Sveriges lantbruksuniversitet
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Faculty of Landscape Architecture,
Horticulture and Crop Production Science

Expression and characterization of phytoglobin genes from sugar beet (*Beta vulgaris* L.*)* in tobacco (*Nicotiana benthamiana)*

Uttryck och karaktärisering av fytoglobin gener från sockerbeta (*Beta vulgaris* L.*)* i tobak (*Nicotiana benthamiana)*

Axel Carl-Vilhelm Ögell Benediktsson

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Abstract

Phytoglobin is a plant globin protein similar to hemoglobin, which is found in all vertebrates. Phytoglobin is categorized depending on its origin and characteristics into 6 different classes: phytoglobin class 0, phytoglobin class 1, phytoglobin class 2, symbiotic phytoglobin, legphytoglobin and phytoglobin class 3. A recent study has identified two class 1 phytoglobin genes, BvHb1.1 and BvHb1.2, in *Beta vulgaris* that has not been functionally characterized so far. In the current study, the synthesized genes BvHb1.1 (BvPb1.1) and BvHb1.2 (BvPb1.2) were cloned into a tobacco mosaic virus based viral vector. The resulted plasmids were sequence-confirmed and transferred into *Agrobacterium tumefaciens* strain GV3101. The transformed *A. tumefaciens* was agrosprayed into *Nicotiana benthamiana* leaves along with a vector (pJL3:p19) containing the tomato bushy stunt virus (TBSV) gene-silencing suppressor (P19). Transiently expressed heterologous proteins in *N. benthamiana* were confirmed by SDS-PAGE and spectrophotometry. The spectrophotometric analysis showed that heterologously produced phytoglobins were functional as both proteins exhibited oxygen (O_2) and carbon monoxide (CO) binding properties. This suggests that the transient expression using viral vector in *N. benthamiana* has great potential to be used for the production of plant proteins at industrial scale.

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1. Introduction

1.1 Hemoglobin and phytoglobin

Hemoglobin is an iron-containing metalloprotein and exists in all vertebrates, several invertebrates and fungi (Maton et al., 1993; Weber and Vinogradov, 2001). As hemoglobin exists in close to all eukaryote groups, it is considered that they are intertwined with bacterial globin (Vinogradov et al. 2011a). Hemoglobin is mainly thought to have an oxygen-carrying function though it has several other functions as well. Hemoglobin found in invertebrates have shown to have functions such as transporting small molecules and ions such as carbon dioxide (CO_2), phosphorus mononitride (NP), hydrogen sulfide (H_2S) and sulfur (S). According to Vazquez-Limon et al. (2012) hemoglobins role as oxygen transporter is a more recent adaptation in vertebrates. Vinogradov and Moens (2008) proposed that the early functions of hemoglobin most likely were enzymatic and oxygen sensing.

Recently, hemoglobin from plants has been renamed into phytoglobins and they are categorized into six different classes depending on their origins and characteristics (Hill et al., 2016). The different types of phytoglobin consist of phytoglobin class 0 (Pb0), phytoglobin class 1 (Pb1), phytoglobin class 2 (Pb2), symbiotic phytoglobin (sPb), legphytoglobin (Lb) and phytoglobin class 3 (Pb3). Pb0 can be located in any plant organ in algae, bryophytes and gymnosperms. sPb are found in N_2-fixing nodules of non-legume plants while Lb has been shown to exist in N_2-fixing nodules of N_2-fixing legumes. Pb1 and Pb2 are both found in any plant organ of angiosperms. Pb1 has an extremely high affinity for O_2 while Pb2 varies between a moderate to high affinity. Pb3 can be found in any plant organ of algae and land plants. The evolution of different types of phytoglobins and new functions has shown to parallel major transitions in plant evolution (Vazquez-Limon et al., 2012). Phytoglobin has

also shown to be species-specific and have stress-related features (Gupta et al., 2011).

Depending on plant family, the type of phytoglobins can differ for example sPbs can be found in the nodules of legumes (Arredondo-Peter et al., 1997; Duff et al., 1997; Bruno et al., 2007; Hunt et al., 2001; Garrocho-Villegas et al., 2007; Vinogradov et al., 2011b). Pb1 and Pb2 are spread across different organs of bryophytes and angiosperms during all development stages. These two classes have also mainly been studied in the crops *Oryza*, *Hordeum vulgare*, *Zea mays*, *Arabidopsis*, *Lotus japonicus*, *Trema tomentosa*, *Solanum lycopersicum* and *Cichorium intybus*. Phytoglobin class 1 and 2 are not found always found together in monocots which seem to lack Pb2. Instead, monocots can have more than one Pb1 and dicots generally have both (Hunt et al., 2001; Garrocho-Villegas et al., 2007; Smagghe et al., 2009). Although exceptions exist, such as legumes who has Pb3 instead of Pb2. It has also been suggested that legumes originally had Pb2 and it evolved into Pb3. Helebestrup et al. (2006) confirmed that Pb1 from *Arabidopsis thaliana* can complement the functions of Pb2 and showed that some functions overlap. In *A. thaliana* seedlings, at least one of them must be present for its survival.

1.2 The role of phytoglobin

There are different roles that have been proposed for phytoglobins. Pb1 has a believed function to alter the hypoxically generated nitrous oxide (NO) levels to boost the vigor of the plant (Igamberdiev and Hill, 2009). On the other hand, Pb2 has a suggested function that increases the oxygen supply for the mitochondrial respiration (Anderson et al., 1996; Spyrakis et al., 2011; Vigeolas et al., 2011). Also, the expression of the gene can change depending on stress factors such as hypoxia, cold, nutrient deprivation and osmotic stress (Dordas, 2009; Taylor et al., 1994; Nie and Hill, 1997; Trevaskis et al., 1997; Sowa et al., 1998; Hunt et al., 2001; Wang et al., 2003; Bustos-Sanmamed et al., 2011). The response of signaling compounds can also be altered depending on different stress factors. Mur et al. (2013) and Hill (2012) suggested that Pb1 and Pb2 could function as a hormone signal transduction by regulating both the plant produced NO and its effect under different stress factors.

Pb1 and Pb2 have shown to differ in expression based on development stages of the plant and tissue during normal growing conditions (Hunt et al., 2001). The two classes also have shown to have a favored distribution between the plant organs.

1.3 BvHb1.1 and BvHb1.2

Few Pb1 have been studied on a gene or protein level so far. Recently, Leiva-Eriksson et al. (2014) reported the findings of 4 genes coding for phytoglobins of classes 1, 2 and 3. Among these, BvHb1.1 (BvPb1.1) and BvHb1.2 (BvPb1.2) belong to class 1 phytoglobins. The total sizes for the genes, including introns are 2.5 kb and 2.2 kb. Also, BvPb1.1 showed similar conserved sites as found in *Oryza* and *Arabidopsis* which indicate its evolution.

Leiva-Eriksson et al. (2014) predicted that the two genes excluding introns with 708 and 513 base pairs and the expressed proteins have a molecular mass of 27 and 19.8 kDa. Due to the long N-terminus of BvPb1.1, it is thought to be a precursor protein, which translocates to the chloroplast where its extension is cleaved by stromal processing peptidase (Richter and Lamppa, 1998). BvPb1.2 was designated as a cytoplasmic protein until the exact location is found. Phytoglobins have also been found in the cytosol, nucleus and the plastids (Ross et al., 2001; Sergelyes et al., 2000; Sainz et al., 2013; Smagghe et al., 2007; Kim et al., 2013).

During normal growing conditions in *B. vulgaris*, BvPb1.1 was most commonly expressed in seeds where BvPb1.2 also had its highest expression level. Hebelstrup et al. (2007) proposed a function of Pb1 in seeds that can explain their high expression levels in seeds. They have a function which maintains energy levels and the reduction-oxidation reaction inside the embryo. BvPb1.2 otherwise had low expression levels in other tissue of the plant. In the reproductive stage, BvPb1.1 has an increased expression in the flowers due to a high demand of energy (Leiva-Eriksson et al., 2014). It was shown that during vernalization in *B. vulgaris*, BvPb1.1 had a gradually increased expression (Leiva-Eriksson et al., 2014).

1.4 Application of phytoglobins

Phytoglobins has several interesting properties and they can have a very specific function due to being species-specific. Henkel-Hanke and Oleck (2007) summarized the use of hemoglobins as artificial blood carriers. There is a possibility to use purified hemoglobin as a replacement for blood transfusions. A possibility of using plants or even phytoglobin exists according to Nélida Leiva-Eriksson in an interview with BBC (2014) written by Pippa Stephens. There are 2 possibilities – the first is to adapt phytoglobin for human use and the second is to use plants as a system to produce human hemoglobin.

The expression of Pb1 has shown to increase NO and ethylene (Dordas et al., 2003; Manac'h-Little et al., 2005) and it is up-regulated under stress conditions. NO functions as an important defense response against pathogens. NO induces the production of salicylic acid or cyclic guanine monophosphate leading to the initiation of genes in the defense pathways (Durner et al., 1998; Wendehenne et al., 2001). Hypersensitive response is a common plant defense response against pathogens which is induced by salicylic acid (Mur et al. 2008). It has been shown that expression of Pb1 is induced when pathogens infect the plant (Seregélyes et al., 2003; Seregélyes et al., 2004; Qu et al., 2006). Shimoda et al. (2005) also mentioned that Pb1 seems to have shielding role of nitrogenase activity in root nodules by scavenging NO. Different studies have shown that the elevated NO expression created by Pb1 increases resistance against pathogens (Mur et al., 2012; Chun et al., 2012). Wally et al. (2013) proposed that Pb1 might also have an important role in plant growth and development by affecting the biosynthesis of the plant hormone auxin during embryogenesis.

1.5 Plant-based expression system and viral vectors

Plant-based expression systems have several advantages for producing a broad range of proteins (Jin et al., 2015). Unlike bacteria, plants have the ability of subcellular targeting, proper folding of proteins, posttranslational modification and the eukaryotic protein modification mechanisms. To produce a correctly folded and fully functional plant protein, a plant-based system is ideal. Hahn et al. (2015) developed a modified method of expression using spray to infiltrate plants instead of syringe/vacuum infiltration. This method has the advantage of being quick and easy to scale-up.

In addition, there are advantages of using viral vectors instead of binary vectors (Lindbo, 2007; Jin et al., 2015). Viral vector creates a transient expression and is different from binary vectors because the trait is not heritable. Viral vectors also have advantages such as obtaining a high expression in a relatively low short time. Many plant viruses have also been designed to express foreign proteins. Also, there are plant viral vectors being developed for industrial-scale expression of heterologous proteins successfully. Binary vectors are used to create a stable expression system (Shah et al., 2013) and require a longer time frame with 10 – 20 % lower expression using an optimized system. A stable expression can have approximately up to ten times lower expression when compared with a transient system.

1.6 Aim

The aim of this study is to (1) clone BvPb1.1 and BvPb1.2 into a plant viral-based expression vector, pJL-TRBO, (2) express the genes transiently in *Nicotiana benthamiana* and (3) characterize the expressed phytoglobin proteins.

2. Material and methods

2.1 BvPb1.1 & BvPb1.2

The synthesized genes BvPb1.1 and BvPb1.2 were cloned into the cloning vector pMA-T. Figure 1 the sequences of both the genes including restriction sites (*Pac*I and *Avr*II). BvPb1.1 is 747 base pairs long and BvPb1.2 is 552 base pairs long. For cloning purposes, both genes used the forward primer.

A:

ttaattaacgccaccatggcttttatgagcaccaagacccctgcttctaacgctacctgcatctctaatgctgtgaccctgaacggtaacttcgcttgcatca
agaagtccaacttcaagagcggtttcaccgagctgtcttgggttaagagagagggtttccagatccagggactgttctgcaagaaccctagcttcatcacc
agcgtgagaaaaagaaacgataacggtggtctgatcgtgaaggctttcagcgaagaacaagaggctctggtggtgaagtcttggaacgctatgaaga
agaacgctagcgagctggctctgaagttcttcttgagggtgttcgagattgctcctaccgctaagaagctgttcagcttcctgagggatagctccgatgatgt
gcctcttgagaagaacccaaagctgaaggctcatgctctgaccgtgttctctatgacttgcgagtctgctgtgaaccttaggaaggctggtaaggttaccgt
gaaagagagcaacctgaaggatcttggaaccacccacttcaagtacggtgtggctgatgagcatttcgaggttgtgaggttcgctctgctggaaactatc
aaagaggctgtgcctgagatgtggtccgctgaaatgaaggaagcttggaaagaggcttacgatcagctggtggctgctatcaagcaagagatgaagtc
tcctgagcaggctctgcaatag**cctagg**

B:

ttaattaacgccaccatgagcttcaccaacgttaactaccctgcttccgatggaaccgtgatcttcactgaagaacaagaggctctggtggtgcagtcttg
gaacgtgatgaagaagaactctgctgagctgggtctgaagctgttccttaagatcttcgagatcgctcctaccgctaagaagatgttcagcttcgttagga
ttccgatgtgcctcttgagcagaaccagaagcttaagggtcacgctatgagcgtgttcgtgatgacatgtaagtctgctgcacagctgaggaaggctggta
aggttacattcggtgagtccagccttaagcacatgggttctgtgcaccttaagtacggtgtggtggatgagcatttcgaggttaccaggttcgctctgctgga
aactatcaaagaggctgtgcctgagatgtggtcccctgagatgaagaatgcttgggctgaggctttcaaccaccttgtggctgctattaagg ctgagatgc
agaggctttctacccagccttag**cctagg**

Figure 1. A: BvPb1.1 sequence with primers marked in red. B: BvPb1.2 sequence with primers marked in red.

2.2 Vectors

In 2007, Lindbo developed a plant viral-based expression vector named pJL-TRBO and is useful to produce recombinant proteins due to a high agroinfection rate, scalable and high protein expression levels. The vector is based on pJL 48 and has a total size of 10606 base pairs.

A vector, pJL3, containing tomato bushy stunt virus (TBSV) gene-silencing suppressor P19 was used alongside the expression vector in the transient *N. benthamiana* expression system. P19 was used to suppress the posttranscriptional gene silencing of the introduced gene.

2.3 Cloning

Escherichia coli strain, NovaBlue was used for the cloning work. Luria-Bertani (LB) medium plates with kanamycin (50 µg/ml) were used to suppress false positive colonies. BvPb1.1 and BvPb1.2 were amplified using restriction digestion, digested with restriction enzymes and ligated into the vector pJL-TRBO. After, it was transformed into *E. coli* strain Nova Blue. The positive colonies carrying the constructs, pJL-TRBO – BvPb1.1 and pJL-TRBO – BvPB1.2, were selected and confirmed by using colony PCR, restriction digestion and sequencing using Eurofins genomics service, Germany. The positive colonies were inoculated and extracted for use in sequencing and transformation into *Agrobacterium tumefaciens*.

Agrobacterium tumefaciens, strain GV3101::pMP90 was used for plant infiltration. *A. tumefaciens* was transformed with the pJL-TRBO – BvPb1.1 and pJL-TRBO – BvPb1.2 constructs and screened using colony PCR. Vector P19 was used alongside the transient expression vectors for RNA silencing suppression (Lombardi et al., 2009). LB plates with kanamycin (50 µg/ml), gentamycin (25 µg/ml) and rifampicin (10 µg/ml) were used to suppress false positive colonies.

Glycerol stocks were prepared for all the constructs in both *E.coli* and *A. tumefaciens* and stored at -80°C.

2.4 Transient expression system

N. benthamiana was used as host plants for the transient expression system and is commonly used in transient expression systems. This is partly due to having several plant viruses which can infect the plant (Goodin et al., 2008). The second reason is that the methods for virus-induced gene silencing and protein expression are developed in *N. benthamiana*. The seeds were sowed in pots and after 2 weeks, a few seedlings were transplanted into new pots. Seedlings are transplanted to increase the amount of plants available for agroinfiltration and are ready about a week later. Figure 2 shows plants growing at different stages. They were grown at 25 °C during the day and 20 °C night period in a long day cycle in a growth chamber. The plants were watered three times per week and nematodes were added once per week to reduce the pathogen pressure on the plants.

Figure 2. Growth chamber with *Nicotiana* plants growing in pots.
Photo taken by: Axel Benediktsson.

A transient expression system functions by creating a temporary expression in the plant by infiltrating them with *A. tumefaciens* carrying the construct. It was done through either agrospray or syringe infiltration of the leaves. The leaves were then harvested after a certain amount of days. Leaves subjected to syringe infiltration were harvested 7 days after infiltration (DAI) and sprayed leaves were harvested at 8 and 10 days after agrospray (DAA). *A. tumefaciens* was suspended in agroinduction media and used to infiltrate the plants. The media was prepared using sterile water with a final concentration of 10 µM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 10 mM Magnesium chloride ($MgCl_2$) and 100 µM acetosyringone.

A. tumefaciens was inoculated the day before infiltration/spray and was grown in LB media containing the kanamycin (50 µg/ml), gentamycin (25 µg/ml) and rifampicin (10 µg/ml) overnight in a smaller volume. The inoculation was diluted 10 times with LB liquid media containing kanamycin (50 µg/ml), gentamycin (25 µg/ml) and 20 µM acetosyringone after 18 hours. Upon reaching an OD_{600} of 1.00, the media were centrifuged and resuspended in agroinduction media with 100 µM acteosyringone. This substance induces the *vir* genes, which make it possible for *A. tumefaciens* to infiltrate *N. benthamiana* (McCullen and Binns, 2006). Infiltration was done by two different methods: agrospray and syringe infiltration. A few plants were left untreated to be used as negative control.

Syringe agroinfiltration forces the bacteria into parenchymal airspace through the stomata on the leaves. It was made easier by leaving the plants with less moisture before the procedure. Agrospray was mediated using surfactant to reduce the droplet size so that an even layer of the *A. tumefaciens* media covers the leaves. Plants subjected to the spray are made more susceptible by watering the plants the same day to keep the moisture level high inside the plant. The effect of agrospray is improved by using a low amount of agro induction media to spread the leaves evenly in the leaves by reducing surface tension. Young leaves before expansion was removed due to difficulties during infiltration. Also, older leaves were removed before infiltration as they exhibit less physiological activity. After the agroinfiltration, leaves from the syringe infiltrated plants were harvested after 7 DAI, 8 DDA and 10 DAA. This was done to compare the expression between methods. The harvested leaves were weighed, packed, and stored at -80 °C until further use.

2.5 Protein extraction and characterisation

To extract the phytoglobin from the leaves, the leaf samples were grinded in a mortar and pestle using liquid nitrogen until a fine powder was obtained. The protein extraction was done by mixing the fine powder and with 2 volumes of sodium phosphate buffer pH 6 (100mM) including 5 %, polyvinylpolypyrolidone (PVPP), 5mM dithiothreitol (DTT) and 1 % protease inhibitor. To partially purify the samples, they were centrifuged and the supernatant was transferred and heated for 3-5 minutes at 60 °C. A part of the sample was left unheated for comparison. When extracting samples for characterisation in Lund, Tris-HCl buffer pH 8.5 (50 mM) including 5% PVPP, 5 mM DTT, 0.1 % protease inhibitor, 10 mM 2-mercaptoethanol and 20 mM sodium metabisulfite was used instead.

Protein electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), was used to separate the proteins on a gel by size and showing the content of the extraction. This was used in conjunction with a control to verify if the plants had expressed the transformed genes. After extraction the samples were loaded in Bolt Bis-Tris plus gels after adding loading buffer and heating them at 70 °C for 10 minutes. The gels were prepared and the standard, negative control and samples were loaded. The samples ran for 45 minutes at 145 volt (V) and the gel was later washed for 3 times in sterile water for 5 minutes each. After this, the gel was stained for 1 hour and destained overnight in sterile water. Destaining was improved by changing the water often.

BCA, bicinchoninic acid, is a method to quantify the amount of proteins in a sample. It requires an extraction with less DTT, 1mM, to not disrupt the results of the BCA. A standard was also made to be able to ascertain the concentration of our samples. 8 different concentrations of bovine serum albumin (BSA) were used at 0, 125, 250, 500, 750, 1000, 1500 and 2000 ug/mL.

Spectrophotometry was used to check the absorbance between 350 to 700 nm of the extracted samples of BvPb1.1 and BvPb1.2. Both the samples were heated immediately after extraction, centrifuged at 10000 rpm for 15 minutes and filtered.

An ion exchange chromatography with a qHP column was done in at Kemi Centrum in Lund with the help of Nélida Leiva Eriksson. This method utilizes an anion exchanger that traps the phytoglobin protein in the column and is later eluted with an elution buffer. Preferably the conductivity should be lower than 5 mS/cM (millisiemens/centimetre), which depends on the amount of ions in the sample. Another experiment was done in Lund at Kemi Centrum using a spectrophotometer to check the functionality of the protein. The method tests the same sample three times to observe the absorbance changes after adding NaD (sodium dithionite) and CO (carbon monoxide) to detect all the three different forms of phytoglobins: oxy, deoxy and carboxy. NaD was used to detect the deoxy form and CO to detect the carboxy form of the phytoglobins.

3. Results

3.1 Cloning of BvPb1.1 and BvPb1.2

The cloning was successful in ligating BvPb1.1 and BvPb1.2 with the vector pJL-TRBO. All the vectors were confirmed by colony-PCR, restriction digestion and sequencing. Figure 3 shows the two genes displaying the right size after a restriction digestion. In lane 2, the band around the 750 bp is the BvPb1.1 insert and in lane 3 the BvPb1.2 insert can be seen around 500 bp. Lane 2-3 shows a one larger band; this is the vector pMA-T. Lane 4 shows pJL-TRBO and the band is a bit bigger than 10 kb. BvPb1.1, BvPb1.2 and pJL-TRBO were later extracted from the gel and used for ligation and transformation. The sequencing results were compared with the sequences in figure 1 and showed that they had a 100% similarity.

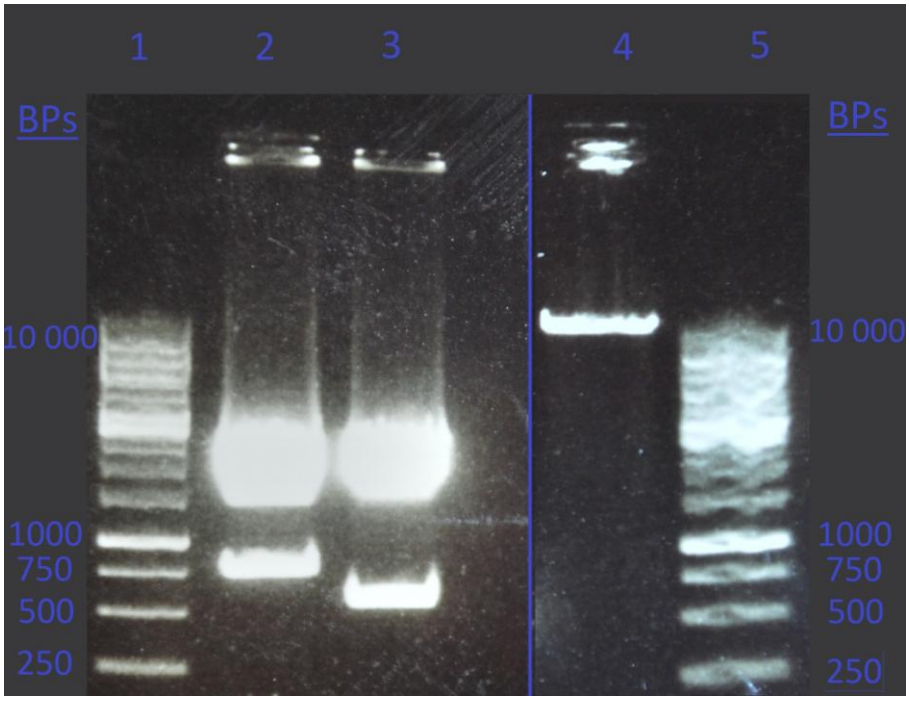

Figure 3. 2 gels showing a restriction digestion of BvPb1.1, BvPb1.2 and pJL-TRBO. The gels are separated by a blue line. 1: GeneRuler 1 kb DNA ladder #SM0313, 2: BvPb1.1, 3: BvPb1.2, 4: pJL-TRBO, 5: GeneRuler 1 kb DNA ladder #SM0313

Figure 4 shows two plates carrying colonies of Nova Blue with BvPb1.1 and BvPb1.2 constructs. No noticeable differences could be made between the different plates and had enough single colonies for making a master plate to identify positive colonies.

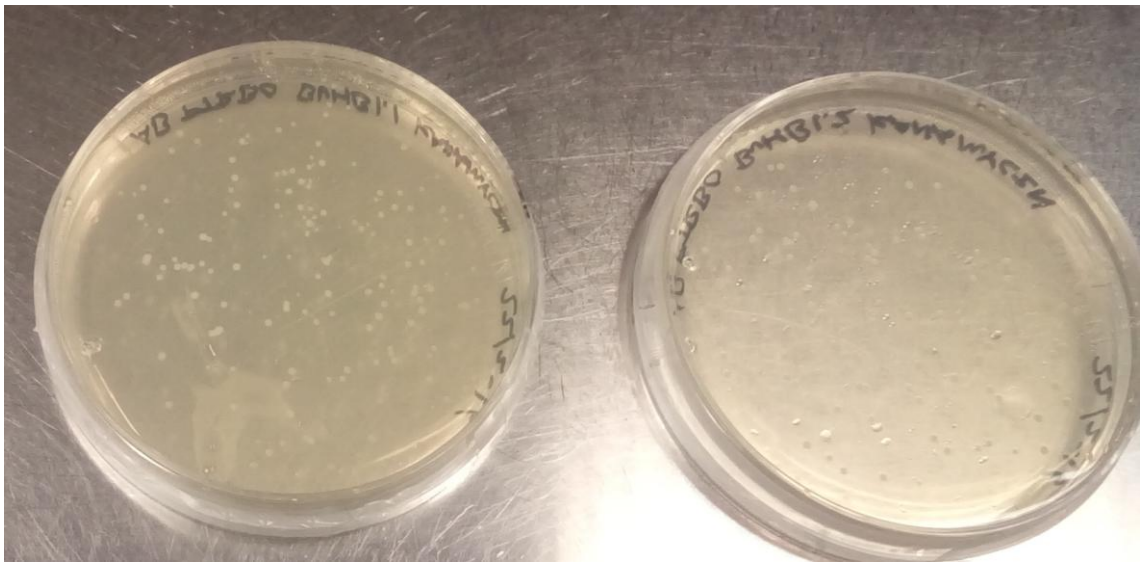

Figure 4. Showing plates with *E. coli* NovaBlue transformed with ligated pJL-TRBO. From left to right: BvPb1.1 and BvPb1.2. Photo taken by: Axel Benediktsson.

3.2 SDS-PAGE & BCA

In figure 5, the lanes 3-8 shows the heated and unheated protein samples from *N. benthamiana* leaves expressing BvPb1.2, when compared with the negative control. The sample clearly has a new band around 17 kDa, which is absent in the negative control. This shows that BvPb1.2 is relatively thermostable and figure 6 shows similar results for BvPb1.1. The band from the syringe infiltrated sample is visually darker, indicating a higher expression. The protein bands produced by samples 8 and 10 DAA varies as the 10 DAA sample produced stronger bands. The 8 DAA samples had lower concentration of the expressed protein, but also seem to have had fewer proteins in general. The other proteins that have increased are likely to be stress proteins produced due to the shock of the agroinfection.

After the initial agroinfiltration, 8 DAA was used exclusively. Syringe infiltration is seemingly more ideal, though due to the time consuming process of infiltrating the leaves, it was not used for further experiments. We can see the size of both proteins closely corresponds with the band corresponding to 17 kDa. Leiva-Eriksson et al. (2014) predicted that the gene BvPb1.1 was 27 kDa and BvPb1.2 was 19.8 kDa in size; and differs from our results.

In figure 6, samples that express BvPb1.1 and BvPb1.2 can be seen. The negative control also shows a weak band before heating and is smaller than the transiently expressed proteins. The band disappears after heating, which separates the band from the expressed protein in both size and thermostability. Both of the expressed proteins seem to be of the same size, 17 kDa, and most likely due to cleavage of chloroplast transit peptide in BvPb1.1. After 5 minutes of heating at 60 °C, the samples using sodium phosphate buffer with pH 6 have almost no other proteins and a weak band left of the expressed protein. When heating for 3 minutes, all bands are stronger and therefore the sample has a higher concentration at the cost of purity.

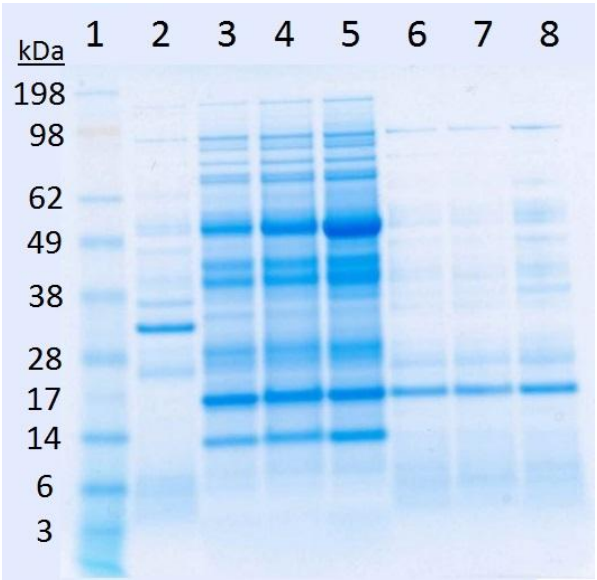

Figure 5. Gel showing the extracted leaves whichwere treated to express BvPb1.2 using sodium phosphate buffer pH 6. Heated samples were in a 60°Cwater bath for 3 minutes. 1: SeeBlue Plus2 Pre-Stained protein standard, 2: negative control, 3: sprayed 8 DAA, 4: sprayed, 10 DAA, 5: syringe infiltrated 7 DAI, 6: sprayed 8 DAA heated, 7: sprayed 10 DAA, 8: syringe infiltrated 7 DAI heated.

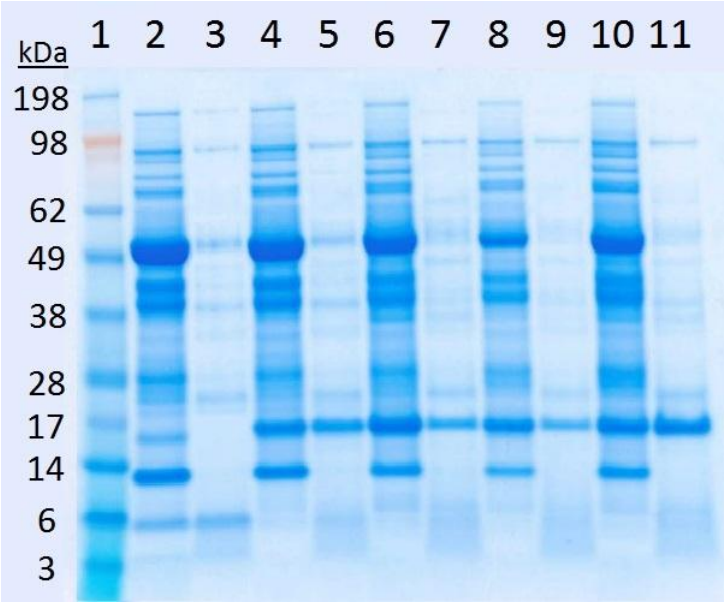

Figure 6. Gel showing the protein extracted from leaves expressing BvPb1.1 and BvPb1.2 sodium phosphate buffer pH 6. Heated samples were in a 60°C water bath for 5 minutes. 1: SeeBlue Plus2 Pre-Stained protein standard, 2: negative control, 3: blank heated; 4: BvPb1.1 expression 10 DAA, 5: BvPb1.1 expression 10 DAA heated, 6: BvPb1.1 expression 7 DAI; 7: BvPb1.1 expression 7 DAI heated; 8: BvPb1.2 expression 10 DAA, 9: BvPb1.2 expression 10 DAA heated, 10: BvPb1.2 expression 7 DAI, 11: BvPb1.2 expression 7 DAI heated.

When using Tris-HCl buffer with pH 8.5, the BvPb1.1 protein is still thermostable and shows a relatively high level of purity as compared to BvPb1.2 (Figure 7). BvPb1.2 however disappears quickly when heated in this buffer. This buffer is does not seem to offer similar protein stability as sodium phosphate buffer. This may be due to presence of high amount of active plant proteases at pH 8.5. The proteins thermostability could also be linked with the location of the proteins in the plant. BvPb1.2 is a cytoplasmic protein rather than the chloroplast targeted BvPb1.1. When heating the BvPb1.2 sample for only 30 seconds it shows a lone weak band and indicates that it is less thermostable.

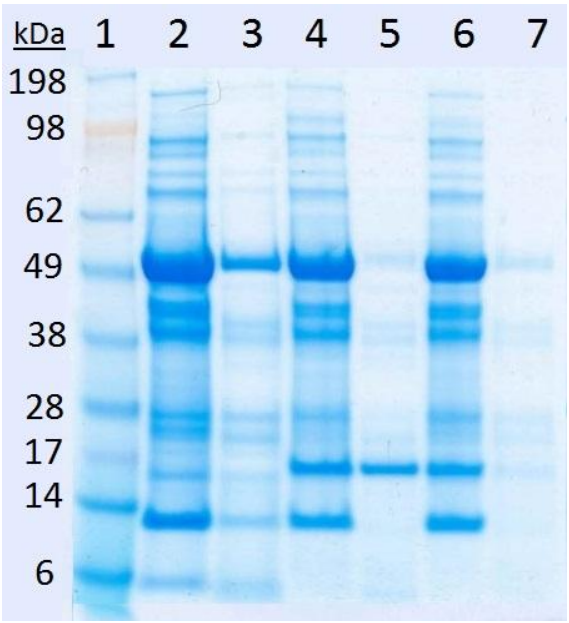

Figure 7. Gel showing the extracted leaves which were treated to express BvPb1.1 and BvPb1.2 using Tris-HCl buffer pH 8.5. Heated samples were in a 60°C water bath for 2 minutes. 1: SeeBlue Plus2 Pre-Stained protein standard, 2: negative control, 3: negative control heated, 4: BvPb1.1 expression 8 DAA, 5: BvPb1.1 expression 8 DAA heated, 6: BvPb1.2 expression 8 DAA, 7: BvPb1.2 expression 8 DAA heated.

In table 1, the BCA results of the samples are in mg protein per gram of leaf sample. The concentration of the highest and lowest sample seems to differ quite widely. BvPb1.1 8 DAA has a 54% higher concentration than BvPb1.2 8 DAA. A difference in thermostability could partly explain this difference and a second being that the expression of BvPb1.1 is up-regulated by the stress factors. Also, in *B. vulgaris* BvPb1.2 had low expression in the plant tissue only excluding the seeds. The protein extracted with sodium phosphate buffer and purified using 5 minutes of heat indicates similar purity as shown in figure 6.

Table 1. BCA results.

Sample	Protein in leaf sample (mg/g)
BvPb1.1 7 DAI	131,446
BvPb1.1 8 DAA	136,038
BvPb1.2 8 DAA	88,478
BvPb1.2 10 DAA	130,954

3.3 Spectrophotometry & ion exchange chromatography

A comparison of the results using spectrophotometry with the samples BvPb1.1 and BvPb1.2 can be seen in figure 8. The factors of BvPb1.1 and BvPb1.2 samples are 8 DAA, 10 DAA and 7 DAI. A peak around 409 nm, 540 nm and 575 nm indicates the presence of phytoglobin. The peak around 409 nm is called Soret peak and the peaks around 540 and 575 nm are Q-bands. We can see an obvious peak for respective samples at 409 nm. Also 2 additional peaks can be seen around 540 and 575 nm. These three peaks verify that our proteins are phytoglobins. BvPb1.1 7 DAI clearly has highest absorbance and shows very clear peaks.

BvPb1.1 and BvPb1.2 samples were also tested with NaD and CO using spectrophotometry to verify the functionality of the proteins. In figure 9 and 10, the shifting peaks show that both proteins can bind and release O_2 and CO. There is a clear difference in the peaks between oxy, deoxy and carboxy forms which makes the forms easy to distinguish. Testing an unheated sample of BvPb1.1 and BvPb1.2 shows less clarity due to the presence of other proteins. The heated sample overall have a lower background where only BvPb1.2 carboxy form differ. This is most likely due to using the same tip for the before and after heating samples when adding the CO.

Ion exchange chromatography was used to further purify an extracted, heated and filtered BvPb1.1 expressing leaf sample. Due to very high conductivity >10 mS/cm, the binding did not occur with the qHP purification. The sample simply had too many ions competing and would need more purification or dialysis to reduce the amount of ions.

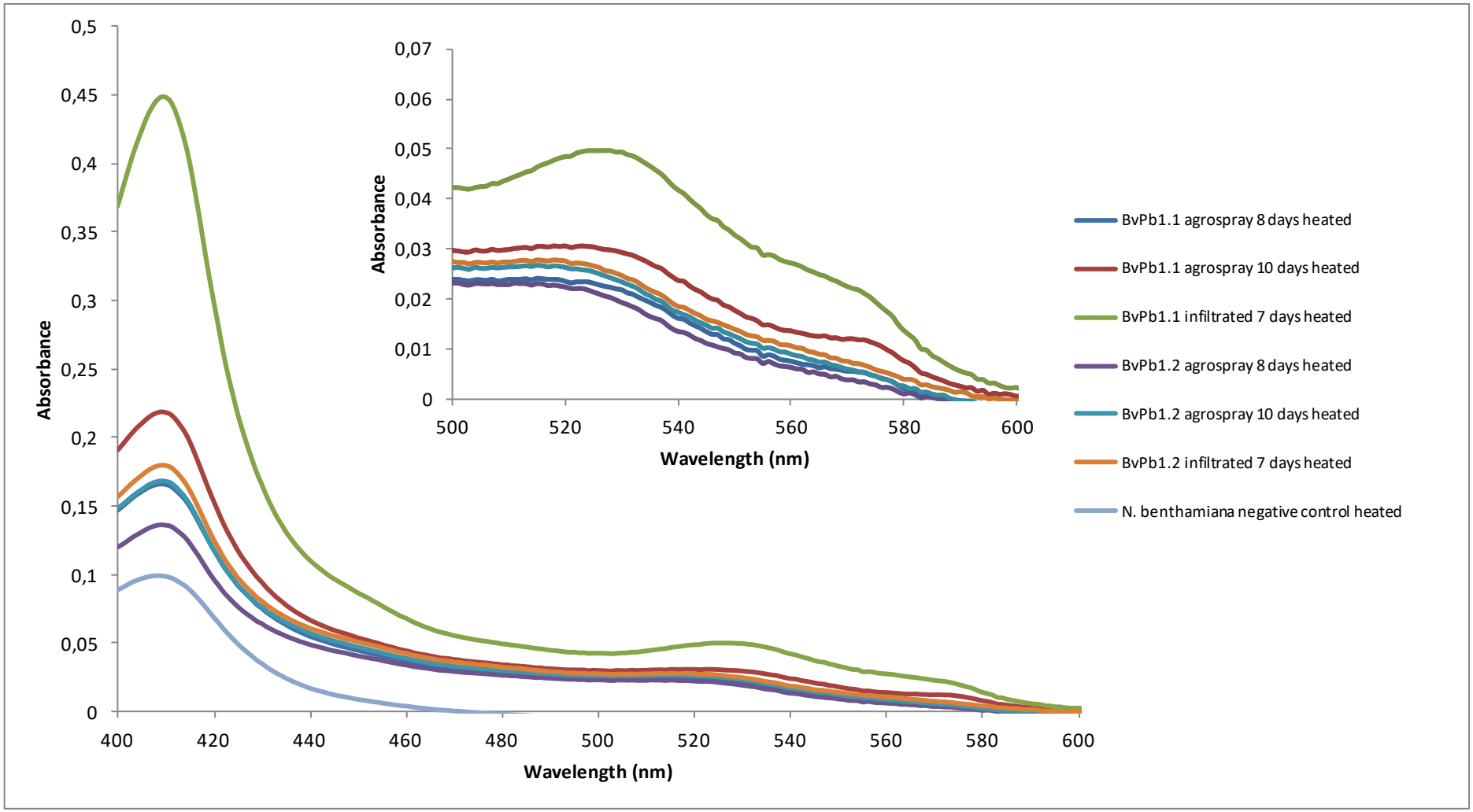

Figure 8. Graph showing the absorbance of partially purified BvPb1.1 and BvPb1.2 after heating comparing different infiltration methods and amount of days.

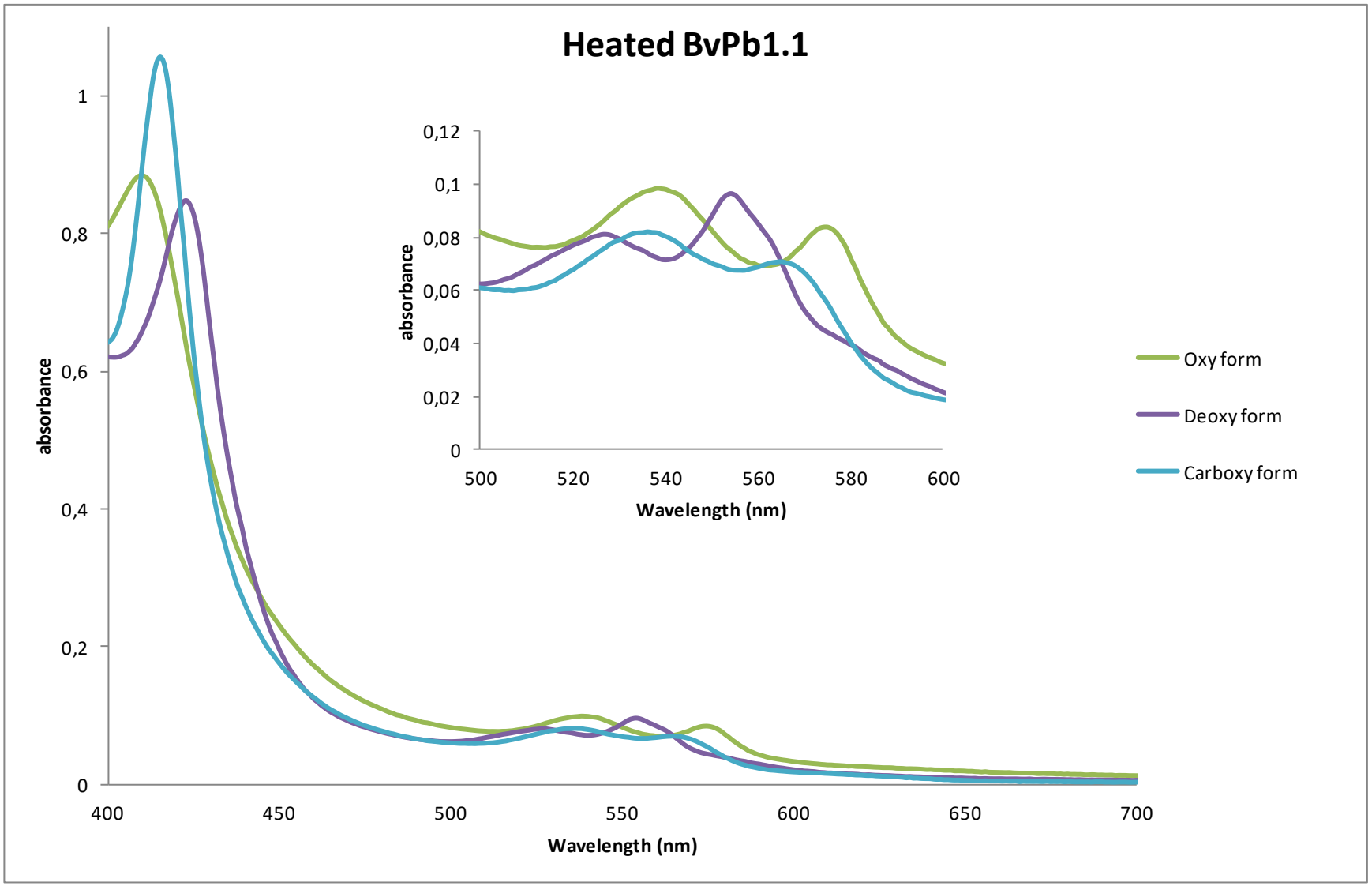

Figure 9. Graph showing the absorbance of the different forms of heated BvPb1.1.

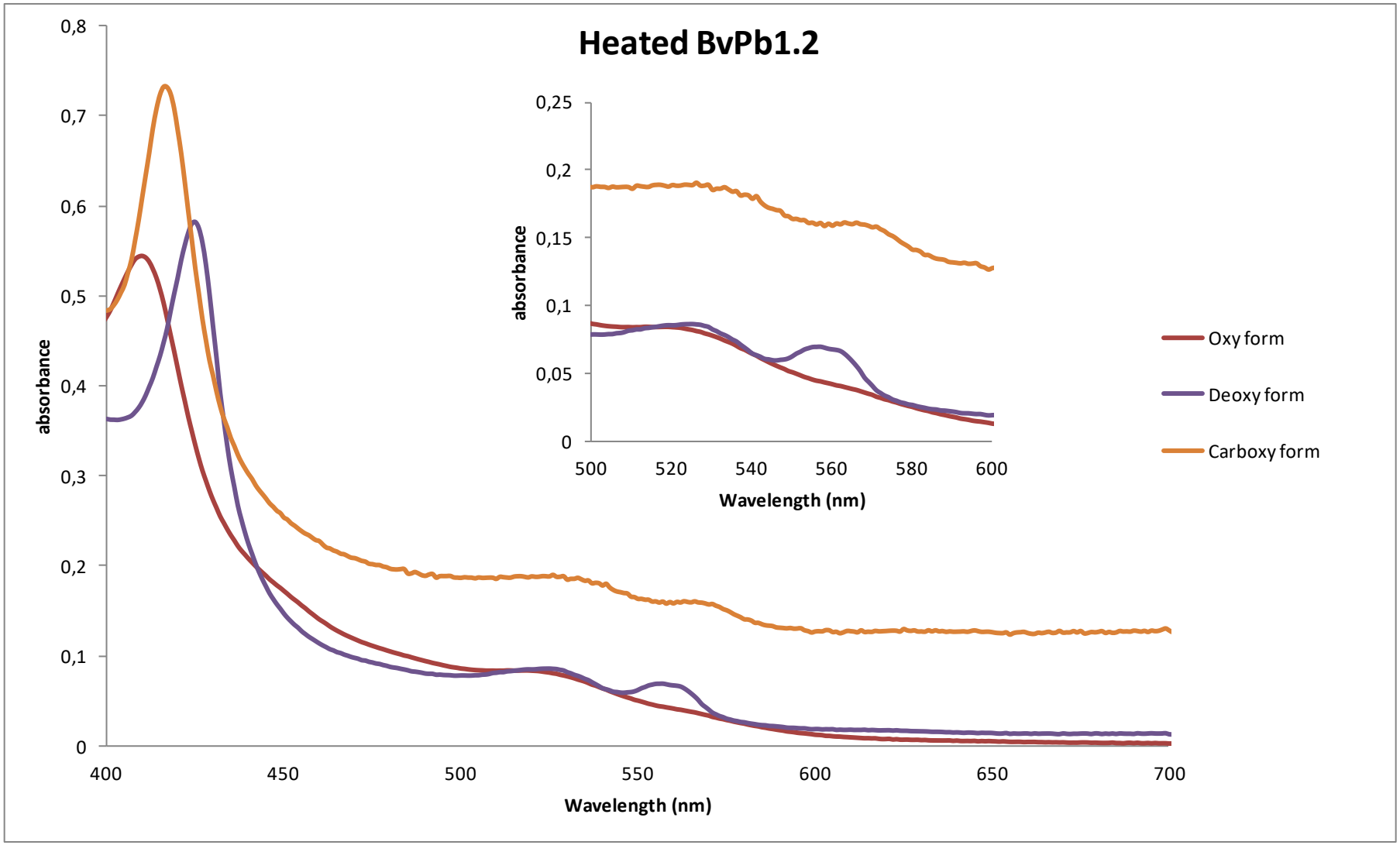

Figure 10. Graph showing the absorbance of the different forms of heated BvPb1.2.

4. Discussion

4.1 Proteins and methods

The genes, BvPb1.1 and BvPb1.2, code for proteins that belong to class 1 phytoglobins and seem to differ from the predicted protein size while over expressing in plants. Both the expressed proteins seem to have the same protein size when produced in tobacco. BvPb1.2 is close to its predicted size of 19.8 kDa and its exact size cannot be accurately distinguished on the SDS-PAGE gel. BvPb1.1 is however smaller than the predicted size which can most likely be explained by Richter and Lamppa (1998). BvPb1.1 is thought to translocate into the chloroplast where its extension (N-terminal transit peptide) is cleaved by stromal processing peptidase reducing its size. Other additional steps in the plants such as proper folding, posttranslational modification and the eukaryotic protein modification mechanisms could have had an effect on the proteins (Jin et al., 2015). Proper folding of the protein might change its structure into more common configuration and reducing its size. Leiva-Eriksson et al. (2014) predicted that the probable location of BvPb1.1 is in the chloroplast and BvPb1.2 is in the cytoplasm. This difference might have an effect on the thermostability of the proteins. BvPb1.1 seems thermostable in figure 7 which could be due to additional posttranslational modifications in the chloroplast.

The form of the phytoglobins in *Beta vulgaris* is largely unknown and could have a specific form or even change regularly between its oxy, deoxy and carboxy form. Using spectrophotometry, the phytoglobins in an extracted sample seem to be in oxy form. This could indicate that phytoglobins are in oxyform in the plant or O_2 could have come from the environment during extraction. The spectrophotometric results showed expected peaks in both BvPb1.1 and BvPb1.2 samples. In figures 9 & 10, BvPb1.1 and BvPb1.2 shows shifting peaks indicating that both proteins are functional. Figure 8 also clearly shows the shifting Soret peak (~409 nm) and the 2 Q-bands (540 & 575 nm) change depending on their forms.

The protein samples look fairly pure in SDS-PAGE gels although they are partially purified. Figure 5, 7 and 8 shows the protein composition of samples expressing BvPb1.1 BvPb1.2 in SDS-PAGE. The protein extracted at 10 DAA seems to have stronger bands than 8 DAA. After the first agroinfiltration, 8 DAA was exclusively used after trying all methods once. When heating the samples, some of the expressed protein can be lost despite relatively high thermostability of phytoglobins. The syringe-infiltrated sample had the highest yield and would be a good alternative as it can be harvested earlier. However, 8 DAA might be preferred if simplicity and purity is more important. Also, agrospray is fast, economical and easy to scale up. In In table 1, a noticeable difference of protein concentration can be seen between 8 and 10 DAA samples expressing BvPb1.2. The 7 DAI and 8 DAA samples expressing BvPb1.1 did not show any clear difference, indicating a plateau of the expressed protein. The results of the gel shown in figure 5 seem to correspond with the BCA results of BvPb1.2.

The BCA assay showed high concentration when testing all of the samples. The samples were all diluted 41 times and despite this, BvPb1.1 expression 10 days DAA and BvPb1.2 7 DAI was outside the range, >2mg/ml in 41x dilution. The assay was done with an extracted sample using sodium phosphate buffer and was heated for 5 minutes. The partially purified sample has comparable purity to the gel shown in figure 6. When the assay was carried out 1mM of DTT was used to obtain a concentration within the acceptable parameters. Another factor that could change the concentration could be when extracting protein from only parts of the leaves; as some portions could have a higher concentration (Bashandy et al., 2015).

Purification using ion exchange chromatography did not work as the partially purified sample had high conductivity.

4.2 Transient expression system

The transient expression system using *N. benthamiana* has been utilized for expressing several different proteins. The current study shows promise for producing phytoglobin in a plant-based transient expression system. As mentioned in an

interview by BBC (2014), phytoglobins produced in plants may have the potential to be used as substitute for blood transfusion. The plant-based expression system could possibly also be used to produce human hemoglobin. The specific protein would need to be tested for expression and functionality. Production of hemoglobin in plants would also need testing for possible changes when produced in a plant. As blood is in constant need for transfusion it would be a practical and scalable solution. Another potential application of the plant-produced phytoglobin would be as a biostimulant to induce the plant defence mechanisms. Many high effective strategies against pathogens in plants are not considered as organic and leave certain crops open to harmful attacks and losses for farmers. This could be an alternative way for organic farmers to shield their crops from biotic stress.

As mentioned by Dordas et al. (2003) and Manac'h-Little et al. (2005) Pb1 such as BvPb1.1 and BvPb1.2 have shown to increase NO and is an important defense response. It induces the production of salicylic acid or cyclic guanine monophosphate which in turn induces the defense genes pathway. There is also possibility to use in hydrophonic system or to water the plants with the extract in green house or open field as plant roots have a potential to absorb the phytoglobins. Paungfoo-Lonhienne et al. (2008) identified two ways roots can use protein. One way is to digest it with proteolytic enzymes and use it as a nitrogen source. The other way is to absorb the whole protein into the root cells most likely via endocytosis. The total amount of protein absorbed compared to digested would need to be assessed. As an inducer, phytoglobin would most likely be used in the beginning of an invasion of a pest to lessen its effect.

If the system would be used for this purpose, a stable transformation could be preferred, as the leaves require no agroinfection between harvests. Low expression levels might deter the use of stable transformation however; there is a possibility to use the whole plant for the extraction process. One of the bigger issues would be the high cost that would follow the production of the protein. Also, its efficacy as defence mechanism inducer is not established so far.

However, thermostable nature of phytoglobin can possess the properties of long shelf life, which would most likely to be considered to launch an industrial product. A higher level of purification could enable even longer shelf life as most of proteases are removed during purification; though it might also increase cost and decrease the concentration of phytoglobin. Climate conditions such as heat can vary quite a lot

depending on location and annual shifts. This could cause the phytoglobins' life expectancy to vary widely depending on location.

These phytoglobins from *B. vulgaris* has not been expressed and tested in a plant-based expression system so far. This means that processes affecting phytoglobin functions might not be completely known or understood. Considering this, the effect of protein such as increasing NO might be altered when producing in bacteria rather than in a plant. There is a possibility for a change in protein functions by choosing another crop species due to possible different end processes.

4.3 Conclusion

This study has shown that the BvPb1.1 and BvPb1.2 can be successfully expressed transiently in *N. benthamiana* and that the proteins produced are functional. The relatively thermostable proteins and can bind and release O_2 and CO *invitro*. It shows the possibility of heterologously producing phytoglobins in a plant-based expression system at industrial level, which has potential to be used as biostimulant to alleviate biotic stresses.

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