,0	17,0
i3i3 HA WT 9B	0,3608	2,49	12,4	1,2	18,8	1,6	18,4	3,2	16,8
i3i3 HA WT 10A	0,3227	2,21	11,1	1,4	18,6	1,8	18,2	3,6	16,4
i3i3 HA WT 10B	0,1105	0,68	3,4	4,4	15,6	5,9	14,1	11,7	8,3
i3i3 HA 9A	0,4166	2,89	14,4	1,0	19,0	1,4	18,6	2,8	17,2
i3i3 HA 9B	0,2414	1,63	8,1	1,8	18,2	2,5	17,5	4,9	15,1
i3i3 HA 10A	0,3427	2,36	11,8	1,3	18,7	1,7	18,3	3,4	16,6
i3i3 HA 10B	0,3418	2,35	11,7	1,3	18,7	1,7	18,3	3,4	16,6