PROJECT CLOSURE REPORT – OTKA NN 85349 (2011-2015)

SUMMARY

Acclimation to diverse climate factors includes avoiding oxidative stress of biological membranes and membrane bound functions. This is achieved by counterbalancing the stress induced production of reactive oxygen species (ROS) with antioxidants. Leaves and their chloroplasts are primary sites of ROS photo-production, thus changes in antioxidant – pro-oxidant balance in chloroplasts are key factors in stress responses. We studied this balance in leaves in response to natural sunlight (sun/shade leaves) or various combinations of photosynthetically active radiation (PAR) and supplemental ultraviolet light (UV) in growth chambers. We used a variety of plants, chosen according to research questions. Transplastomic tobacco lines, which were used in addition to wild type tobacco and other plants were created by the Irish collaborating partner. Plastid (in contrast to nuclear) transformation proceeds through homologous recombination, providing more stable and homogeneous plant material.

We found that: (1) Singlet oxygen neutralizing antioxidants facilitate acclimation to high intensity sunlight. (2) Acclimation to UV was less determined by base levels of antioxidants than by their inducibilities in response to the irradiation. (3) Solar UV is capable of converting metabolic hydrogen peroxide to hydroxyl radicals via direct photo-cleavage. In accordance with the above results, we showed that (4) the primary line of antioxidant defence in UV-irradiated tobacco leaves is centred on lowering plastid hydrogen peroxide concentration. (5) The synergistic nature of solar UV with other abiotic stress factors is realised via hydrogen peroxide. Conclusions are detailed below.

Results were published in eight peer reviewed journal publications (plus one under review) and also served as the basis of two PhD dissertations, one completed (Petra Majer, 2013) and one being written (Gyula Czégény, 2016).

RESULTS

(1) SINGLET OXYGEN NEUTRALIZING ANTIOXIDANTS FACILITATE ACCLIMATION TO HIGH INTENSITY SUNLIGHT

Both high photosynthetically active radiation (PAR) and ultraviolet radiation (UV) are capable of causing photooxidative stress, but leaves are equipped with an array of protective mechanisms making life under full sunlight possible. Comparing acclimation strategies of *Tilia platyphyllos* leaves we found that sun leaves were better protected against stress than shade leaves by having (i) more efficient regulated non-photochemical quenching, (ii) a higher capacity to neutralize singlet oxygen, a reactive oxygen species known to be capable of promoting oxidative damage by excess PAR and (iii) containing more UV absorbing pigments. HPLC–MS^n analysis showed both quantitative and qualitative differences in higher flavonoid contents: sun leaves contained 4.2-times more flavonoids than shade leaves and the quercetin:kaempferol ratio was also higher in the former. Interestingly, experiments with pure test compounds showed that although multi hydroxylated flavonols (such as quercetin and myricetin) improved the singlet oxygen targeted antioxidant potential of leaves, these compounds did not have better UV-B absorption than monohydroxylated ones (kaempferol derivatives). Tilia was used in this study due to the plants' richness in flavonoids and the availability of leaves exposed to two distinct light regimes throughout their development.

The significance of the above results is that we have shown how responses to two different components of sunlight (high intensity PAR and UV) are distinct in dominant ROS: singlet oxygen being the major factor in the former (Fischer et al. 2013, Kálai et al. 2013, Kovács et al. 2014) and while other, type-I photodynamic products are more important in UV exposure (see topics 3-4 below). On the other hand, the two pathways are connected via non-enzymatic flavonoid antioxidants also present at chloroplasts (Saunders and McClure 1976). In this way, our data suggest that responses to high intensity PAR and to solar UV-B are closely connected and that UV-inducible flavonols play a key role in the successful acclimation of sun leaves to high PAR as efficient singlet oxygen antioxidants (Majer et al. 2014a). Although further studies on plant polyphenols proved promising, these were beyond the scope of OTKA NN85349 and work in this project was focussed on other chloroplast antioxidants.

(2) ACCLIMATION TO UV IS LESS DETERMINED BY BASE LEVELS OF ANTIOXIDANTS THAN BY THEIR INDUCTION IN RESPONSE TO THE IRRADIATION

The onset of oxidative stress is characterized by a shift in the balance of antioxidants and ROS by an increase in the latter. Because maintaining high antioxidant activities in the absence of stressor is taxing metabolic pathways, a key issue of acclimation to stress conditions is whether and to what extent stress responses rely on existing high antioxidant capacities or on rapid induction of these. To this end, greenhouse grown tobacco plants were exposed to both PAR and supplemental ultraviolet irradiation and changes in their photosynthesis (gas exchange and electron transport) as well as general and ROS specific antioxidant activities were measured. Two groups of plants, which were different in their leaf antioxidant capacities due to one of them having been acclimated to high PAR before the UV treatment, responded differently. High light pre-treated leaves lost approximately 25% of photosynthetic activity during the UV exposure and showed no change either in the amounts of UV-absorbing pigments or antioxidant levels. On the other hand, leaves which were exposed to UV irradiation without the preceding high light acclimation had 60% lower photosynthesis by the end of the treatment, and increased antioxidant activities (Majer et al. 2014a).

A similar study was conducted comparing the impact of UV on photosynthesis, photosystem II photochemical yields and antioxidant responses of younger and older leaves of green-house grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. Grapevine was used in this study, because horizontally trained shoots provided leaves of different several developmental stages on the same plant. Supplemental UV irradiation corresponded to 8.95 kJ m^{-2} d^{-1} global (280-400 nm) or 8.04 kJ m^{-2} d^{-1} UV-B (280-315 nm) biologically effective dose. UV irradiation was applied daily and its effects were evaluated after 4-days. Younger (1-3 weeks-old) leaves and older (4-6 weeks-old) leaves were affected differently: UV irradiation decreased their photochemical yields to 78% and 56%, respectively. Unlike older leaves, younger leaves responded by an increase in UV-B absorbing pigment, anthocyanin and total phenolics contents. UV irradiation increased total antioxidant capacities in younger leaves but not in older ones. Younger leaves were also different in their ability to increase specific hydroxyl radical and singlet oxygen neutralizing capacities in response to the supplemental UV irradiation, which was reported for the first time (Majer et al. 2014b).

These two sets experiments showed that the ability of acclimation to supplemental UV is not necessarily determined by base levels of antioxidants but rather by their inducibilities in response to stress. These results also highlighted the importance of hydroxyl radicals in UV stress, and prompted studies listed below.

(3) UV-B COMPONENTS OF SUNLIGHT ARE CAPABLE OF GENERATING HYDROXYL RADICALS FROM HYDROGEN PEROXIDE

We have presented theoretical (quantum chemistry calculations) and experimental (in vitro action spectrum) evidence for the ability of solar UV wavelength to convert hydrogen peroxide to hydroxyl radicals. Such photo-cleavage by high energy (160 nm) UV has been known for material physics for decades (Harbour et al. 1974), the novelty of our finding is that UV-B (280-315 nm) components of sunlight reaching and penetrating into photosynthetic tissue are also capable of promoting the reaction. Thus the oxidative effect of UV-B in leaves is at least twofold: (i) it increases cellular hydrogen peroxide concentrations, to a larger extent in pyridoxine antioxidant mutant pdx1.3-1 *Arabidopsis* and; (ii) is capable of a partial photo-conversion of both 'natural' and 'extra' hydrogen peroxide to hydroxyl radicals. (Czégény et al. 2014). In this study, *Arabidopsis* plants were used, due to the availability of pdx1.3-1 plants with lower hydroxyl radical antioxidant pyridoxine (vitamin-B2), but experiments were also continued using transplastomic tobacco (topics-4 and 5).

Because experiments also demonstrated UV-inducible production of hydrogen peroxide in chloroplasts, the above study implied that successful acclimation to UV should involve neutralizing either hydroxyl radicals or their precursor ROS in this organelle. In this way, research questions spiralled back to the original hypothesis of our study on the central role of chloroplast antioxidants.

(4) THE PRIMARY LINE OF ANTIOXIDANT DEFENCE IN UV-IRRADIATED TOBACCO LEAVES IS CENTRED ON LOWERING PLASTID HYDROGEN-PEROXIDE CONCENTRATION

In the following experiments, greenhouse grown tobacco plants were exposed to supplemental UV centred at 318 nm and corresponding to 13.6 kJ m^{-2} d^{-1} biologically effective UV-B (280-315 nm) radiation. Six days of this treatment decreased photosynthesis by 30% and leaves responded by a large increase in UV-absorbing pigment content and antioxidant capacities. UV-stimulated defence against ROS was strongest in chloroplasts, since activities of plastid enzymes FeSOD and APX had larger relative increases than other, non-plastid specific SODs or peroxidases. In addition, non-enzymatic defence against hydroxyl radicals was doubled in UV treated leaves as compared to controls. In UV treated leaves, the extent of activation of ROS neutralizing capacities followed a peroxidases > hydroxyl-radical neutralization > SOD order. These results demonstrated that highly effective hydrogen peroxide neutralization was the focal point of surviving UV-inducible oxidative stress (Majer et al. 2014b)

To explore whether peroxidases could be partly relieved by non-enzymatic plastid antioxidants, we used transplastomic tobacco lines expressing glutathione reductase in combination with either dehydroascorbate reductase or glutathione-S-transferase. Both transplastomic lines exhibited better tolerance to supplemental UV-B (280-315 nm), corresponding to 7.7 kJ m^{-2} d^{-1} biologically effective, than wild type plants. After 10 days of this UV treatment both maximum and effective quantum yields of PSII decreased in the wild type by 10% but were unaffected in either of the transformed lines. Activities of all the enzymes examined were increased by UV-B in all lines. However, the observed more successful acclimation required less activation of peroxidases in the doubly transformed plants than in the wild type and less increase in non-enzymatic hydroxyl radical neutralization in the dehydroascorbate reductase plus glutathione reductase than in either of the other lines. These results highlight the fundamental role of efficient glutathione, and especially ascorbate, recycling in the chloroplast in response to exposure of plants to UV-B (Czégény et al. 2016).

(5) A NEW HYPOTHESIS: THE SYNERGISTIC NATURE OF SOLAR UV WITH OTHER ABIOTIC STRESS FACTORS IS REALISED VIA HYDROGEN PEROXIDE

An emerging new paradigm in plant UV research is recognising that solar UV by itself in not a stress factor but rather a developmental signal (Hideg et al. 2013). On the other hand, even ambient UV may manifest as stress factor, when it is combined with other abiotic stress conditions. Our results may explain how the synergy of environmental UV radiation with other stress factors leads to oxidative stress: Cellular hydrogen peroxide concentrations are known to be increased by a variety of abiotic and biotic factors and in our hypothesis their UV-B mediated photo-conversion into more harmful hydroxyl radicals would create the synergic effect. The above experiments support this hypothesis (Czégény et al. 2014, Majer et al. 2014b).

In addition, this model would argue against a direct signalling role of hydrogen peroxide in adaptation to UV, and in fact to any stress condition occurring under field conditions, in UV rich sunlight. A diverse role of various ROS in stress response signalling has already been suggested (Sabater and Martin 2013) and our assumption may also be relevant to this model, although confirmation certainly needs further studies.

PERSPECTIVES

Research realized in the framework of OTKA NN 85349 highlighted new perspectives, such as (i) the need to explore ROS specific antioxidant properties of leaf flavonoids, (ii) the unique nature of signalling in stress conditions occurring in sunlight and (iii) the interconnections of acclimative and stress responses to PAR and UV. These topics will be explored in the future, subject to the availability of funding.

UNSCHEDULED PROJECT EVENTS

Halfway through the project, at the beginning of project year-3, research was transferred from BRC Szeged to the University of Pécs where the PI became employed full time and Gyula Czégény participating as PhD student has been accepted to the Biology Doctoral School of UP as second year student from 1st of September 2013. Unfortunately, the financial administration of UP found it difficult to accommodate that the student's PhD scholarship is to be paid from the OTKA grant, the project was overcharged for personal costs during the first few months and the situation only normalized by the 1st of January 2014. Apart from this administrative issue, the transfer of research was without difficulties. The Faculty of Sciences at UP generously provided the infrastructure left behind in BRC and also purchased a growth chamber with built-in supplemental UV sources which were not available at Szeged earlier. This latter was especially helpful, as government regulations in Hungary on research including transgenic plant material became stricter since the project was conceived and submitted. This change in political environment affected the research negatively, but work was re-scheduled without losing competitiveness.

Collaboration with the Irish partner was essential, as the source of transplastomic tobacco lines for the research. Additional collaboration became available through the Hungarian PI's participation in a COST Action, which was chaired by the Irish PI. This made research visits and some of the conference participations for the two Hungarian PhD students possible as COST-financed short term science missions (STSM) and allowed re-allocation of financial resources into other, more expensive conferences.

This is part of the reason why a relatively smaller proportion of OTKA funds were spent directly on student exchange than originally planned. The other is again caused by a UP administration issue, which does not allow paying daily stipend to PhD students attending conferences abroad, even though they are on OTKA payroll. This problem can only be resolved by having a full stipend paid to the PI and private sharing. Administration issues at UP which overrule OTKA regulations and thus hamper project work has been brought up by the PI as well as other senior lecturers, but without effect so far.

EDUCATION

The project was realized by the PI and her two PhD students, who participated on OTKA payroll: Petra Majer (1st of September 2010 – 31st of August 2013) and Gyula Czégény (1st of September 2012 – 31st of December 2015). The two PhD students co-authored four and three (respectively) of the projects publications in peer reviewed journals. Gyula Czégény was employed as junior research fellow for a four months extension of the project (1st of September 2015 – 31st of December 2015) and this period was used to complete and submit the last project publication, which is his third one. This publication is still under review at the time this closure report is due. Both PhD students presented results at domestic meetings and also at international conferences. Project results became parts of PhD theses: Petra Majer defended her thesis at the PhD School of Szeged University in 2013 (summa cum laude) and the thesis of Gyula Czégény is scheduled to be submitted to the Biology PhD School of the University of Pécs during 2016.

Undergraduate UP students also participated in some sub-projects; one of them even earned co-authorship in a forthcoming publication (Dóra Pávkovics, in Czégény et al. 2016). Examples from among emerging results were also mentioned in connection to regular UP lectures taught by the PI. Collaborative work involved two PhD students from Ireland, one of them visited UP on Irish project budget, and a second one sent samples for analyses to us. Results of this work are still being combined with experiments ongoing in Ireland and are expected to be published later.

PUBLIC DISSEMINATION

The PI gave two invited talks at events organised in connection to the International Year of Light 2015 (one at the Pécs regional group of HAS and the second organised at national level by the Biology Division of HAS) as well as a third, public lecture on the occasion of Fascination of Plants Day. Main results of OTKA NN 85349 were reported at all three events, explaining their relevance to recent climate issues, and financial support from OTKA was acknowledged both verbally and on projected slides.

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