Dehydrin patterns in wheat leaves during severe drought and recovery

Master's Thesis

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Stefan Eichenberger

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Supervisor:

Prof. Dr. Urs Feller
Institute of Plant Sciences and Oeschger Centre for Climate Change Research

Co-Supervisor:

Prof. Dr. Jürg Fuhrer
Agroscope Reckenholz-Tänikon Research Station ART and Oeschger Centre for Climate Change Research

Abstract

Plants suffering from drought go through adaptations to survive such periods. One of these adaptations is to produce proteins called dehydrins, the functions of which are still under investigation. In this study, dehydrins of wheat (*Triticum aestivum* L. cultivar Arina) as an important crop plant were analyzed under heavy drought conditions and during the following recovery phase. The method of immunoblotting was used to determine changed dehydrin patterns. Several dehydrins with molecular weights of 180 (double band) 58, 42, 24 and 20 kDa were detected in plants grown on soil (stressed by withholding water). The clearest bands were always observed in young leaves. Dehydrin levels decreased again during a subsequent recovery phase.

Introduction

Influence of climate change on vegetation is an important issue today and will most likely become more important in the future, as models predict that global climate is getting warmer and extreme climatic events will occur more frequently. Many different aspects to be considered include how plants can react and recover from more abundant storms, heat waves or in our case, what is their physiological response on long periods of drought i.e. drought stress (Saier, 2007).

Plants have evolved several different strategies to survive periods of drought. A first one is to avoid damage by completing the life cycle before the stress can harm the plant (drought escape). Plants can further achieve the ability to maintain tissue hydration by morphological and physiological adaptations (desiccation postponement) or still function during drought (desiccation tolerance) (Taiz &Zeiger, 2003). These physiological adaptations take place, on the one hand, on a transcriptional level, where genes responsible, for example, for photosynthesis are down regulated while several others involved in the detoxification of reactive oxygen species (ROS) are up regulated. On the other hand the translation of mRNA of stress related proteins or enzymes is changed as well. Enzymes in biosynthesis pathways of osmolytes are translated more often leading to higher concentrations of certain sugars, amino acids (prolin) or quarternary ammonium compounds (glycine betaine), all of which increase the osmotic potential inside the cell and thereby preventing water loss to the apoplast (Ingram and Bartels, 1996). Another mechanism to increase drought resistance is by increasing the concentration of a special group of proteins, the late embryogenesis abundant (LEA) proteins. As the name suggests, these proteins are usually produced during late embryogenesis, but have also been

shown to occur during all sorts of osmotic stresses like high salinity, heat, cold, or drought. (Bray, 1997).

Dehydrins are part of these LEA proteins (group II) and are built up by many charged and polar amino acids without cystein and tryptophan ever occurring (Wahid and Close, 2007). They are widely spread in higher plants, algae, yeasts, and cyanobacteria. Apart from being induced by stress, dehydrins are also known to be produced in response to the phytohormone abscisic acid (ABA) (Allagulova et al., 2003).

It was found that dehydrins consist of three highly conserved segments: The K, the Y and the S segment, named after each predominant amino acid (K: lysine, Y: tyrosine, S: serine). The K segment (EKKGIMDKIKEKLPG) is the only common feature of all dehydrins. It occurs in the different proteins in numbers of 1 to 12 repeats and is found near the C-terminus. According to computer-aided analysis, the sequence can form an amphipathic α-helix. These helices are predicted to be a key component of the binding of dehydrins with other proteins. Not very much is known about the Y-segment [(V/T)DEYGNP] except that it is usually localized near the N-terminus. By comparing it with other known sequences, high similarity with a chaperon binding site could be detected. However, so far no interactions between chaperons and dehydrins have been identified. The S-segment consists of a different number of serine residues, which were shown to be phosphorylatable in vitro. This phosphorylation is suggested to play a role in dehydrin signal peptide interaction, leading to translocation of the dehydrin to the nucleus (T. J. Close1996, Allagulova et al., 2003). A classification of dehydrins has been introduced based on these three segments, grouping most dehydrins into the five groups: Y_nSK_n, K_n, K_nS, SK_n, and

Y_nK_n. *In vitro* experiments with these subclasses showed that the Y_nSK_n–type could bind to lipid vesicles containing acidic phospholipids, while the K_nS–type was shown to bind to metals (Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) and hydroxyl and to protect lipid membranes against peroxydation. Another proposed function of dehydrins is a cryoprotective activity by SK_n- and K_n–type, since an accumulation of these proteins is observed especially in and around vascular tissues of cold stressed plants (Rorat, 2006). Additional functions are still under investigation (Allagulova et al., 2003). Dehydrins are primarily distributed in the cytosol and the nucleus, but also occur in the vicinity of plasma membranes, vacuoles and mitochondria (Rorat, 2006).

As one of the major crop plants, wheat (*Triticum* aestivum) is of special importance with respect to world nutrition since 21% of the world's food depends on this one plant. In a changing global climate, it becomes increasingly important to adapt the crop to new environmental conditions (Ortiz, 2008). Therefore, it is crucial to have a holistic understanding of stress tolerance. Studies on different cultivars of wheat revealed a significant correlation of the presence of dehydrins and yield of dry matter (Lopez et al., 2003).

So far, no one has compared dehydrin accumulation in the different leaves. In addition, all previous immunological experiments were conducted with antibodies against the K-segment only.

In our experiments, plants from the species *Triticum aestivum* cultivar *Arina* were used, grown under controlled conditions in a growth chamber on soil and in hydroponic culture. The aim was to examine the dehydrin pattern under drought stress and its recovery with different antibodies. Since samples had been taken on a

leaf basis, it was possible to investigate the distribution of dehydrins within the plant. The question was asked if dehydrins could serve as molecular markers for drought stress after a period of recovery.

Material and Methods

Plant material:

All main experiments were performed with plants from the species *Triticum aestivum* L. cultivar Arina. Grains were placed in a container on several layers of paper soaked with deionized water and kept in the dark for five days. During this time seedlings germinated and grew coleoptiles of about 5cm. Then the cover of the container was removed for seedlings to produce a first leaf. Seven days after germination seedlings were planted either in hydroponic cultures (after removing the endosperm) or on soil (with the endosperm).

For the test of the antibodies, stressed plant material from *Dactylis glomerata* was used as well as material from *Triticum aestivum*.

Plants in hydroponic culture:

Plants were grown in a room under controlled temperatures of 24°C/17°C (14 h day) with a photosynthetic photon flux of 121 $\mu mol*m^{-2}*s^{-1}$ (Spectrum Technologies, Inc, Logan, UT). Twelve young plants were transferred into pots containing 150 ml of diluted nutrient solution (one part standard nutrient solution, one part deionized water, standard nutrient solution: 8 g K_2PO_4, 7.5 g $MgSO_4*7H_2O$, 3.17 g $Ca(NO_3)_2*4H_2O$, 0.88 g KNO_3, 0.28 g Fe-EDDHA, 2 mg $MnCl_2*4\ H_2O$, 3 mg H_3BO_3, 0.5 mg $ZnSO_4*7H_2O$, 0.3 mg $CuSO_4*5H_2O$, 0.5 mg $Na_2MoO_4*2H_2O$, 0.15 mg $Ni(NO_3)_2*6H_2O$) for the first growth period of eight days. Roots and nutrient solutions were kept dark by covering them with an inverted pot, with the plants growing out of

the holes in the bottom (Figure 1). Control plants were kept on sufficient amounts of standard nutrient solution, while stressed plants were transferred to 150ml nutrient solution containing 100g/l of polyethylene glycol (PEG) to simulate drought stress. Some plants were kept in this medium until the end of the experiment (stressed plants: S), while others were transferred to standard nutrient solution after the fourth sampling date (stress-recovery plants: SR). To investigate stress response at different developmental stages, stress was applied at two different times. In a first series, stress was started 15 days after germination, and samples were taken on days 0, 4, 8, 12, 16 and 20 of stress (series a). In a second series plants were grown for 25 days (seven days of germination, eight days on one-to-one diluted nutrient solution and ten days on standard nutrient solution) and samples were taken on days 0, 3, 6, 9, 12 and 16 (series b). Same leaves of four plants were combined to one sample. Of each sample one replicate was taken.

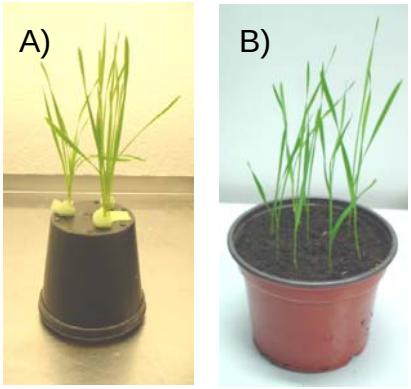

Figure 1: Wheat plants. A: Plants in hydroponic culture, twelve plants per pot in 150 ml nutrient solution. B: Plants grown on soil, nine plants per pot with 270 g of soil (dry weight).

Plants on soil:

Plants were grown in a growth chamber under controlled temperature of 24°C/16°C (14 h day) with a photosynthetic photon flux of 138 μmol*m^{-2}*s^{-1} (Spectrum Technologies, Inc, Logan, UT). Pots were irrigated with distilled water and nine seedlings were planted with grains about 5 mm in the soil (Landerde, Ricoter,

Aarberg, Switzerland). All plants were well watered for 13 days. Control plants were continuously irrigated, while for stressed plants irrigation was stopped 20 days after germination when plants were at the four-leaf developmental stage. Half of them were kept dry until the end of the experiment (stress plants: S), while for the others irrigation was started again after the third sampling date (27 days of stress) to investigate recovery from drought stress (stress-recovery plants: SR). Samples were then taken after 0, 21, 27, 35 and 43 days. Same leaves of four plants were combined to one sample. Of each sample one replicate was taken.

Protein extraction and sample treatment:

Protein extractions were made in 5 ml of extraction buffer (20 ml 100mM Na-phosphate-buffer, 1 g polyvinyl-polypyrrolidone (PVPP), 100 μl β-mercaptoethanol, 80 ml H_2O). The plant material was disintegrated with a polytron (Kinematica GmbH, Luzern, Switzerland) for 20 seconds on position five and then for five seconds at full speed. Then extracts were filtered through two layers of Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA). From this protein extract 200 μl was added to 100 μl of concentrated sample buffer (2 ml 0.5M Tris-HCL (pH 6.8), 1.6 ml glycerol (87%), 0.32 g sodium dodecyl sulfate (SDS), 0.7 ml Brilliant Blue R-250, 0.8 ml β -mercaptoethanol) and was incubated in a boiling water bath for 5 minutes (total protein samples, T-samples).

Heat treatment and dialysis:

Different treatments were tested. Heating the sample for ten minutes at 80°C (Volaire, 2002) appeared to remove some of the unwanted proteins, but the most efficient treatment was the one applied by Wahid and Close (2007) where samples were heated up to 95°C for five minutes. Two ml extract was centrifuged and the

supernatant incubated in a boiling water bath for five minutes to remove heat-sensitive proteins. Then the extract was centrifuged and samples were dialyzed in a tube of cellulose (Sigma Chemical Company, St. Louis), kept in PEG solution (50% g/v). After about half an hour, some water had diffused into the surrounding solution, reducing the volume to about 0.5 ml. Of this protein extract 200 μl were used to produce samples (treated samples: D-samples). All samples were stored at –20°C prior to SDS-PAGE and immunoblotting.

Protein content of total extract and treated samples was quantified with a dye-binding assay (Bradford, 1976) at a wave length of 578 nm.

SDS-PAGE and immunoblotting:

Different primary antibodies were used for the experiments. Both, a commercially available antibody against the K-segment (Deh-AB) and antibodies against the K (K-AB), the Y (Y-AB) and the S-segment (S-AB), produced in rabbits, were tested.

Main proteins (Light harvesting complex II, large and small subunit of Rubisco) were investigated with an SDS-PAGE (12% (w/v) acrylamide, 0.75 mm). Immunoblotting with 1.5 mm thick gels was performed according to Mitsuhashi and Feller (1992) to investigate dehydrins. Primary anti-dehydrin antibodies and secondary IgG-solutions were diluted 1:1000.

Results

Testing of antibodies:

Antibodies were tested first. Plant material (*Dactylis glomerata* and *Triticum aestivum*) known to show dehydrin expression was used with all antibodies. As shown in Figure 2, the blots with the four antibodies showed different patterns.

K-AB: The K-AB mainly showed a response with control samples of *T. aestivum* and at sizes at which no dehydrins were detectable with any other antibody (48, 38, 30 and 15 kDa).

Deh-AB: The commercially available Deh-AB showed bands with both plants around 58, 45, 40, 23, and a double band around 20 kDa. The band at 23 kDa occurred with all samples, including controls, suggesting that it is either a constitutively expressed dehydrin or an interference with another protein. The size and the strength of the signal led to the hypothesis that it could be an interference with Light Harvesting Complex II (LHCII). Further investigation of this band showed it to be membrane bound. The fact that dehydrins are soluble proteins and LHCII is the most abundant protein bound to membranes was a further indication for this 23 kDa band to be LHCII. After the heat treatment, there still remained a band at around 24 kDa, first thought to be the debris of LHCII. When compared with blots from the Y-AB, it was identified as a dehydrin. Another double band around 36 kDa could be detected in *D. glomerata*, while *T. aestivum* showed a double band around 180 kDa and another band at around 42 kDa.

Y-AB: The Y-AB showed some similar features compared to the Deh-AB. There were bands at around 55, 27, 24, and a double band around 20 kDa for *T. aestivum*. The band at 55 kDa was more striking in control samples. As no dehydrin was detected with the Deh-AB against the K-segment, this band could not be a dehydrin. Since it is at the size of the large subunit of Rubisco (LSU), it is possible, that this band is an interference with LSU. *D. glomerata* did not show this band at 55 kDa, but a further distinct band appeared around the size of 38 kDa.

S-AB: With the S-AB no band was detectable for *D. glomerata* and only weak bands which also occurred in control samples of *T. aestivum*.

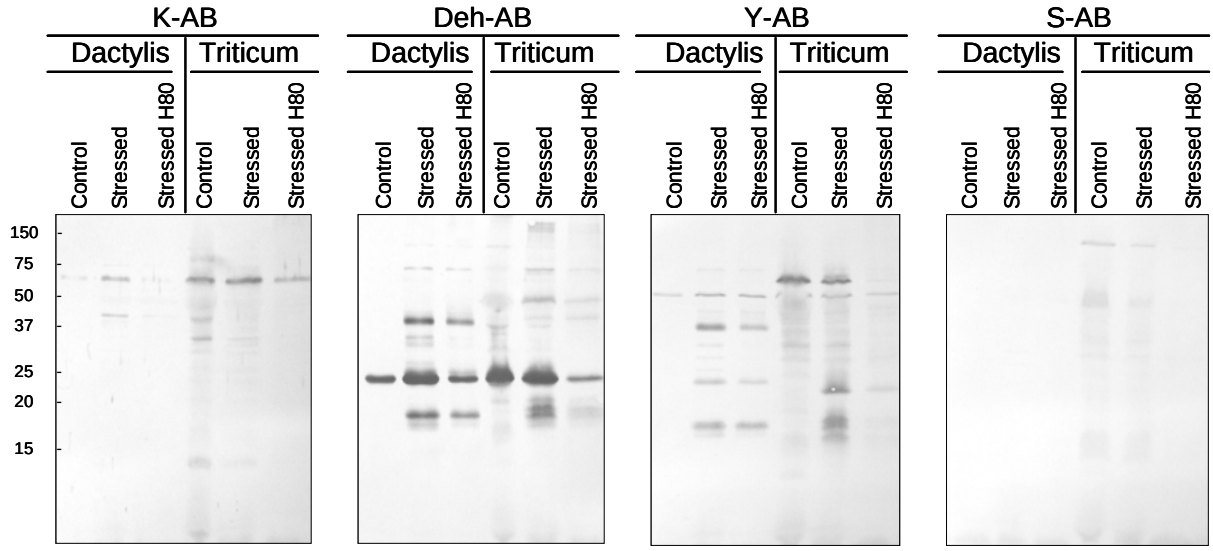

Figure 2: Identical blots, developed with the four different antibodies against the K-segment (K-AB and Deh-AB), the Y-segment (Y-AB) and the S-segment (S-AB) of dehydrins. Samples of *D. glomerata* and *T. aestivum*, known to show dehydrin expression were blotted. To remove heat sensitive proteins, Stressed H80-samples were heated 10 minutes at 80 °C after protein extraction.

K-AB and S-AB were not suitable to use, and all further experiments were performed with the Y-AB against the Y-segment and the commercial Deh-AB against the K-segment.

Plants on soil:

First experiments had shown, old leaves (leaf 1, data not shown) did not produce high concentrations of dehydrins, which is why the fourth leaves were extracted and blotted at all the different sampling dates to see the time course of dehydrin production (Figures 3 and 4). Three Dehydrins (40, 24 and 20 kDa) could be detected with both the Deh-AB and the Y-AB, the band at 20 kDa perhaps even being a double band. Additionally, with the Deh-AB there was a double band at the size of around 180 kDa, one at 58 kDa and another weak signal at the size of 42 kDa. Since not every dehydrin contains a Y-segment, it is reasonable that certain bands occur only with the Deh-AB but not with the Y-AB. For the bands at the size of 23 kDa with the Deh-AB and of 55 kDa with the Y-AB see paragraph testing of the antibodies.

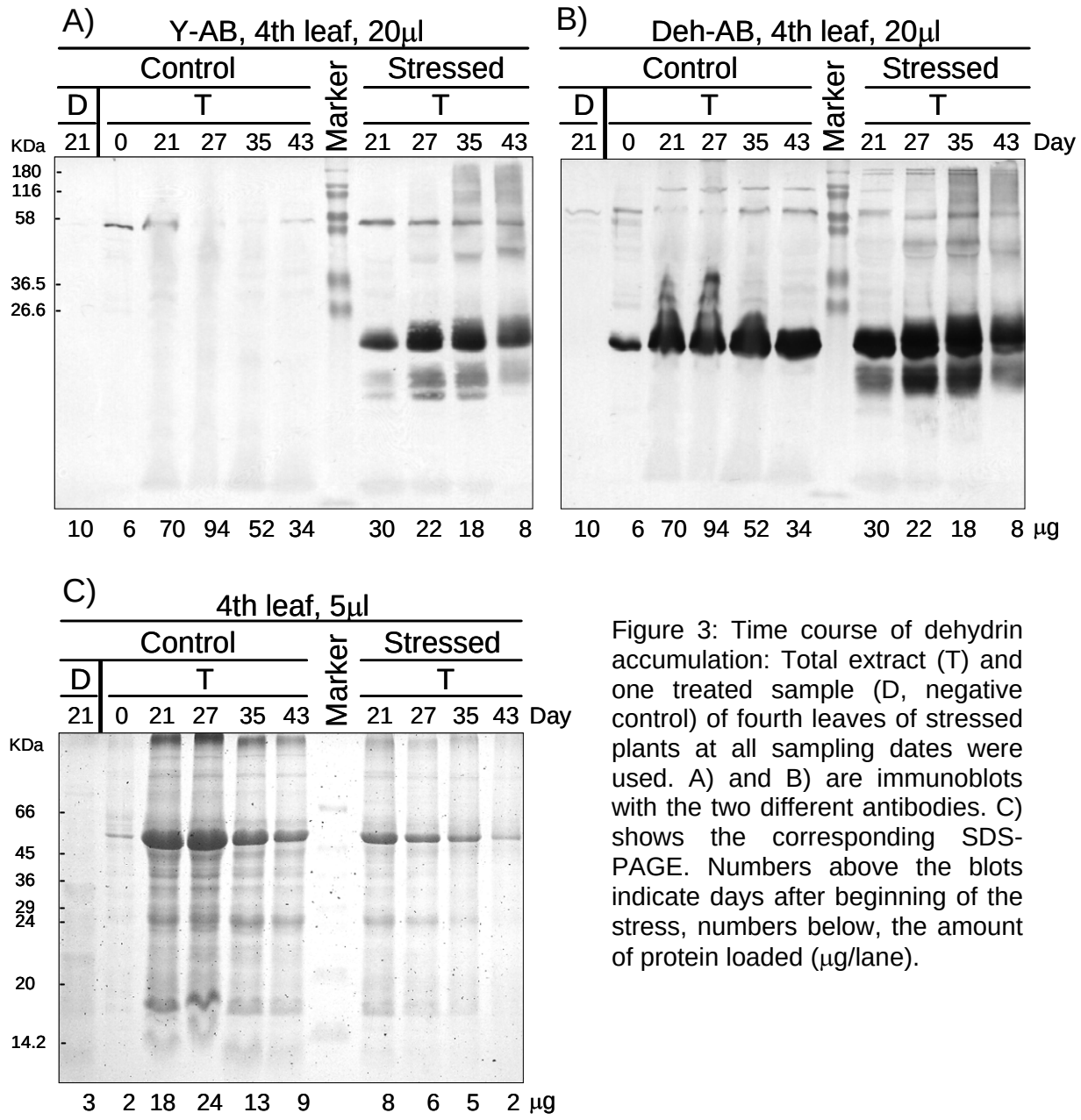

Figure 3: Time course of dehydrin accumulation: Total extract (T) and one treated sample (D, negative control) of fourth leaves of stressed plants at all sampling dates were used. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

The immonoblots revealed that the plant accumulated most dehydrins before the third sampling date (27 days without water), then remained more or less at the same level until the forth date (35 days), before contents of dehydrin started to decline again. At the first sampling date, especially with treated samples, some bands were also visible for control plants (see Figure 4).

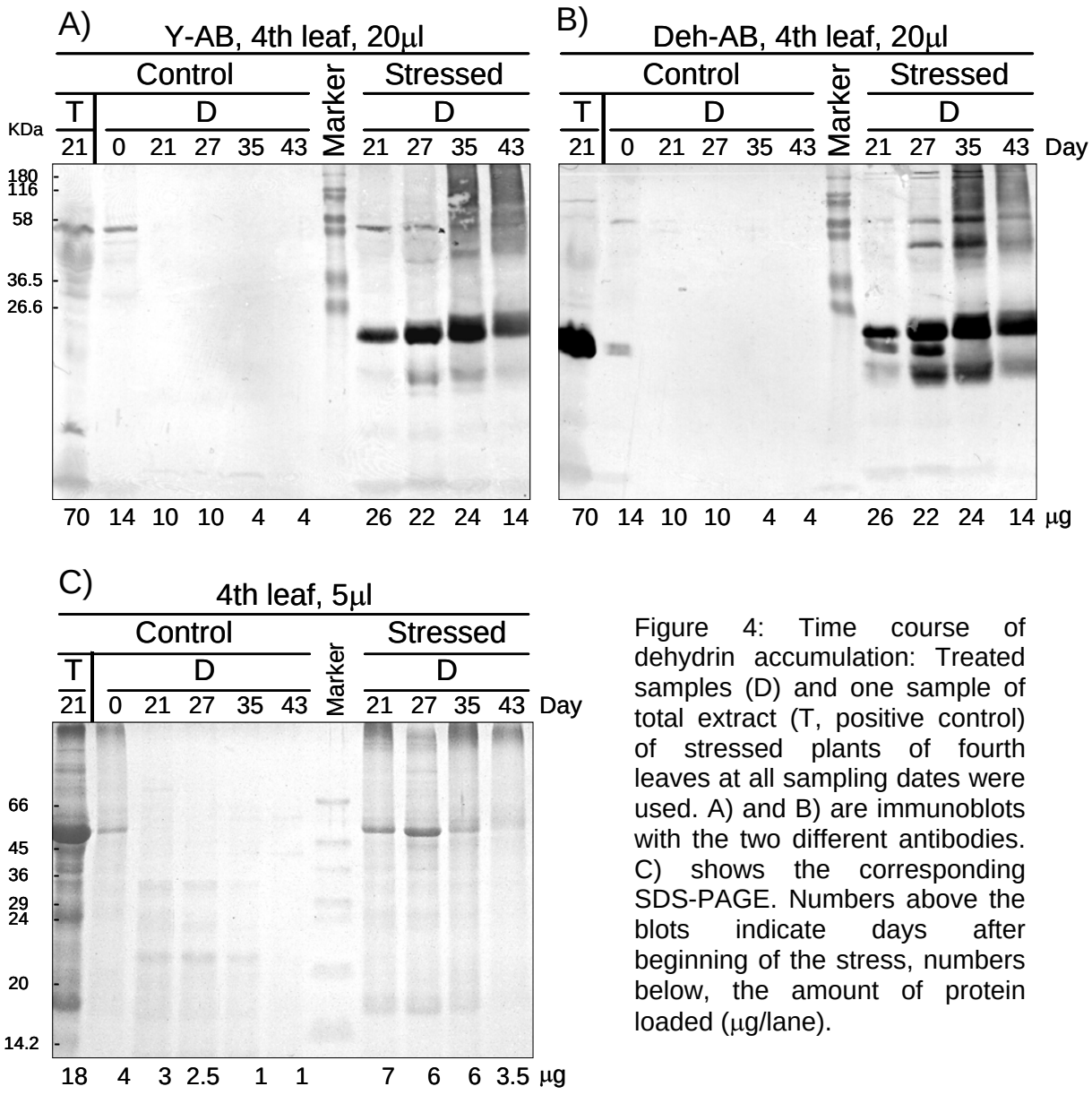

Figure 4: Time course of dehydrin accumulation: Treated samples (D) and one sample of total extract (T, positive control) of stressed plants of fourth leaves at all sampling dates were used. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

To estimate the distribution of dehydrins within the plant, all leaves from the third sampling date were blotted (Figure 5). For both antibodies very similar results were obtained. There was a clear tendency towards dehydrin production in younger leaves. Leaves three and four again showed the four dehydrins of 58, 40, 24 and 20 kDa. Additionally another band occurred at around 27 kDa. Total extracts of control plants showed a lot of background. In the treated sample of leaf 4 of control, no dehydrins were detectable with our antibody.

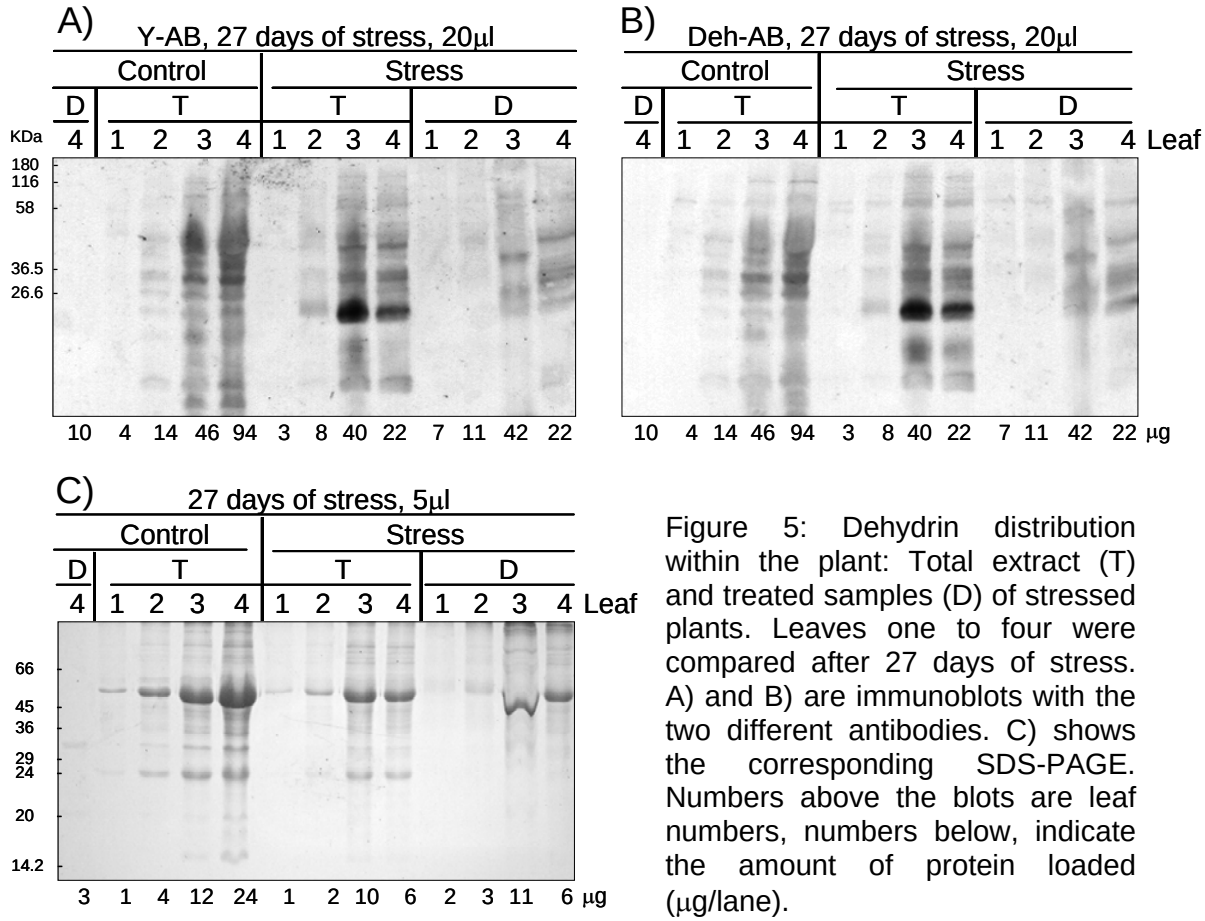

Figure 5: Dehydrin distribution within the plant: Total extract (T) and treated samples (D) of stressed plants. Leaves one to four were compared after 27 days of stress. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots are leaf numbers, numbers below, indicate the amount of protein loaded (µg/lane).

The blots in the stress-recovery experiment exhibited very similar bands with the Deh-AB and the Y-AB. By looking at leaf four from day 21 until the end of the experiment at day 43 of stress, it could be seen how dehydrin concentration of the same five dehydrins (58, 40, 27, 24 and 20 kDa) rose until maximum stress after 27 days without water (Figure 6). At that time, recovery was initiated, which appeared on the blots as a decrease (T-samples) and by total removal (D-samples) of dehydrins already 8 days after stress was relieved. The T-samples during times of recovery still showed expression of all the dehydrins except the one at 24 kDa. Control samples again showed considerable background, which was totally removed in the D-sample by the treatment.

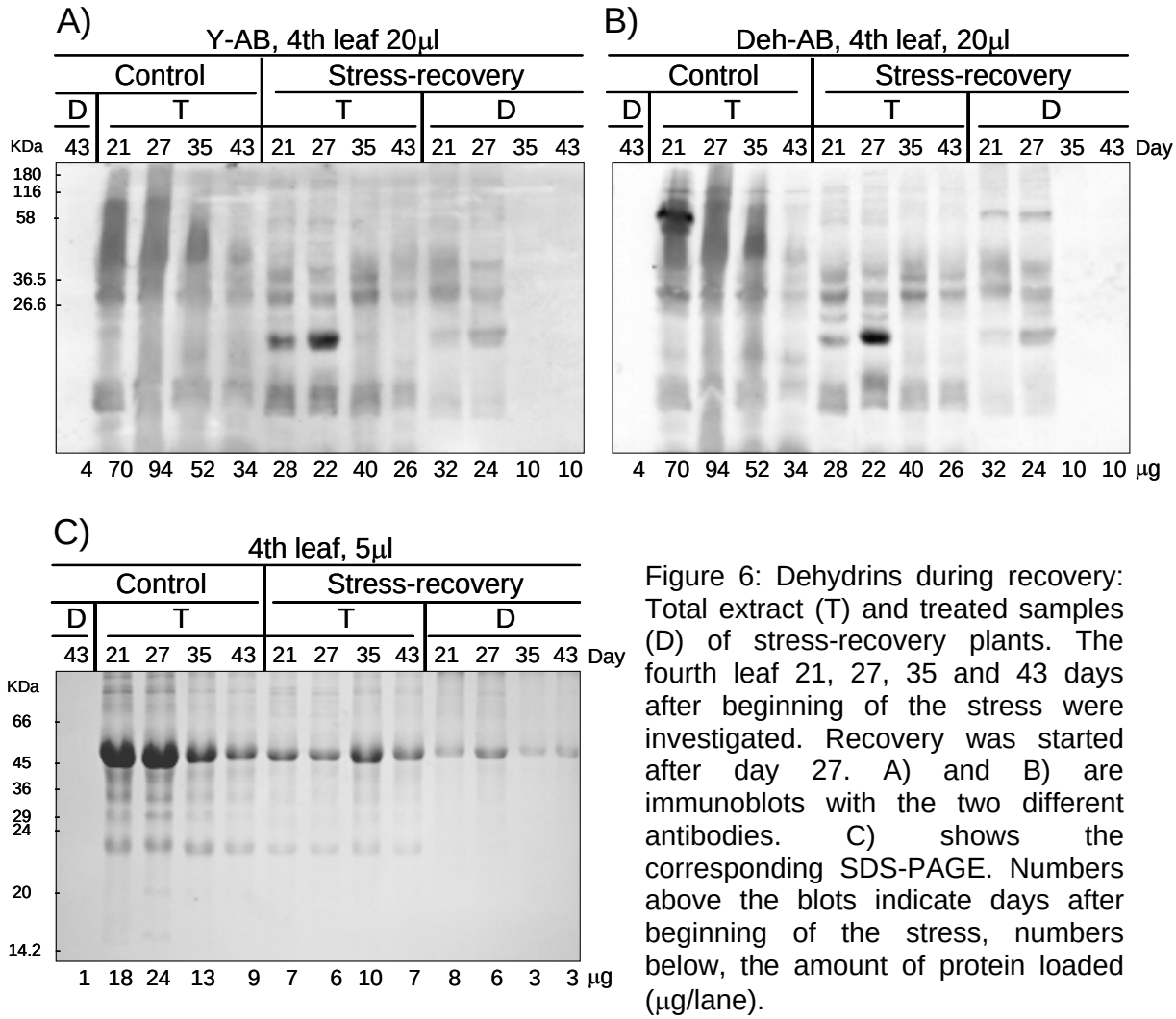

Figure 6: Dehydrins during recovery: Total extract (T) and treated samples (D) of stress-recovery plants. The fourth leaf 21, 27, 35 and 43 days after beginning of the stress were investigated. Recovery was started after day 27. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

Plants in hydroponic culture: Series a

The plants investigated in this experiment were at the two-leaf developmental stage when stress was initiated (15 days after germination). Uptake of nutrient solution and concentrations of PEG for the stress and stress-recovery experiments are shown in Figure 7. As the amount of nutrient solution declined, PEG concentration and therefore the stress on plants increased. For the stress recovery experiment, PEG solution was replaced by a normal nutrient solution on day 12. After that, these plants were kept on sufficient amounts of nutrient solution until the end of the experiment with the solution getting replaced a second time on day 17.

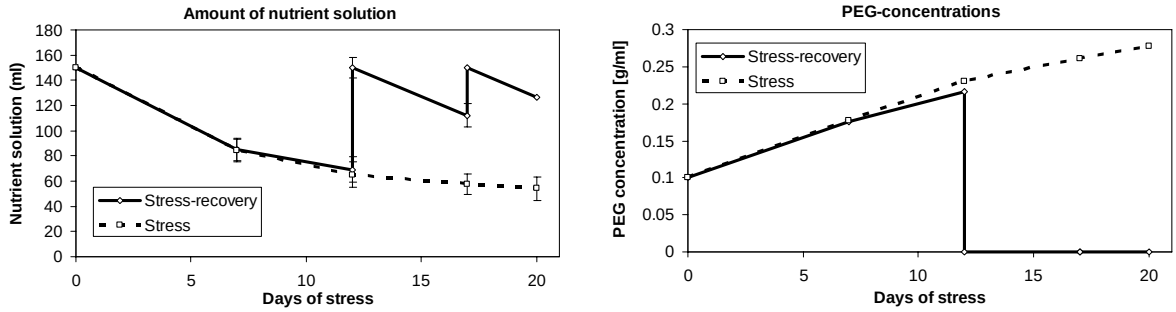

Figure 7: Amounts of nutrient solution (left) and the corresponding PEG-concentrations (right), for the stress experiment (dotted lines) and for the stress-recovery experiment (black lines).

To show the distribution of dehydrins within the plant, all leaves after 20 days of stress were investigated (Figure 8). The two antibodies presented a similar response, with Deh-AB showing clearer bands than the Y-AB. Four dehydrins (58, 24 and a double band around 20 kDa) were detected in the sample of leaf three, where most proteins were expressed. Total extract samples of control plants showed two faint bands, which disappeared in the D-sample.

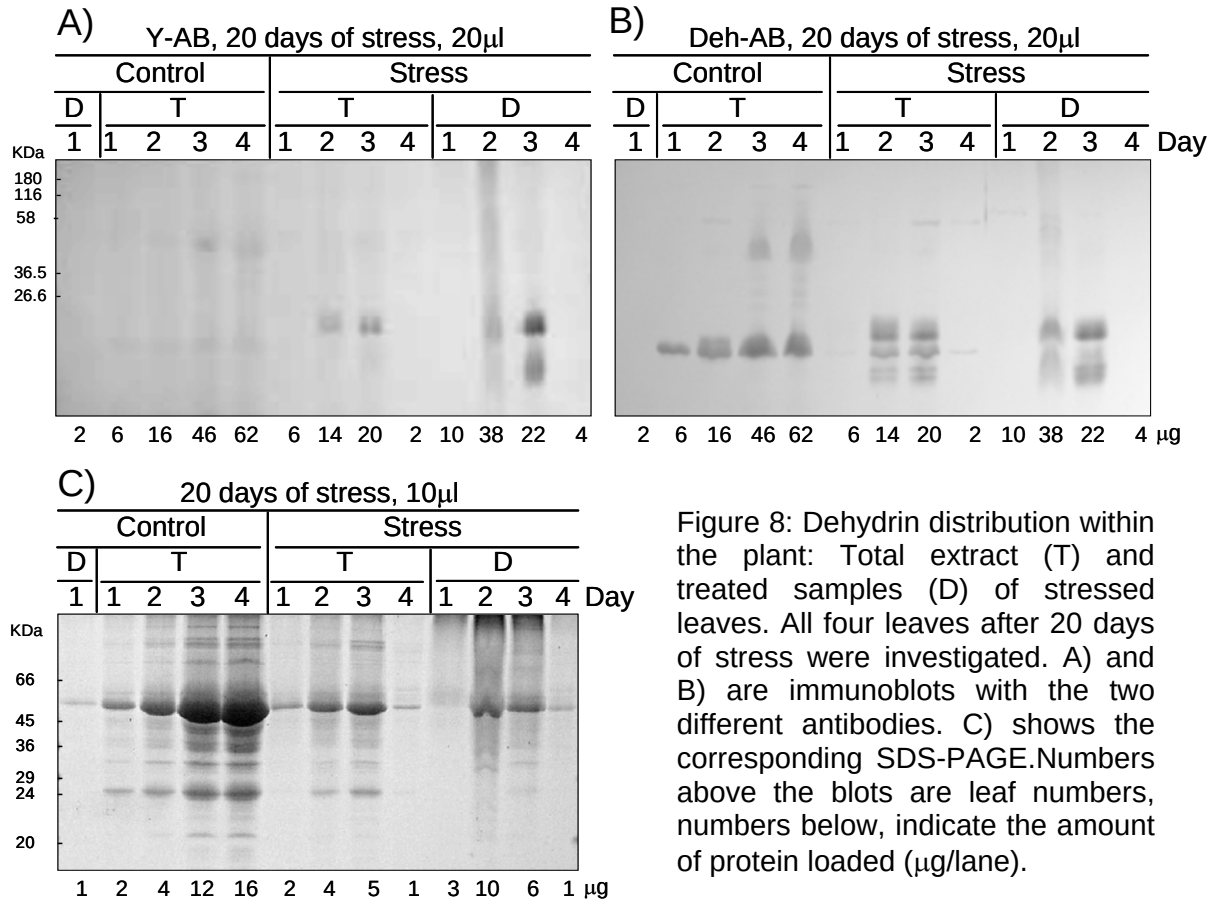

Figure 8: Dehydrin distribution within the plant: Total extract (T) and treated samples (D) of stressed leaves. All four leaves after 20 days of stress were investigated. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE.Numbers above the blots are leaf numbers, numbers below, indicate the amount of protein loaded (µg/lane).

Investigating the behavior of dehydrins during the recovery phase, the third leaves were blotted on days 12 (start of recovery), 16 and 20. Like in the previous results, dehydrins were observed at sizes of 58, 24 and 20 kDa (double band). All proteins declined over time and were almost gone after eight days of recovery (Figure 9).

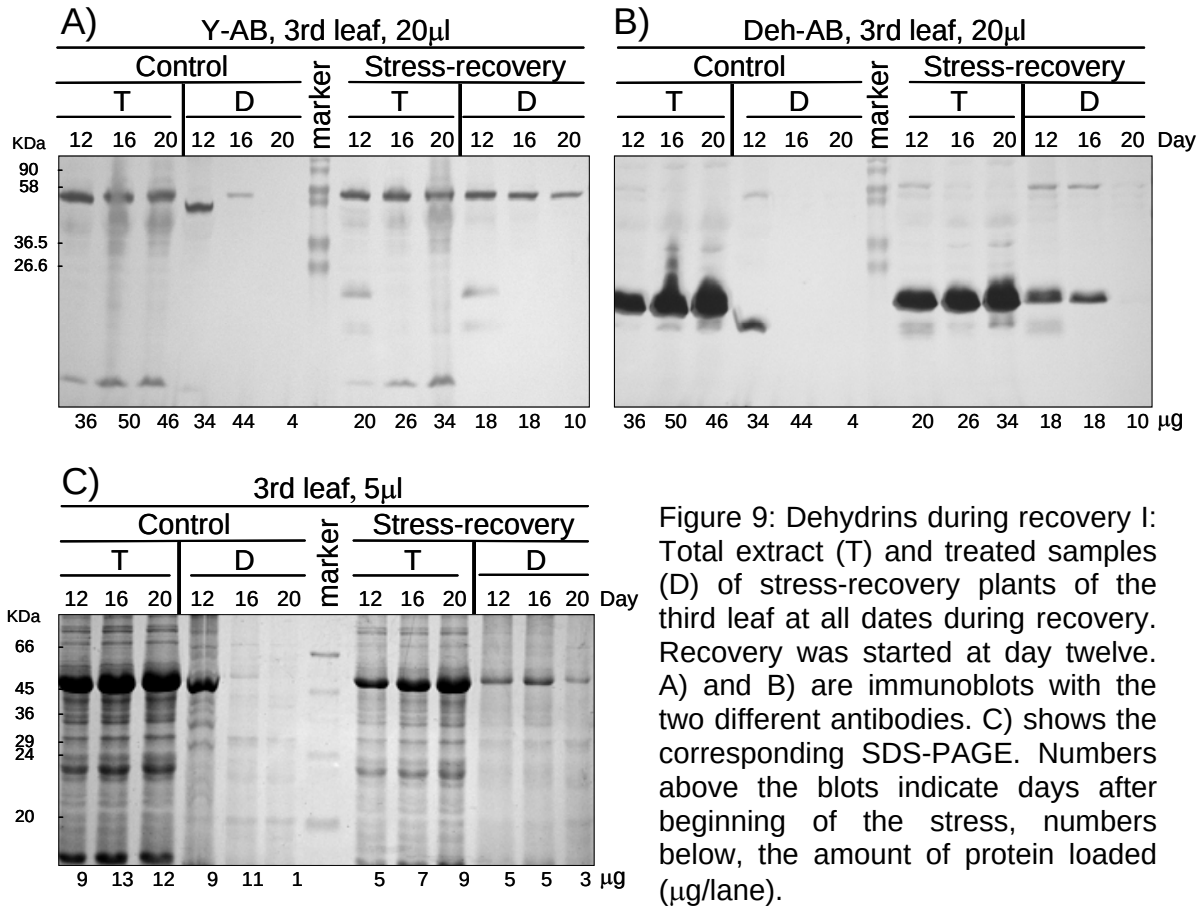

Figure 9: Dehydrins during recovery I: Total extract (T) and treated samples (D) of stress-recovery plants of the third leaf at all dates during recovery. Recovery was started at day twelve. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

To be able to compare, how the different leaves responded to this recovery, identical blots were made with the fourth leaves and very different results were obtained (Figure 10). Instead of decreasing amounts, dehydrins of fourth leaves increased during the recovery phase. Especially the Deh-AB showed a very clearly increasing signal at the size of 24 kDa and a second less clear one at the size of 58 kDa.

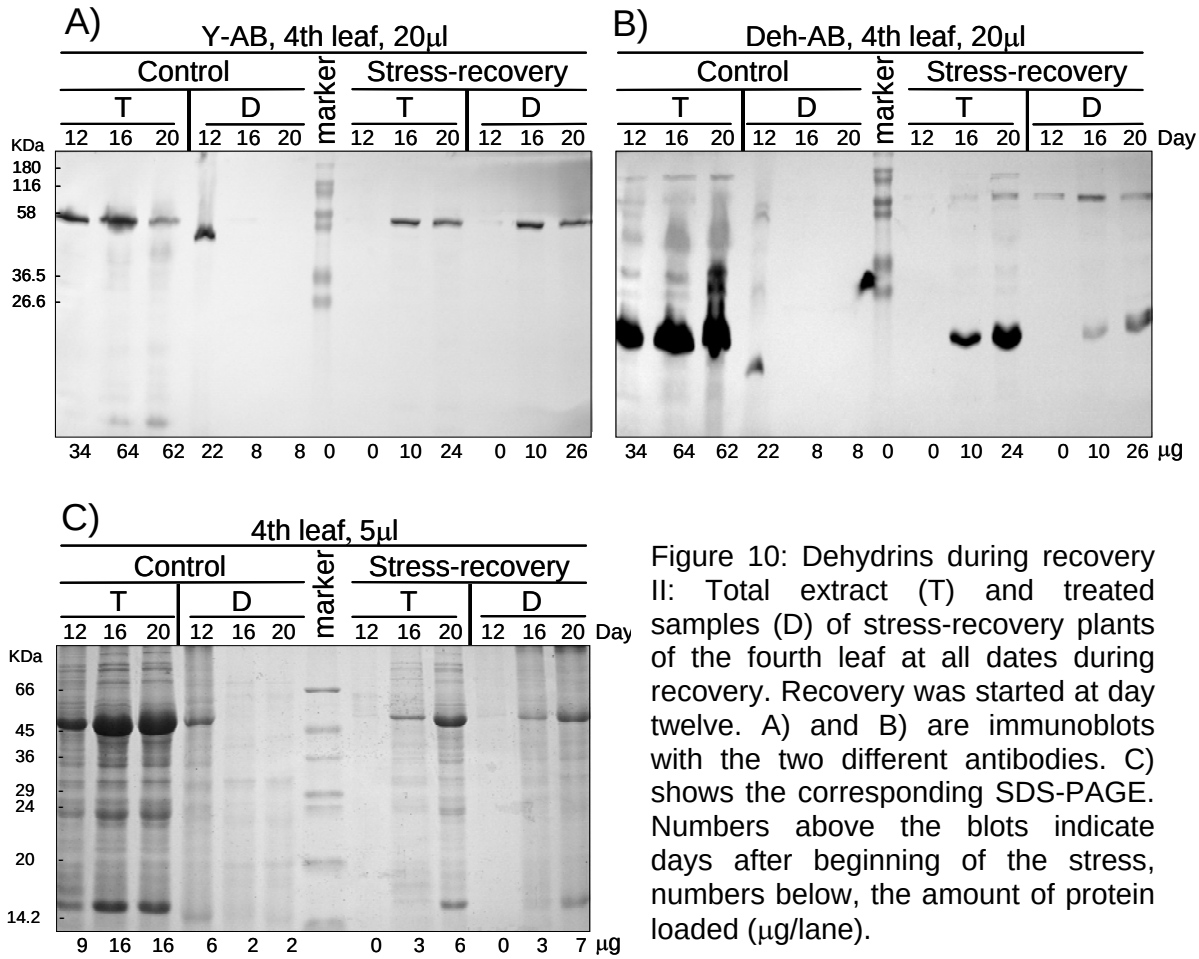

Figure 10: Dehydrins during recovery II: Total extract (T) and treated samples (D) of stress-recovery plants of the fourth leaf at all dates during recovery. Recovery was started at day twelve. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

Plants in hydroponic culture: Series b:

Stress was started 25 days after germination, when the plants were at the four-leaf developmental stage. Since their leaf area and, therefore, presumably, their level of transpiration was higher, the solution was taken up more quickly, resulting in higher PEG concentrations, which means a higher stress compared to series a. Recovery phase was initiated after nine days of stress, by exchanging PEG-solution with standard nutrient solution (Figure 11). This solution got replaced a second time on day twelve.

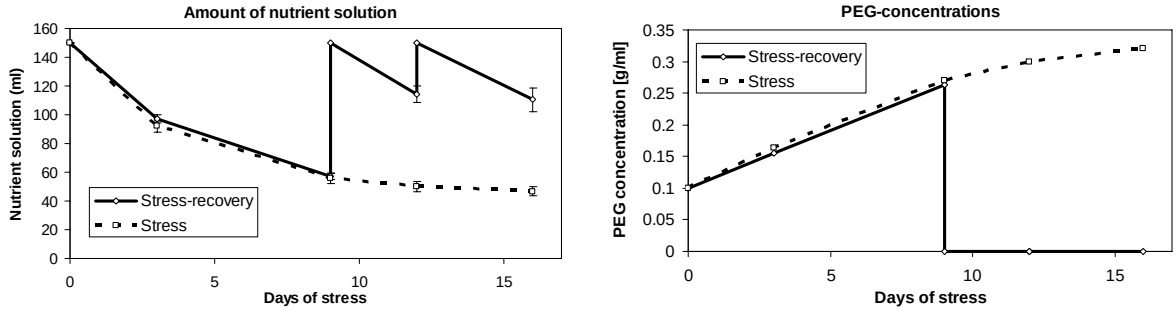

Figure 11: Amounts of nutrient solution (left) and the corresponding PEG-concentrations (right), for the stress experiment (dotted lines) and for the stress-recovery experiment (black lines).

To get an idea when exactly dehydrins were produced during the experiment, the different sampling dates were blotted of the youngest leaf (leaf four) already occurring in the beginning of the stress. Besides the band at 23 kDa (see paragraph: testing of antibodies) no bands could be detected in the blots of total extracts (Figure 12). Background in control plants was again totally removed after D-treatment.

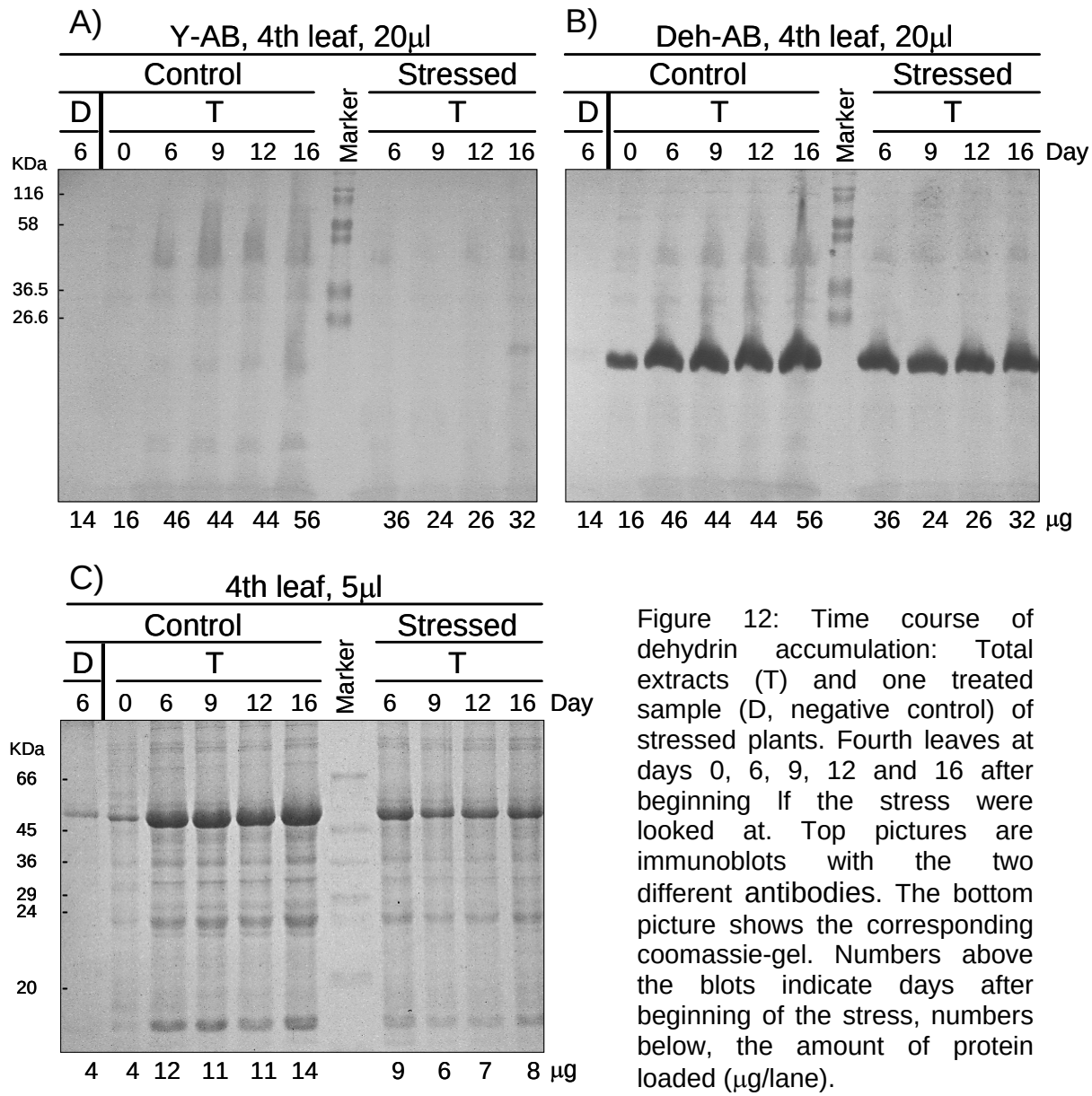

Figure 12: Time course of dehydrin accumulation: Total extracts (T) and one treated sample (D, negative control) of stressed plants. Fourth leaves at days 0, 6, 9, 12 and 16 after beginning lf the stress were looked at. Top pictures are immunoblots with the two different antibodies. The bottom picture shows the corresponding coomassie-gel. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

In corresponding blots of D-samples only two weak bands around 24 and 58 kDa could be detected (Figure 13). The T-sample serving as positive control, showed the strong band at 23 kDa. The blots again revealed there to be some dehydrins in control samples of the fourth leaf at the first sampling date (Figure 13, control plants, 0 days after beginning of the stress). All later control samples did not show these bands. This finding is comparable to the results obtained with the plants on soil (see Figure 4).

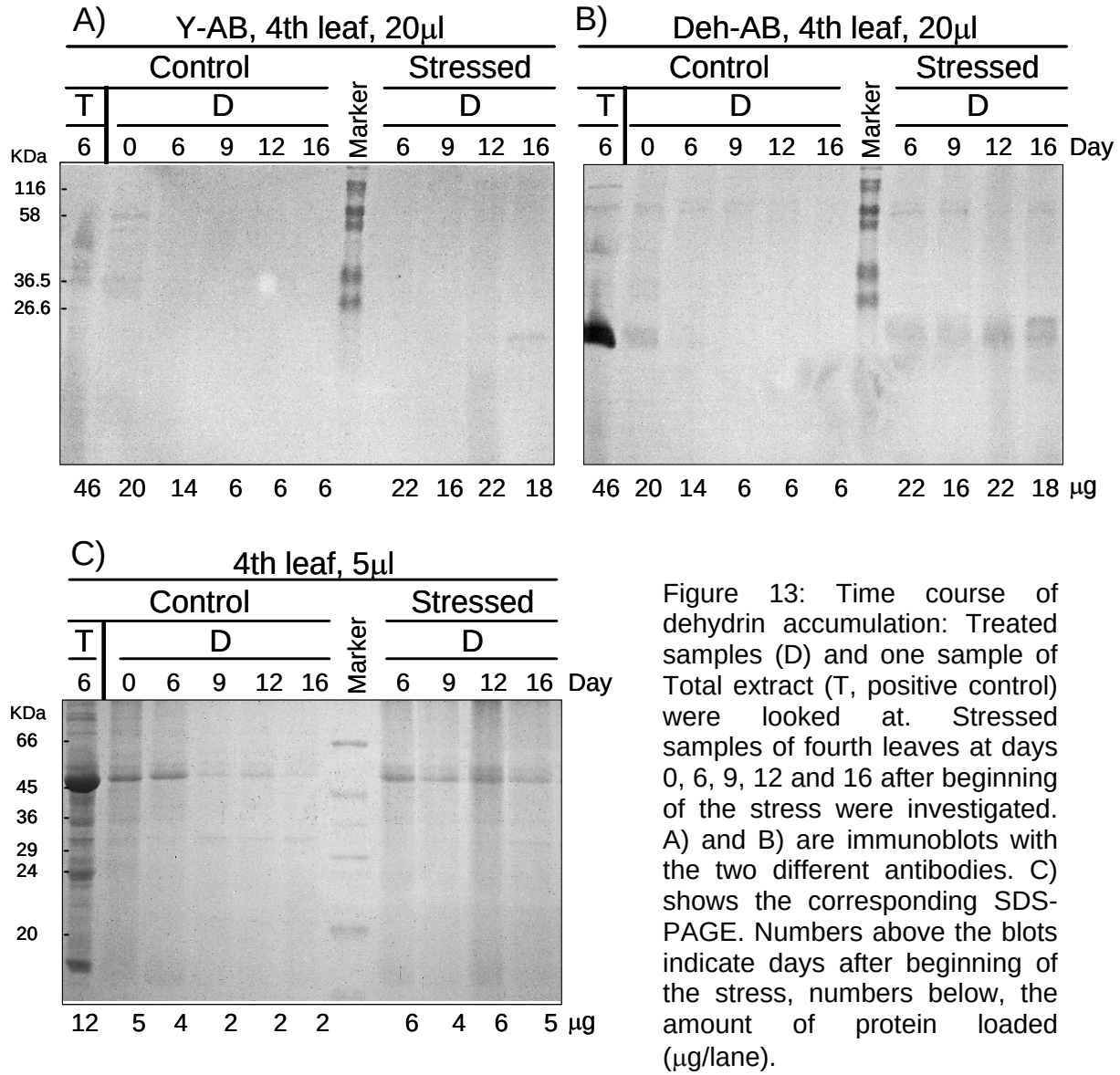

Figure 13: Time course of dehydrin accumulation: Treated samples (D) and one sample of Total extract (T, positive control) were looked at. Stressed samples of fourth leaves at days 0, 6, 9, 12 and 16 after beginning of the stress were investigated. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

Having seen the sixth sampling date (16 days of stress) to show most dehydrins, this date was investigated more closely by looking at the distribution of dehydrins within the plant (leaf one to five). Total extracts of control plants showed a lot of background, which was removed by the D-treatment. For stressed leaves only one band around 24 kDa was detected in young leaves (Figure 14).

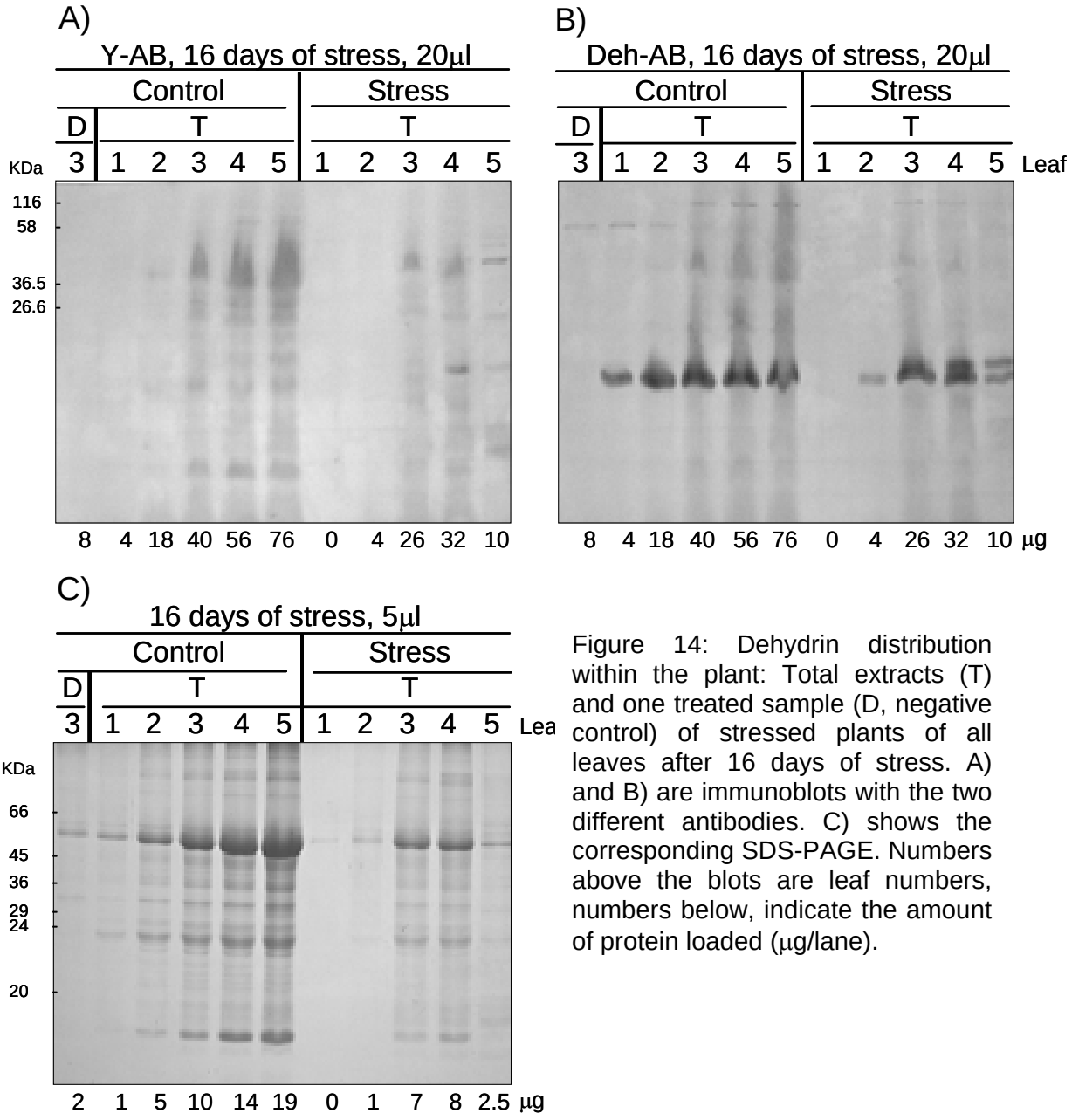

Figure 14: Dehydrin distribution within the plant: Total extracts (T) and one treated sample (D, negative control) of stressed plants of all leaves after 16 days of stress. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots are leaf numbers, numbers below, indicate the amount of protein loaded (µg/lane).

In corresponding blots of D-samples, there was a clear band at 58 kDa which also occurred in old leaves of control plants. Besides that there were some unclear bands in young leaves (leaf 5) occurring with both antibodies (Figure 15).

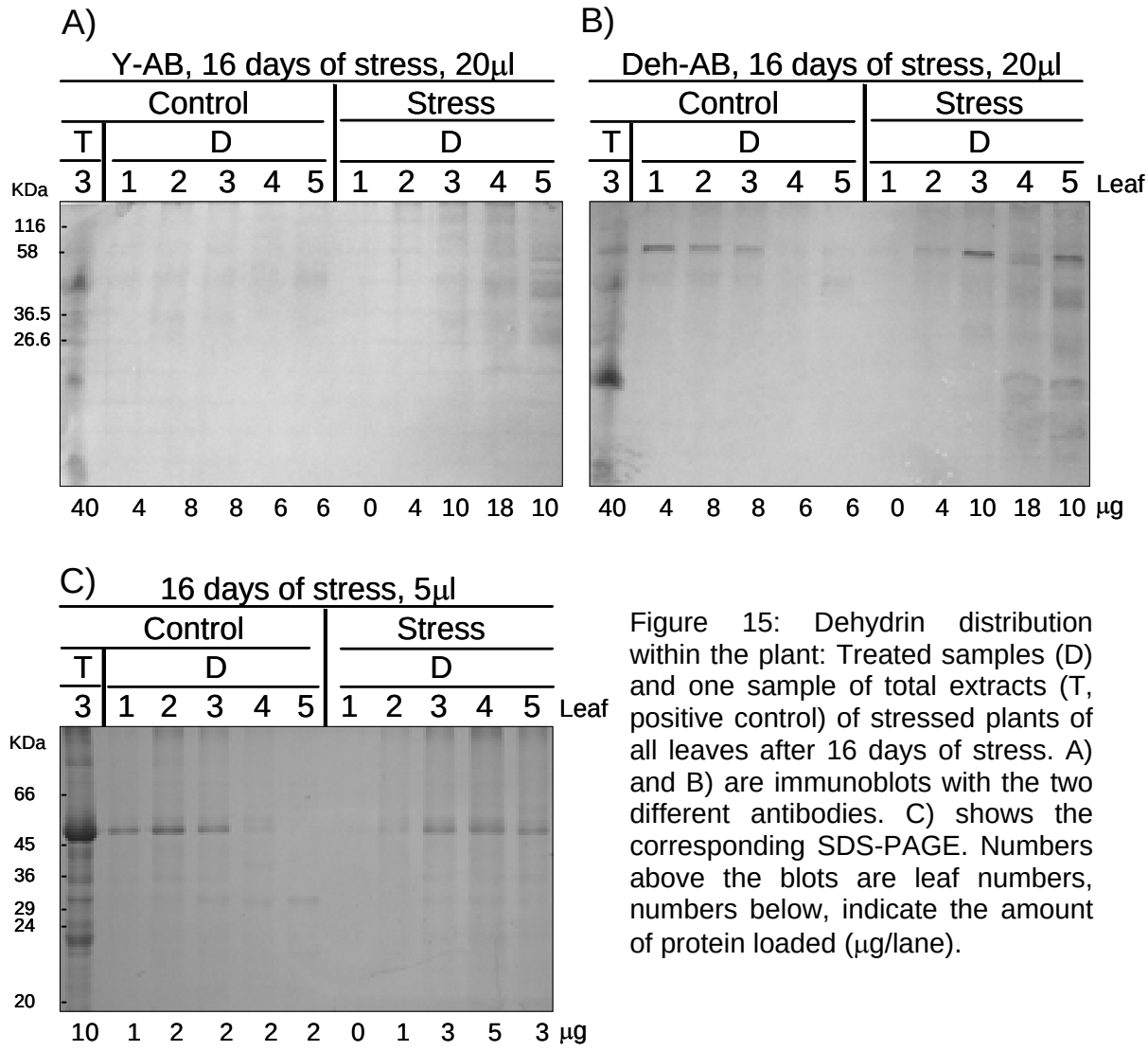

Figure 15: Dehydrin distribution within the plant: Treated samples (D) and one sample of total extracts (T, positive control) of stressed plants of all leaves after 16 days of stress. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots are leaf numbers, numbers below, indicate the amount of protein loaded (µg/lane).

To try to elucidate the recovery phase, samples of fifth leaves from day 9 to 16 were blotted (Figure 16). Besides a lot of background in the samples of the total extracts, only one clear band appeared at the size of 58 kDa. This protein also occurred in control samples but with a different temporal trend. While in control plants the band vanished after 16 days of stress, it rose to a maximum in the samples of stress-recovery plants.

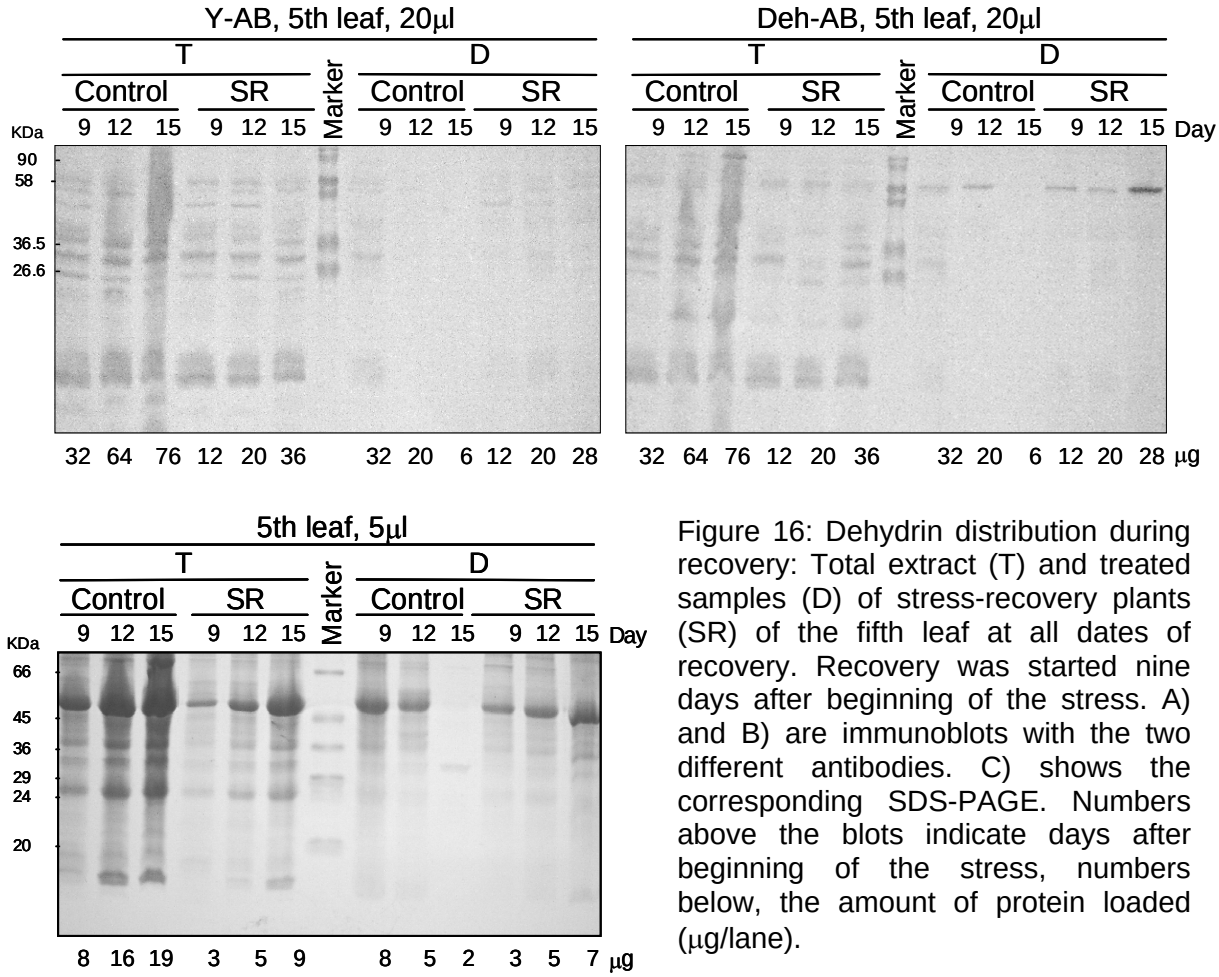

Figure 16: Dehydrin distribution during recovery: Total extract (T) and treated samples (D) of stress-recovery plants (SR) of the fifth leaf at all dates of recovery. Recovery was started nine days after beginning of the stress. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

Root samples only showed expression of one dehydrin at the size of about 60 kDa (Figure 17). The band only present in treated samples occurred with control as well as with stressed roots but with a different temporal trend. While the band heavily decreased from day 9 to 16 in the control, it remained constant in the stressed samples or became even slightly stronger. The band of the stress-recovery root (SR) showed the same band as well. The occurring band seems to be at a lower level for control plants as they are for stress and stress-recovery plants.

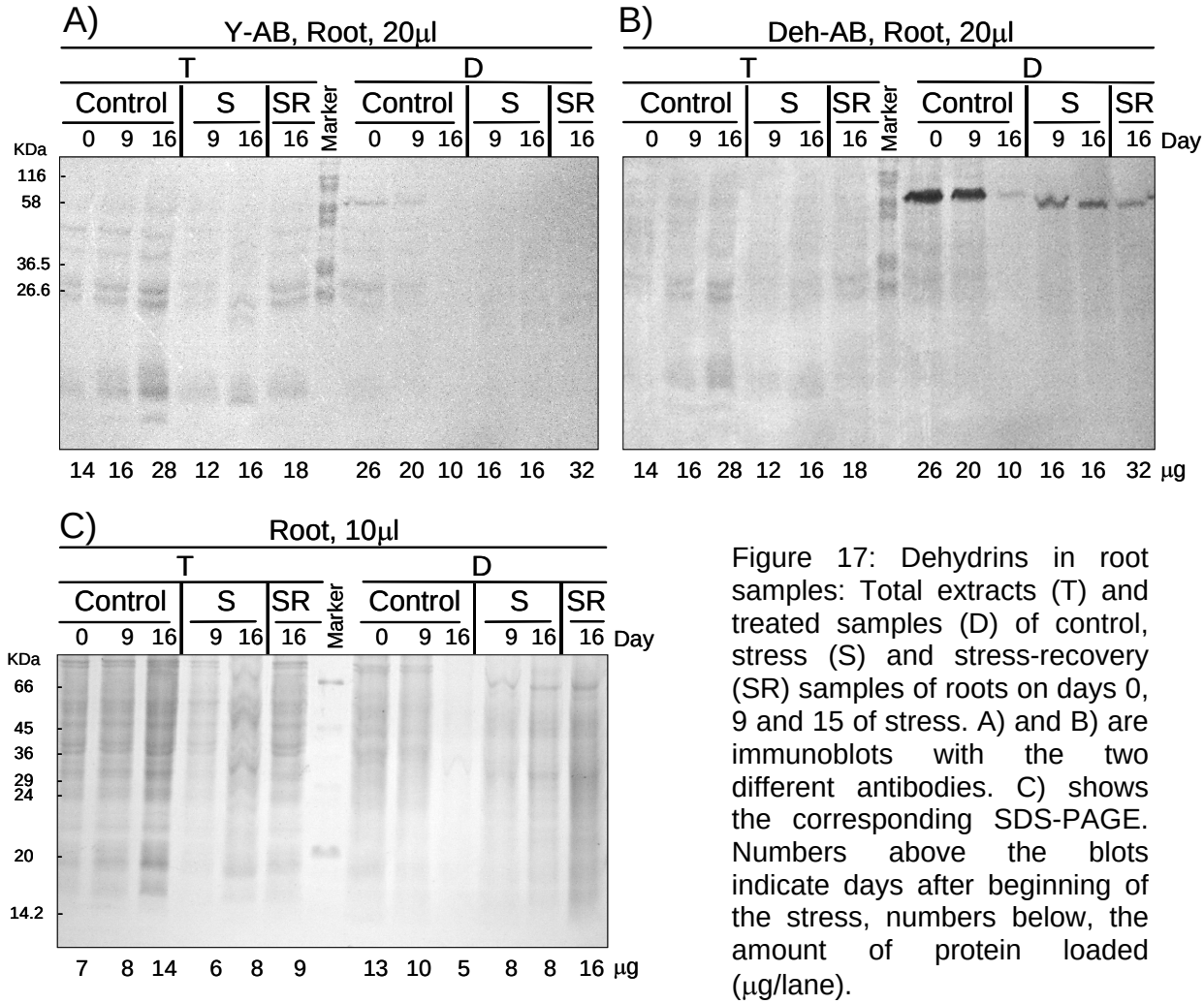

Figure 17: Dehydrins in root samples: Total extracts (T) and treated samples (D) of control, stress (S) and stress-recovery (SR) samples of roots on days 0, 9 and 15 of stress. A) and B) are immunoblots with the two different antibodies. C) shows the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

S-Anti body:

Only with the blots of the strongest response (Dehydrin pattern with soil plants, Figure 4), the S-AB was used again to see, if there was just a problem of detection limit (Figure 18). Certain bands were detectable very weakly and only after a second development a response of the stressed compared with control samples could be detected. All bands were at places where no dehydrins were detectable with any other antibody (90, 48, 38, and 30 kDa). Even this blot shows that there is some dehydrin expression of control plants at the beginning of the stress at day zero.

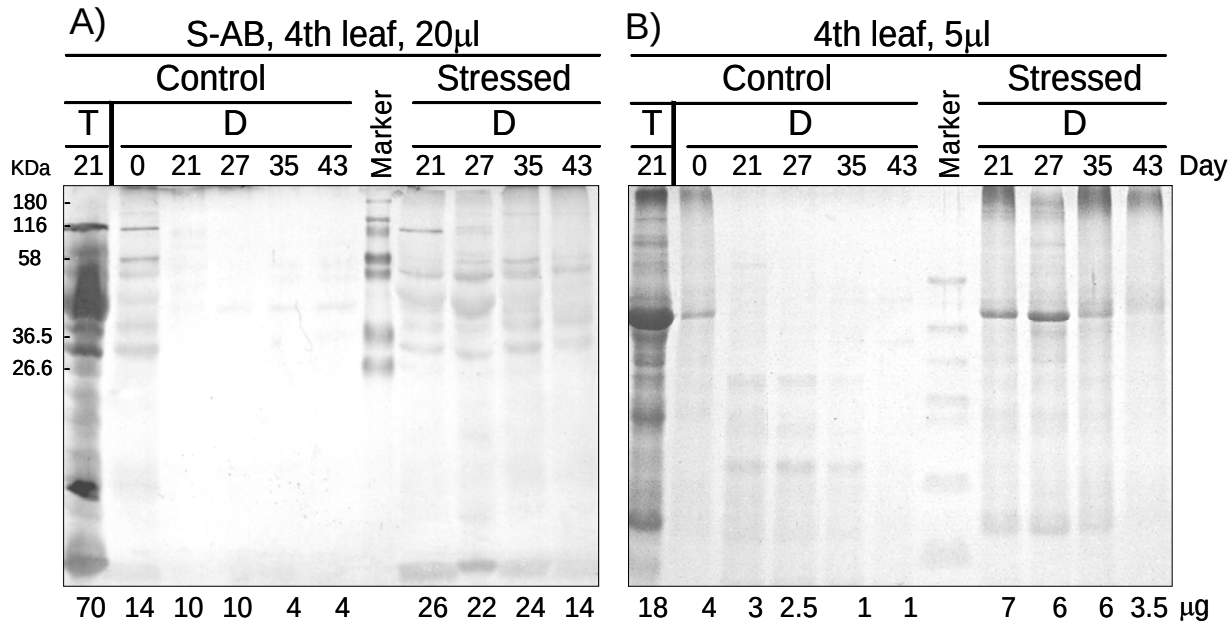

Figure 18: Time course of dehydrin accumulation of soil plants with the S-AB: Fourth leaves of stressed plants, total extracts (T) and treated samples (D) were investigated at day 0, 21, 27, 35 and 43 after beginning of the stress. A) picture of the immunoblot developed with the S-AB and B) the corresponding SDS-PAGE. Numbers above the blots indicate days after beginning of the stress, numbers below, the amount of protein loaded (µg/lane).

Protein content after Treatment:

All experiments showed that protein of total extracts (T) was higher in control than in stressed plants. However, after D-treatment values for stressed samples remained higher, than they did for controls, (Figure 19). This finding was independent of whether plants were grown on soil or in hydroponic culture.

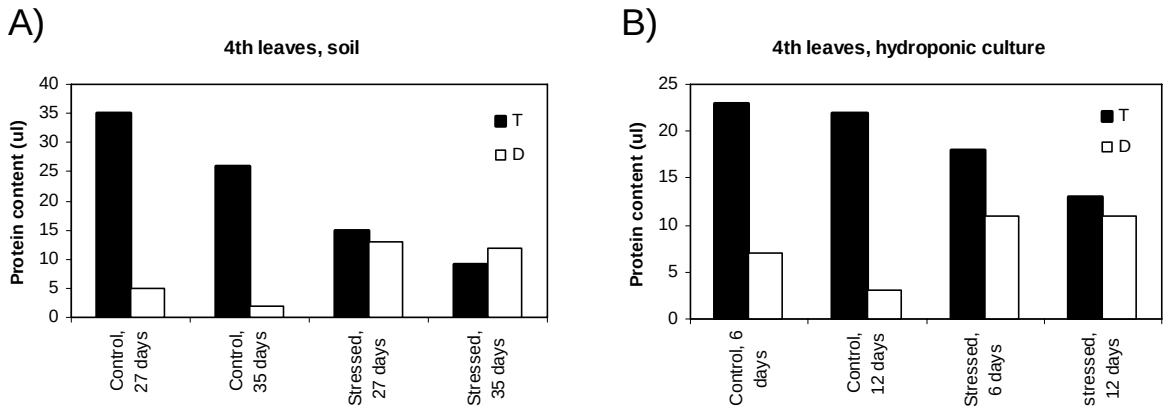

Figure 19: Protein contents determined by a Bradford Assay with plants on soil (A) and on hydroponic culture (B). T: total extract samples, D: treated samples

Discussion

Several dehydrins with molecular weight of 180 (double band), 58, 42, 24 and 20 (double band) kDa were found in wheat. The strongest ones at 24 and 20 kDa were detectable with the Deh-AB and the Y-AB. Therefore, these two proteins consist of at least one K- and one Y-segment and correspond with dehydrins found by Lopez et al (2003). The four larger dehydrins were detectable with the Deh-AB only, implying that either the Y-AB was not sensitive enough or that these dehydrins belong to groups without a Y-segment. Since the S-AB was not sensitive enough, it was impossible to decide whether there was an S-segment.

The plants grown on soil with drought induced by withholding water were presumably closest to field conditions. Withholding water slowly increased the stress, allowing the

plant to metabolically respond with no artificial nutritional problems. In hydroponic culture, artefacts resulting from the PEG treatment or from nutrient deficiencies could not be excluded. The three dehydrins at 58 (only with K-AB), 24 and at 20 kDa were detected in plants grown on hydroponic culture (series a) as well, but with a clearly weaker response compared to the plants on soil. Since the bands were weaker, it was possible to see that the band at 20 kDa was a double band. The smallest number of dehydrins (with a very weak intensity) was detected in plants grown in hydroponic culture when stress was initiated at a later stage (series b). Possible explanations are a) the rapid uptake of water which increased the stress due to higher concentrations of PEG and b) the concentrations of nutrients (e.g. nitrogen) which decreased during the experiment.

Dehydrins were predominantly produced in young leaves. No dehydrins were detected in senesced leaves implying that they had been degraded in the course of senescence. In root samples, only one clear band appeared at 60 kDa in control and stressed plants of treated samples. It was detectable with the Deh-AB and, weakly, also with the Y-AB implying that this dehydrin consists of at least one K-and one Y-segment.

Dehydrin levels in previously stressed wheat leaves decreased again during a recovery phase of eight days. These findings indicate that dehydrins accumulate during drought and degrade again after rewatering. Differing results for plants in hydroponic culture may be explained by rapidly changing water potentials or/and by low levels of nitrogen.

Protein contents in stressed plants remained higher after heat treatment compared to controls (Figure 19). Thus, some changes must have occurred during adaptation to drought stress. Ingram and Bartels (1996) state that osmolytes like proline or trehalose but also dehydrins occur at higher levels in plant material exposed to drought. These compounds may promote an enhanced hydration of proteins in the living cell and presumably also in protein extracts. The importance of such factors for the tolerance of extracted proteins to the heat treatment remains unclear.

In conclusion, up to seven different dehydrins could be detected after an extended drought period. Levels of dehydrins decreased again during a subsequent recovery phase. An enhanced tolerance to the heat treatment of total proteins for stressed plant material was demonstrated. However, the question of the physiological function of dehydrins remains to be answered.

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Project Querco: Influence of drought stress and elevated temperature on young oak trees

Internship report

Swiss Federal Institute for Forest, Snow and Landscape Research (WSL)

Birmensdorf

Presented by

Stefan Eichenberger

2009

Supervisor:

Prof. Dr. Urs Feller
Institute of Plant Sciences and Oeschger Centre for Climate Change Research

Co-Supervisor:

Prof. Dr. Jürg Fuhrer
Agroscope Reckenholz-Tänikon Research Station ART and Oeschger Centre for Climate Change Research

Advisor:

Dr. Matthias Dobbertin
Swiss Federal Institute for Forest, Snow and Landscape Research (WSL)

Introduction

The internship at the WSL (Swiss Federal Institute for Forest, Snow and Landscape Research), Züricherstrasse 111, CH-8903 Birmensdorf took place from February to July 2009 in the project Querco. The work was supervised by Urs Feller while Matthias Dobbertin and Madeleine Goerg-Günthardt acted as co-supervisors.

My two main tasks were to investigate the expression of drought-specific proteins (Dehydrins) on leaf material from 2008 and 2009 and upon the onset of the growing period, measure different phenological and physiological parameters. The aim of the internship was to apply the knowledge of protein metabolism gained during the master thesis with wheat to young oak trees in the project Querco and, by making different kinds of measurements on the trees, to learn more about the applied work of scientists within a national research centre.

Detection of Dehydrins in oak leaves

Introduction

Considering changes in the European climate predicted by most recent models, it has to be assumed that the annual average temperature will increase between 2.3 °C and 5.3 °C by the end of the century. Additionally, models predict a larger probability of extreme climatic events with a decrease in the number of wet days, along with a decrease in precipitation for summer months in Switzerland (IPCC, 2007; Fuhrer et al. 2006). Since it has been shown that for example drought can have an important influence on species distribution in forests (Bigler et al. 2006), the influence of a predicted future climate on tree species in a forest ecosystem is an important issue to be investigated.

Oaks (*Quercus Sp*) are renowned to be highly drought resistant, resulting in a competitive advantage (Leuzinger et al.2005). Project “Querco” aims to investigate the three predominant oak species in Switzerland (*Q. robur*, *Q. petrea* and *Q. pubescens*) considering their adaptation to a predicted warmer climate.

One of various ways plants can adapt to a changing environment is to change their protein metabolism. Many species have been shown to accumulate dehydrins in response to different osmotic stresses including drought (Close 1996, Volaire 2002). For leaves of oaks seedlings (*Quercus ilex*) the expression of a dehydrin-like protein has been reported (Turco et al. 2004) and gene expression in embryos and young seedlings of *Q. robur* under osmotic stress conditions have been investigated (Sunderlikova et al. 2009). No studies of protein expression in leaves have been published on the species examined in the Querco project so far.

The aim was to establish an extraction protocol for proteins of oak leaf material and immunologically detect dehydrins in drought stressed leaves.

Leaf material from 2008

Material and methods

Plant material:

Five year old oaks, growing in the Open Top Chambers (OTC) of the WSL, were subjected to different climatic treatments (control, drought, elevated temperature and combination of drought with elevated temperature). Of each treatment there were four chambers. Leaf samples of *Q. petrea* and *Q. robur* were collected after a long period of drought in August 2008 and stored at -80° C for further analysis. Additional leaves were collected from *Q. pubescens* in November 2008, after the trees had experienced a period with temperatures below 5° C. For comparison, winter buds from *Q. robur* and *Acer pseudoplatanus* were collected in February 2009.

Protein extraction:

To isolate dehydrins from leaves and buds, two extraction protocols were tested: (i) extraction of soluble protein in a detergent free extraction buffer (20 mM $NaPO_4$; 1% w/v PVPP; 0.1% v/v β-mercaptoethanol; pH 7.5) and (ii) a direct extraction of total protein in SDS containing buffer (4% SDS; 5% β-mercaptoethanol; 50 mM Tris-HCl; proteinase inhibitor; pH 6.8). Protein concentrations were determined according to Zaman and Verwilghen (1979). Before loading the samples on the gel, 0.5 µl saturated Coomassie solution and 5 µl of glycerol were added to 50 µl of the protein extract.

Protein enrichment:

Soluble protein extracted with detergent free medium was precipitated with 80% acetone or dialyzed using a cellulose membrane in order to increase protein concentration and remove interfering substances.

SDS-PAGE and Immunoblotting:

Protein samples, containing equal amounts of protein, were separated on 12% SDS minigel according to the method of Laemmli (1970). Separated proteins were blotted onto a PVDF membrane. Dehydrins were detected using a rabbit anti dehydrin antibody (1:1000; Agrisera, Vännäs, Sweden; Product number: AS07 206A) and a secondary gold-labeled goat anti rabbit antibody (1:1000; British Biocell, UK). The gold labeling was enhanced using a silver enhancement kit (British Biocell, UK).

Results

All attempts to extract soluble proteins using detergent free medium were not successful as the amount of protein in the extracts was very low (less than 0.25 μg/μl). To overcome this problem, precipitation and dialysis were performed. Since there was little success with these approaches, all further experiments were conducted using SDS containing extraction buffers. Therewith, protein extractions yielded in concentrations from 2 up to 5 μg/μl. Loading equal amounts of protein, SDS-PAGEs showed a separation of total protein for all samples except the bud sample of *Q. robur* (Figure 1). Some protein became separated but a large fraction seemed to be retained at the very top of the gel. The Immunoblot showed a binding of the anti-dehydrin antibody to a small protein at the size of around 17 kDa in all samples. Only the bud sample of *A. pseudoplatanus* displays clearly other bands, the two main ones at sizes of about 50 and 20 kDa.

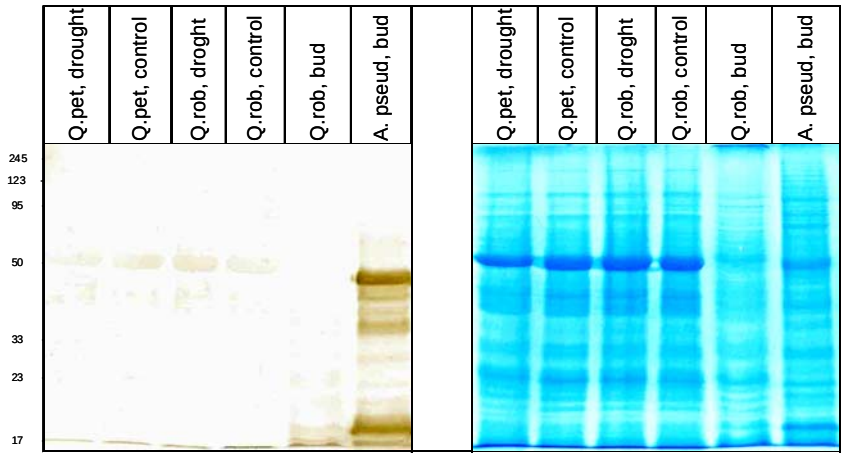

Figure 1: Immunoblot (left) and SDS-PAGE (right) of plants grown in the OTC's (*Q.petrea* and *Q. robur*) and a bud sample of *A. pseudoplatanus* from the grounds of the WSL in Birmensdorf.

Two provenances of control samples of *Q. petrea* (C = Corcelles and G = Gordevio) and *Q. robur* (B = Bonfol and T = Tägerwilen) were compared with the most heavily stressed plants from the combination treatment (Figure 2). The Immunoblot revealed the band around 17 kDa with a stronger band for the late harvested *Q. pubescens* than for the 2 other species. No other bands were detectable for neither of the provenances.

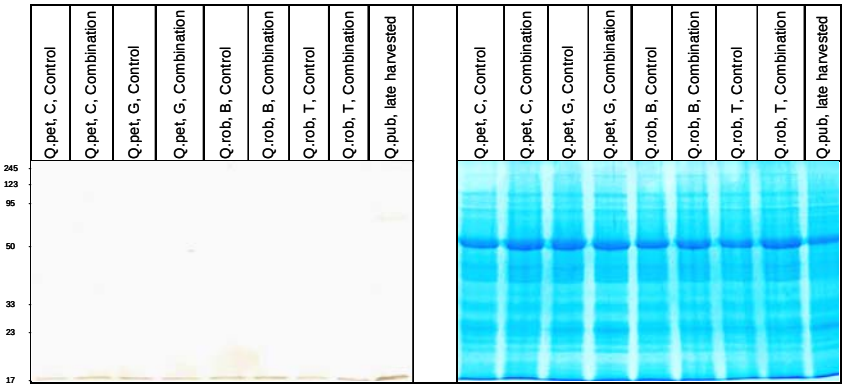

Figure 2: Immunoblot (left) and SDS-PAGE (right) of *Q. petrea* (provenances C and G), *Q. robur* (provenances B and T) and *Q. pubescens* grown in the OTC's.

Discussion

Different protocols to extract proteins from oak plant material were tested and an extraction protocol established using SDS in the extraction buffer. One reason why protein concentrations were very low without SDS might be the high concentration of

phenolic compounds, interfering with the extraction. The bud sample of *Q. robur* seemed to form large protein complexes that lodged at the top of the gel. Phenols, being very abundant in bud scales, are suspected to interfere with the extraction and possibly polymerize the protein. The band at the size of 17 kDa occurred with all samples independent of species or treatment. This gives reason to believe this 17 kDa band not to be a dehydrin but a product of a cross reaction with some other small protein. No other dehydrin, not even in the bud sample of *Q. robur,* could be detected for any oak sample. Possibly the drought treatment during the growth period was not strong enough to make the plant accumulate dehydrins. Another possibility is that the antibody does not recognize the oak K-Segment, a segment known to be highly conservative (Close 1997). As shown in figure 1 the antibody is able to detect dehydrins in maple buds and, according to the manufacturer, is supposed to detect dehydrins in tobacco (*Nicotiana tabacum*) and scots pine (*Pinus sylvestris*). Taking all these species into account it seems highly unlikely that the antibody should not work for oak. To verify the results it could be interesting to use another anti-dehydrin antibody for our oak samples.

For the oncoming season it is planned to look at dehydrin expression under even more severe drought stress and, if dehydrins are detectable, the subsequent recovery phase.

Leaf material from 2009

Material and methods

Chlorophyll fluorescence:

A series of non destructive measurements was carried out to determine how much stress the plants suffered under the different treatments. As part of that, chlorophyll fluorescence measurements were carried out in the morning between 9 and 12 am and in the afternoon between 2 and 5 pm with a portable chlorophyll fluorometer PAM 2000 (Walz Mess- und Regeltechnik, Effeltrich, Germany). Maximum quantum yield of PSII (F_v/F_m) of dark adapted leaves was determined. These measurements were always performed on the same leaves of *Q. petrea* provenance Corcelles (QpeC), *Q. robur* provenance Tägerwilen (QroT) *and Q. pubescens* provenance Leuk (QpuLk) at 3 different tree heights and means were calculated for the 12 values from the 4 trees of the same treatment. These values are an indicator of plant photosynthetic performance. For well irrigated plants a value of about 0.83 can be expected and stress leads to decreased values (Maxwell and Johnson, 2000).

Predawn leaf water potential:

To determine the predawn leaf water potential a Scholander Bomb (MMM Mosler Tech Support, Berlin, Germany) was used. One leaf of each of the 3 investigated provenances (QroT, QpeC and QpuLk) was collected in each chamber and the water potential determined immediately after harvesting. Means from the 4 trees of the same treatment were calculated. Additionally the fresh and the dry weight were determined to calculate relative water content.

Plant material:

From each chamber 2 leaves of the six year old oaks (QpeC, QroT; and QpuLk) were harvested after a long period of drought on June 29th, two days before rewatering on the 1st of July. Until the harvest, only 6.7 l/m^2 of water had been given to plants of drought and combination treatment (Compared to 353.3 l/m^2 for control plants). Predawn leaf water potentials for plants of combination treatment had by then decreased below -3 MPa (see Figure 4). Fluorescence data measured in the afternoon showed a slight decrease of maximum quantum yield of PSII (F_v/F_m) (see Figure 3). After collecting, samples were stored at -80°C for further analysis.

Protein extraction was carried out on plants from control and combination chambers as described above. SDS-PAGE and immunoblotting were performed with 14% SDS gels and nitrocellulose membranes were used for blotting. Leaf samples of all three *Quercus* species and a sample of *Acer pseudoplatanus* were extracted. To verify the results, another antibody raised against the Y-segment (another conservative sequence of dehydrins) of wheat (Y-antibody) was used for immunoblotting (1:1000). For further verification of the results, another development kit (Opti-4CN Substrate Kit, Biorad, Laboratories, CA, USA) was used with secondary antibody concentration of 1:3000.

Results

Chlorophyll fluorescence:

From the sampled trees, values of maximum quantum yield of PSII (F_v/F_m) measured in the afternoon showed a decrease for plants of provenance QroT and QpeC of combination treatment with ongoing stress (Figure 3). Only a small decrease was observed for QpuLk. An increase for all species and treatments could be seen the week before rewatering but total recovery was only detectable after reirrigation.

Values measured in the morning only showed a small difference between treatments (Data not shown).

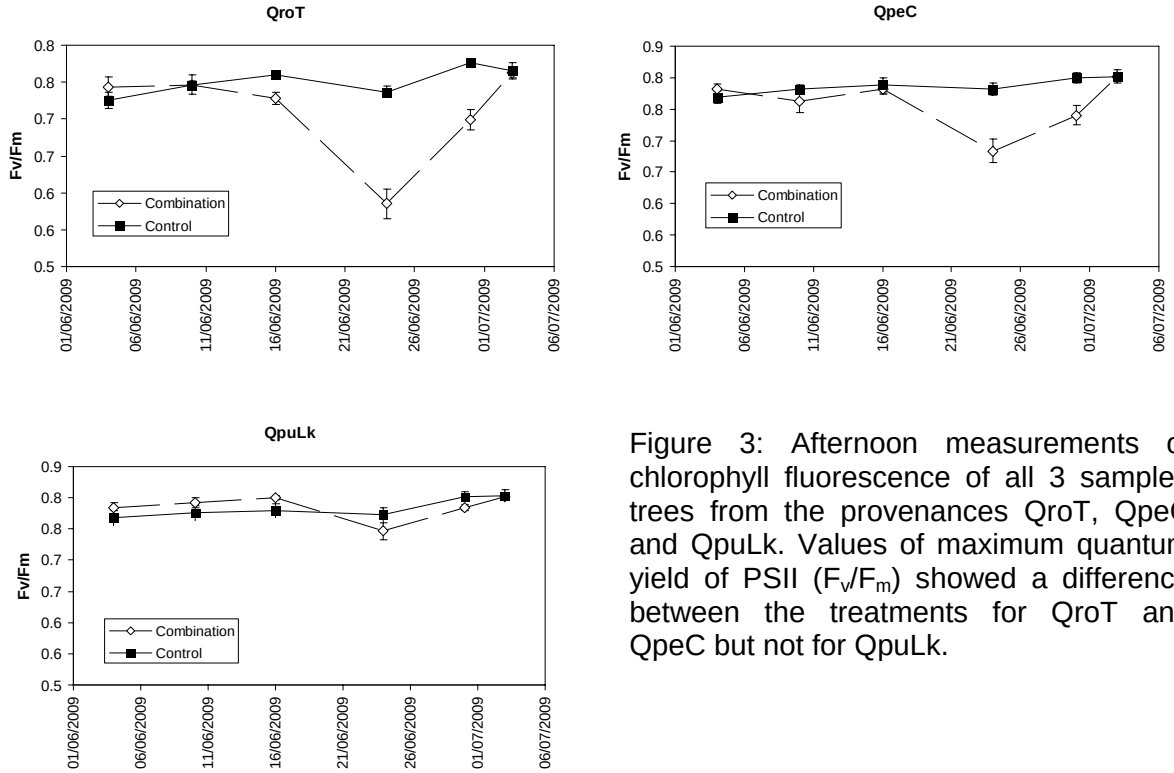

Figure 3: Afternoon measurements of chlorophyll fluorescence of all 3 sampled trees from the provenances QroT, QpeC and QpuLk. Values of maximum quantum yield of PSII (F_v/F_m) showed a difference between the treatments for QroT and QpeC but not for QpuLk.

Predawn leaf water potentials:

Predawn leaf water potentials clearly showed decreasing values for plants of combination treatment with minimum values below -3 MPa for all 3 provenances at the time of maximum stress (see Figure 4). Control plants remained at values around -0.2 MPa during the same period.

SDS-PAGE and Immunoblotting:

Separation of total protein for all samples could be seen with the SDS-PAGEs (Figure 5). Only the *Acer* sample did not seem to separate well with a lot of background. The Immunoblot showed two proteins at the size of around 40 and 50 kDa for all of the *Quercus* samples with all the treatments. Only the bud sample of *A. pseudoplatanus*

displayed other bands at sizes around 50 and 20 kDa. Other faint bands could be detected at sizes around 37, 18 and 17 kDa.

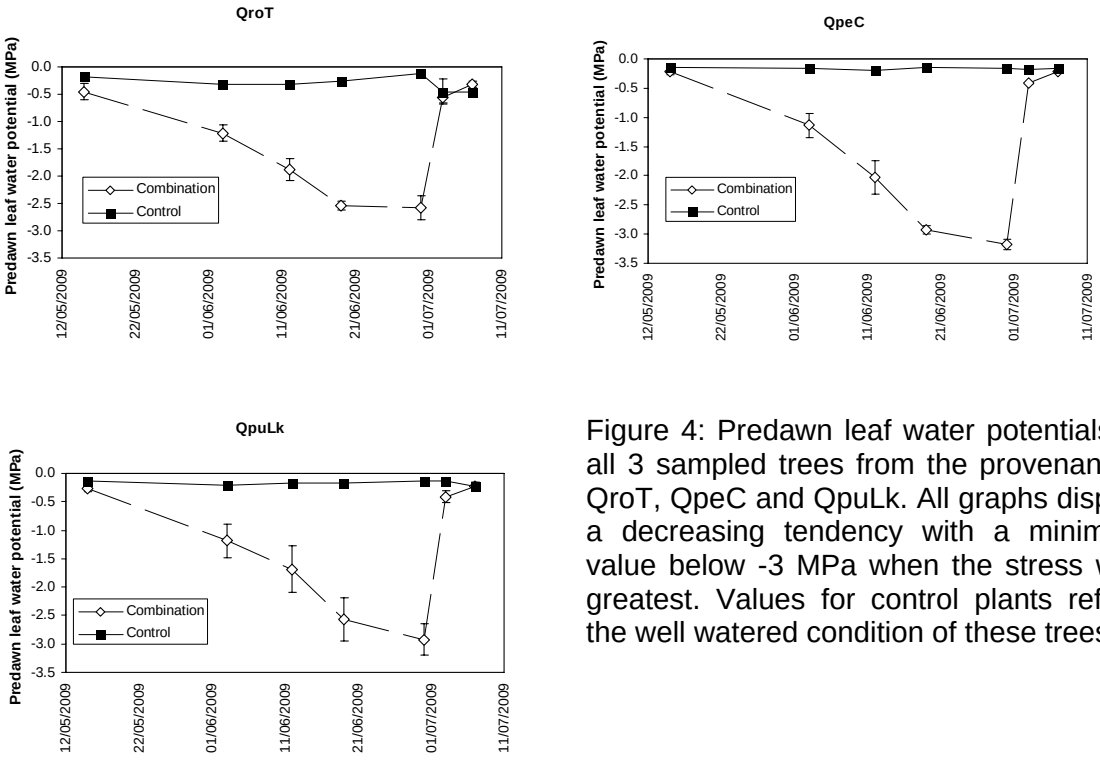

Figure 4: Predawn leaf water potentials of all 3 sampled trees from the provenances QroT, QpeC and QpuLk. All graphs display a decreasing tendency with a minimum value below -3 MPa when the stress was greatest. Values for control plants reflect the well watered condition of these trees.

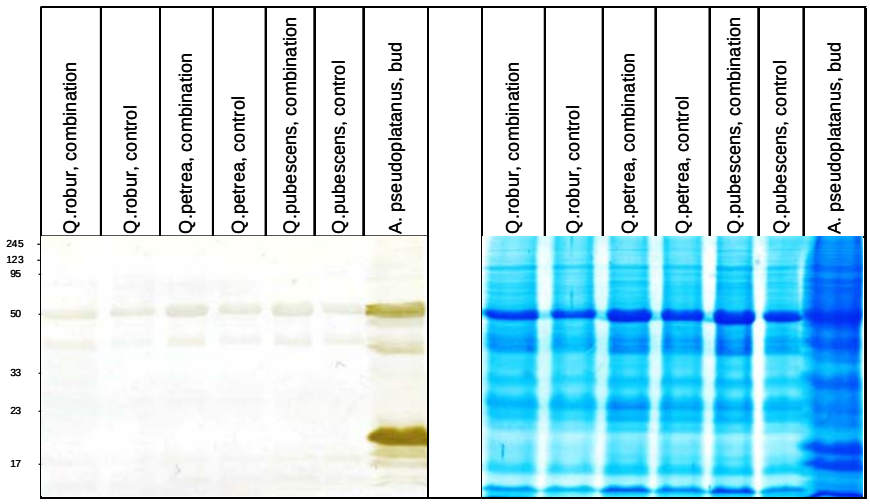

Figure 5: Immunoblot (left) and SDS-PAGE (right) of QroT, QpeC QpuLk grown in the OTC's and a bud sample of *A. pseudoplatanus* from the grounds of the WSL in Birmensdorf.

In a further approach immunoblots were performed with two different antibodies and with a different development kit (Opti-4CN Substrate Kit (Biorad, laboratories, CA, USA; Figure 6). The result remained the same: some background at the size of around 50 kDa but no clear bands for any *Quercus* leaf samples were detected. For the bud sample of *A. pseudoplatanus* the protein of around 20 kDa was detectable with both antibodies.

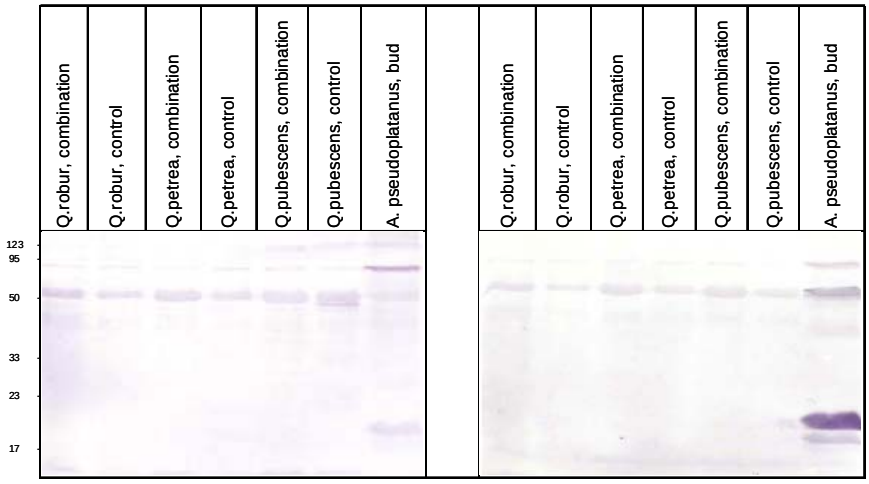

Figure 6: Immunoblots developed with two different primary antibodies: The Y-antibody (left) and the Deh-antibody (right). Additionally, another development kit was used with the secondary antibody bound to a peroxydase.

Discussion

Predawn leaf water potentials display the continuous decrease of water availability to the drought stressed trees. Additionally, the fluorescence data are an indication that photosynthesis is affected by the treatment. Interestingly, the values of maximum quantum yield of PSII already increased again for all species and treatments before the stress was actually stopped. One reason could be the cloudy, rainy weather the week before and on the day of the measurements, June 30th. Total recovery of water status could be seen with both parameters already two days after rewatering.

The immunoblots show a very similar picture compared to the blot derived from leaf samples of 2008. In all oak leaf samples, only background of the large subunit of Rubisco and of another protein around 40KDa was detectable. The sample of *A.*

pseudoplatanus again showed the dehydrins around 20 and 50 kDa. To verify these results another antibody (Y-antibody) plus a different development kit was used with the same result: no dehydrins in oak leaves of any of the investigated species could be detected. Since it was impossible to detect dehydrins in any of the oak samples, a small uncertainty remains the primary antibodies used does not recognize oak dehydrins. Alternatively, it is possible that the stress still was too weak for the oaks to accumulate dehydrins. As a last possibility, the three oak species investigated may have other defense and adaptation mechanisms and do not accumulate dehydrins in leaves.

Phenological Data during 2009

To document possible impact of the different treatments on the trees, the phenological development of each tree was recorded. The greening of leaves, bud burst and leaf unfolding were determined for all 864 trees. Further parameters were to estimate the day when a tree had reached at least 50% of its total leaf area and to measure shoot length as soon as growth started. In early spring these measurements were conducted every 3 to 4 days and, after leaf unfolding was completed shoot lengths were recorded weekly. These data were collected over the last three years and will be analyzed at the end of the growing period 2009.

Further tasks

Stem diameter:

During winter stem diameters were measured to be able to calculate the annual basal area growth.

Weighing of total leaf material:

In autumn 2008 the leaves of each tree had been harvested and dried and weight as one part of total biomass production. Already before this harvest, some trees had lost a part of their foliage which was collected for each quarter chamber and weighed separately.

Grinding of leaves for nutrient determination:

Ten leaves harvested in autumn 2008 were pooled from 2 trees of the same provenance and soil compartment. The task was to grind this leaf material to determine the elemental composition. The samples were extracted by high pressure digestion (240^0C; 120 bar) and analysed in duplicates (spread < 10 %) by ICP-OES in the central laboratory of the WSL (accreditation according to ISO 17025). The following elements were determined: N, C, Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S and Zn.

Removing of caterpillars:

During early shoot development, infestations of caterpillars (*Periclista lineolata*, *Operophtera brumata* and *Apethymus seotinus*) occurred. Before using insecticides they were mechanically removed. After recognizing that certain provenances were

preferentially attacked, one campaign of removal was documented with the result that almost three quarters of all infested trees were from *Q. robur* and below 10 percent from *Q. pubescens*.

Conclusions to the Internship

During my 6 months at the WSL I applied what I had learned during my master thesis on the leaf material of the oak species. Different ways of protein extraction from oak leaves were tested. Attempts to accumulate protein by precipitation failed because the precipitated protein was insoluble. When a protocol was set up to extract protein from the leaves, it was still impossible to detect dehydrins by immunoblotting. It had therefore to be tested if our extraction worked with other species (*Acer pseudoplatanus*, *Tilia cordata*). During all these steps I gained a lot of experience in the handling of proteins.

The phenological measurements in the OTC's were something totally new for me. I spent a lot of time measuring shoots and assessing the bud stages. Through this I became conversant with the growth pattern of oaks and started to recognize different diseases and pests associated with them (mildew, lice, caterpillars, gall wasps). During the physiological measurements I also gained some experience in the handling of the PAM 2000 Fluorometer, and the Scholander bomb together with some measurements with the Li-Cor.

To sum up, I greatly improved my working skills in the lab and field and received a good insight into the work of researchers at a national research institute.

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Declaration

under Art. 28 Para. 2 RSL 05

Last, first name: Eichenberger Stefan

Matriculation number: 02-116-671

Programme: M.Sc. in Climate Sciences

Bachelor ☐ Master ☐ Dissertation ☐

Thesis title: Dehydrin patterns in wheat leaves during severe drought and recovery

Thesis supervisor: Prof. Dr. Urs Feller

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text. In accordance with academic rules and ethical conduct, I have fully cited and referenced all material and results that are not original to this work. I am well aware of the fact that, on the basis of Article 36 Paragraph 1 Letter o of the University Law of 5 September 1996, the Senate is entitled to deny the title awarded on the basis of this work if proven otherwise.

Bern, August 6th 2009

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Signature