

SEKCIJA 3: STRUKTURA IN FUNKCIJA
SESSION 3: STRUCTURE AND FUNCTION

TROL integrates chloroplast redox signalling

S. Jurić¹, H. Lepeduš², K. Hazler-Pilepić³, B. Jeličić¹, T. Bionda¹ and H. Fulgosi^{1*}

¹Department of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, HR-10000 Zagreb, Croatia, ²Department of Biology, J.J. Strossmayer University, Gajev trg 6, HR-31000 Osijek, Croatia, ³Faculty of Pharmacy and Biochemistry, HR-10 000 Zagreb, Croatia
*corresponding author (fulgosi@irb.hr)

Introduction

Thylakoid membranes of chloroplasts catalyze the fundamental process of photosynthetic energy conversion. The dual genetic origin and the enormous physiological versatility, in particular its ability to manage short- and long-term changes in the light environment, are outstanding characteristics of this specialized biomembrane. Various mechanisms can regulate the distribution of excitation energy between the two photosystems, and other can convert excess excitation energy into thermal energy. A significant number of auxiliary enzymes are believed to be involved in these physiological processes. Diverse protein kinases and phosphatases, chaperones, a substantial number of proteases that catalyze regulated protein degradation, and other protein components are required during biogenesis of the photosynthetic multisubunit complexes.

In this work, we used reverse genetics approach to identify novel auxiliary thylakoid component, entitled TROL, which is necessary for sustaining efficient photosynthetic electron transport under conditions of elevated light intensity.

Materials and Methods

Full length At4g01050 cDNA was obtained from Arabidopsis Biological Resource Center, USA and fully sequenced. Gene silencing cassette was constructed by a 3'-5' insertion of the cDNA into pGPTV binary vector. Construct was introduced into *Arabidopsis thaliana* plants by Agrobacterium-mediated floral infiltration. Selection of transformants was performed using glufosinate ammonium resistance. Arabidopsis T-DNA insertional inactivation lines were obtained from Nottingham Arabidopsis Stock Centre, UK. Position of the insertion was verified by sequencing. Homozygous plants were identified via PCR. Plants were grown under 16 hour light regime in a growth chamber. Overexpression of a 23 kDa portion of the protein was done using pRSET system. Recombinant protein was purified by using Ni-NTA affinity matrix (Qiagen). Production of polyclonal antiserum was performed by Pineda (Germany).

Yeast two-hybrid screen was performed according to manufacturer's procedure (Stratagene). *In vitro* chloroplast protein import assays were performed as described in (Fulgosi et al., 1998). Localization studies using YFP fusion were performed on isolated protoplasts using Leica confocal microscope TCS SP2 AOBs. Standard co-immunoprecipitation and Western analyses were performed on various sub-chloroplast fractions. *In vivo* chlorophyll *a* fluorescence kinetics was measured by using mini-PAM (Walz).

Results and discussion

Molecular characterization of TROL

We have used proteomics approach to obtain N-terminal amino acid sequence of a hitherto uncharacterized chloroplast protein. The sequence was used to search Arabidopsis genome database and an ORF, which encodes a hypothetical polypeptide of 466 aa residues with a predicted thylakoid targeting presequence, was identified. Extensive bioinformatics analysis revealed the existence of putative rhodanese (thiosulfate sulfurtransferase)-like domain and a repetitive module possibly involved in binding of ferredoxin:NADP⁺ oxidoreductase (FNR). FNR catalyses the final electron transfer of oxygenic photosynthesis from ferredoxin to NAD(P) (Carrillo and

Caccareli, 2003). Accordingly, the protein was named TROL (thylakoid rh_odanese-like). Localization studies using C-terminal YFP fusion revealed chloroplast localization of TROL. *In vitro* chloroplast import assays confirmed its thylakoid localization and have demonstrated that TROL possesses characteristics of an integral membrane component. Furthermore, TROL is most likely exclusively located in non-appressed thylakoid regions. To confirm the assumption that TROL is involved in anchoring of FNR, the sequence module at the C-terminus of TROL was used as bait in two-hybrid screen using FNR gene as a target. Following yeast transformation, the expression of both protein products resulted in a number of colonies showing β -galactosidase activity. Furthermore, thylakoid membranes were solubilised with 1% Triton X-100, and TROL antibodies were used for immunoprecipitation. FNR was significantly enriched in the immunoprecipitated fraction, whereas the preimmune serum of TROL did not co-precipitate detectable amounts of FNR.

TROL is necessary for photosynthesis

To gain insight into the function of TROL, we have used transgenic Arabidopsis lines in which At4g01050 gene was inactivated by an insertion of a T-DNA element into the last intron at position 2278. Insertion leads to a complete depletion of TROL protein, as tested by Western analyses of cellular extracts from homozygous plants. To be able to verify results obtained from analyses of TROL knock-out plants, we have generated antisense lines in which TROL protein accumulation was lowered. In low-light conditions, both knock-out and antisense lines did not show any visible phenotype. We have investigated photosynthetic performance of wild-type leaves and both transgenic lines by using Pulse-Amplitude fluorometry and saturation pulse method. Knock-out and antisense lines had severely lowered relative electron transport rate (ETR) at light intensities exceeding $250 \mu\text{mol}_{\text{PHOTONS}} \text{m}^{-2}\text{s}^{-1}$. Simultaneously, the amount of nonphotochemical quenching (NPQ) increases, indicating enhanced dissipation of absorbed light energy as heat. Thus, TROL deficient thylakoids direct only a small fraction of absorbed light to photosystems.

In summary, we conclude that TROL represents a thylakoid membrane docking site for the assembly of a ternary complex between FNR, ferredoxin and NAD(P). Deletion of TROL leads to overreduction of thylakoid membranes, indicating that formation of such an anchored complex is required for oriented interaction between electron donor and acceptor in order to accommodate their respective redox centres in optimal orientation for efficient electron transfer. It is tempting to speculate that TROL is a key element in regulation of thylakoid redox poise, thereby influencing a wide range of redox signals originating from photosynthetic membranes.

References

- Fulgosi H., Vener A., Altschmied L., Herrmann R. G. and Andersson B. (1998). *EMBO Journal*. 17 (6), 1577-1587.
Carrillo N., Caccareli E. A. (2003) *Eur. J. Biochem.* 270, 1900-1915.

Dendrochronology and its use in biology and cultural studies

K. Čufar^{1*}, M. Zupančič¹, J. Gričar¹ and M. de Luis Arrillaga²

¹ Biotechnical Faculty, University of Ljubljana, Rožna dolina, Cesta VIII/34, SI-1000 Ljubljana, Slovenia, ² University of Zaragoza, Dept. Geografía y O.T., C/ Pedro Cerbuna 12, 50009 Zaragoza, Spain

* corresponding author (katarina.cufar@bf.uni-lj.si)

Introduction

Dendrochronology is generally based on investigations of tree rings in the wood on an annual scale. The analysis can include measurements of ring widths, early- or late-wood widths and proportions, density profiles throughout the annual ring, analysis of wood structure etc. To produce exactly dated series of tree ring parameters we principally need adequate reference chronologies for the tree species, region, and time span of interest. The annual rings in the wood archive information on factors affecting the tree in different periods of its life. The possibility to use this archived information is manifold. In the recent time many studies have been dedicated to dendroclimatology studying the effects of climate on wood characteristics. Such investigations triggered new research in tree biology, physiology and ecology, including studies of wood formation by the cambium on cellular and intra-annual level. Such studies contributed to new innovative applications in cultural heritage and archaeology.

The objective of the invited lecture is to show some possibilities to use dendrochronology in different disciplines exemplified with own research results.

Material and methods

Different types of samples (cores, discs) and categories of wood (wood from living trees, historical objects, and water-logged archaeological wood) have been smoothed in order to observe structure on cellular level under a stereo microscope. Annual ring widths, and in some studies widths of early- and late-wood, were measured to the nearest 0.01 mm using a measuring table LINTAB connected to a personal computer. Data acquisition and processing was done with TSAP/X and TSAP WIN programmes. Measurements on the cellular level were done on smoothed samples or microscopic sections with help of image analysis system LUCIA.

Results and discussion

Our first dendrochronological studies were focused in silver fir (*Abies alba* Mill.) decline (Čufar 1997). As a result several local and one regional reference chronology were constructed (Levanič and Čufar 1997). They were used to investigate wooden objects important for cultural heritage (Čufar and Lozar-Štamcar 2004, Perčič et al. 2004). Tree ring patterns were used to model and predict mortality of affected trees (Bigler et al. 2004). Since the “window” to receive and archive environmental information is only open when the cambium is active (Frankenstein et al. 2005), basic investigations of wood formation by the cambium (Gričar et al. 2005) helped to define the duration of cambium activity which is important for physiological interpretation of tree-ring characteristics. Experiments of controlled heating and cooling of stem portions in mature Norway spruce (*Picea abies* Karst.) trees contributed to improve basic knowledge on affects of temperature to cambium activity (Gričar et al. 2006). Investigations in larch (*Larix decidua* Mill.) proved that international cooperation is needed to construct longer reference chronologies and to better understand past timber use (Levanič et al. 2001).

Tree-ring records are also important from an ecological perspective and to evaluate tree response to climatic or environmental factors. Effects of climatic (floods, droughts) or non-climatic disturbances (forest fires, human activities) on forest areas can be also

investigated by using information included in tree-rings. The climatic variability has different effects on tree growth in different tree species and localities, therefore we extended our investigations to pines *Pinus halepensis* and *Pinus pinea* from different sites in Spain (Raventós et al., 2001).

The investigation in broadleaved tree species are of importance for environmental studies in hilly SE Slovenia and to investigate the wood from the distant past, like the wood from the pile dwellings from Ljubljansko barje that mainly existed between 4000 and 2500BC (Velušček and Čufar, 2002, Čufar and Velušček 2003). The most important species in this respect are oaks (*Quercus* sp.) and beech (*Fagus sylvatica* L.).

Oaks in Central Europe and in Slovenia mainly represented by sessile oak (*Quercus petraea*) and pedunculate oak (*Q. robur*) are very important in dendrochronology. The oak chronology from south Germany exceeds 10,000 years (Friedrich et al. 2004) and is the longest tree-ring chronology in the world. In Slovenia we long time considered that oak is suboptimal for dendrochronological research and that its tree-ring variation is mainly affected by local factors (Čufar and Levanič 1999, Čater and Levanič 2004). New local chronologies of oak and investigation of historic timbers from SE Slovenia enabled us to construct a 548-year long regional chronology spanning the period AD 1456-2003. The tests for teleconnection – i.e. for its agreement with oak chronologies from distant sites, proved that oak from Slovenia may correlate with chronologies from up to 700 km distant sites in various parts of Europe. This indicates that oak growth in Slovenia is after all driven by a supra-regional factor. The analyses also showed that it has got great dendroclimatological potential.

Recently, special attention has also been dedicated to beech (*Fagus sylvatica* L.) our frequent and important tree species. It is growing on great variety of sites and is therefore interesting for dendroecological and dendroclimatological studies. Beech wood is in some locations preserved in historic buildings, which is unique in Europe. Therefore a construction of a longer regional chronology is possible. The current length of the beech chronology of SE Slovenia is 360 years (Čufar, unpublished). It is composed of wood from old trees and historical constructions. The response analyses showed that beech has got great potential for climatological research (Čufar, De Luis, unpublished). At the end we will present various possibilities to use oak and beech chronologies to investigate wooden objects of the Slovenian cultural heritage.

References

- Bigler Ch., Gričar J., Bugmann H., Čufar K. (2004). *For. Ecol. Manage.* 199, 183-190.
- Čufar K. (1997). *Zb. gozd. lesar.* 52, 165-186
- Čufar K., Levanič T. (1999). *Phyton (Horn)* 39 (3), 113-116.
- Čufar K., Lozar Štamcar M. (2004). *Argo* 47 (2), 74-80.
- Čufar K., Velušček A. (2003). *Zb. gozd. lesar.* 71, 137-158.
- Čater M., Levanič T. (2004). *Ekológia (Bratisl.)* 23 (4), 353-365.
- Frankenstein C., Eckstein D., Schmitt U. (2005) *Dendrochronologia* 23, 57-62.
- Friedrich M., Remmele S., Kromer B., Hofmann J., Spurk M., Kaiser K.F., Orsel C., Küppers M. (2004). *Radiocarbon* 46(3), 1111-1122.
- Gričar J., Čufar K., Oven P., Schmitt U. (2005). *Ann. bot.* 95, 959-965.
- Gričar J., Čufar K., Zupančič M., Koch G., Schmitt U., Oven P. (2006). *Ann. bot.* in print.
- Levanič T., Čufar K. (1997). *Dendrochronologia* 15, 183-190.
- Levanič T., Pignatelli O., Čufar K. (2001). *Dendrochronologia* 19 (2), 221-229.
- Perčič D., Čufar K., Zupančič M. (2004). *Kamniški zb.* 17, 269-274
- Raventós J. De Luis M., Gras M.J., Čufar K., González-Hidalgo J.C., Bonet A., Sánchez J.R. (2001). *Dendrochronologia* 19, 211-220
- Velušček A., Čufar K. (2002). *Arheol. vestn.* 53, 59-67.

The effect of aerial pollution on cytokinins in forest trees

J. Winwood¹, M. D. Collier¹, A. O'Brien¹, L.J. Sheppard² and D. E. Hanke^{1*}

¹University of Cambridge Department of Plant Sciences, Downing Street, Cambridge CB2 3EA, UK, ²Centre for Ecology and Hydrology, Edinburgh Research Station, Bush Estate, Penicuik, Midlothian EH26 0QB, UK

*corresponding author (david.hanke@plantsci.cam.ac.uk)

Introduction

Phytohormones mediate between the environment upstream and the downstream developmental responses of plants that enable them to survive or exploit changes in the environment. This process of acclimation is of greater survival value to long lived perennials, and so forest tree species are programmed with an extensive suite of acclimatory responses. Cytokinins (CKs) have a special role as the prime activators of the developmental activity of shoot apical meristems and leaves. By contrast, abscisic acid (ABA) inhibits these activities, generally opposing the response to CKs. The interactions can be shown to potentiate many acclimatory responses. This system of control evolved over geological time before human intervention in the environment, and there is no *a priori* reason why it should be robust enough to accommodate anthropogenic pollution. We are interested to discover whether the changing chemistry of the gas phase around trees is influencing their morphology by way of effects on the phytohormone signals controlling morphology

Materials and methods

Physiologically mature Sitka spruce (*Picea sitchensis* [Bong.] Carr) trees in a commercial plantation on the Pentland Hills, near Edinburgh, Scotland, were mist sprayed with combinations of S, N and acidity from bud burst until December for a three year period (details in Collier et al. 2003).

60 years old planted beech (*Fagus sylvatica* L.) trees in Kranzberg forest near Freising, Germany, were fumigated with ozone using a free-air system to maintain the concentration of ozone in canopies of treated trees at about two fold ambient ($2xO_3$) throughout the growing period from April/May until October for a two year period (details at www.casiroz.de).

Cytokinins were analysed as in Collier et al. (2003), and ABA using a protocol based on the method of Quarrie et al. (1988).

Results and discussion

Spruce and N

For Sitka spruce on the Pentland Hills, after three years of treatment only needles of neutral N treated trees contained significantly higher levels of cytokinin than those of trees receiving no spray. Needle contents were 5 times those of controls. Addition of acid S could suppress the effects of added N. Needles from neutral N treatments also contained significantly higher levels of biologically active cytokinins and forms that can be transported in the xylem/phloem. Significantly lower levels of active cytokinins were present in needles sprayed with N in combination with acid sulphate. The results demonstrate that the needle cytokinin content may be used as sensitive and selective bioindicator of the early stages in N perturbation in coniferous trees.

Beech and O₃

For beech in Kranzberg Forest, in general, ozone treatment resulted in lower levels of CK in leaf samples and elevated levels in root and leaf xylem samples, but the effect of ozone was not as marked as the effect of sampling time. The cytokinin content of leaves did not alter detectably in samples taken at intervals during a 24h cycle. In 2003 and 2004 the cytokinin content of leaf samples followed a similar seasonal pattern, with a dip from June to July rising to peak values in September before falling again. There were

no detectable differences in the pattern of leaf content vs time for different types of CK, except for the O-glucosides, the leaf content of which was greater than other CKs, and tended to accumulate towards the end of the season, consistent with the vacuolar sequestration of this CK metabolite. In leaf samples, the content of aromatic types was equal to that of the isoprenoid types. As in other plant species aromatic types are minor or trace components, this is a remarkable finding. In root samples, the level of aromatic types was no different from the leaves, but that of the isoprenoid types was much higher. In 2004, the leaf cytokinin content for 2xO₃ trees was lower than for controls at ambient levels of ozone (1xO₃) at all sampling times, though in 2003, a drought year, this effect was obvious only in September and October, which we attribute to the increase in stomatal conductance after the end of the drought. The effect of ozone was greater for leaves in the sun crown than for leaves in the shade crown. By contrast, the root and xylem contents of cytokinin for 2x trees were greatly elevated over the values for 1xO₃ trees early in the growing season, less so for later samples. All groups of cytokinins were affected in the same way by ozone treatment. In 2004 there was no sign of the entire season's accumulation of O-glucosides, a large amount of the hormone, in leaves from 2xO₃ trees. We propose that O₃-associated CK destruction in leaves reduces CK-mediated root growth suppression. The resulting increases in root growth lead to increases in CK export from the root, although O₃-associated CK destruction in the leaves appears to nullify this increase in signal. The consequence is a shift in the root/shoot ratio towards more root.

In general, ozone treatment resulted in elevated levels of ABA but the effect of ozone was not as strong as the effect of sampling time. Analysis of leaves from the sun crown collected at 9 time points during a 24h cycle in July 2004 showed that elevated O₃ caused an increase in ABA content at all time points, but the effect was greater during daylight and maximal around midday. All other samples were collected around midday. In both 2003 and 2004, ABA content of leaves from sun and shade crown increased from June to July and decreased from July to September and October. At 50 nmol g⁻¹FW, the July maximum was greater in 2003, a drought year, than in 2004. In 2003, levels of ABA in phloem followed the same seasonal pattern as in leaves, but no seasonal pattern was detected in 2004, or for xylem in both years. The ABA content of 2xO₃ sun leaves was higher than for 1xO₃ sun leaves in both 2003 and 2004, but no consistent effect was detected for leaves in the shade crown, xylem or phloem samples.

Conclusions

Changes in the chemistry of the ambient gas phase typical of anthropogenic pollution alter the pattern of phytohormones in forest trees. Monitoring these hormonal shifts will help us predict the long term effects of rising levels of chemical pollutants in the atmosphere.

Acknowledgements

MDC and JW thank BBSRC (UK) for CASE studentships. The work on beech was part of the Project "CASIROZ – The carbon sink strength of beech in a changing environment: Experimental risk assessment by mitigation of chronic ozone impact", which was supported by European Commission - Research Directorate-General, Environment Programme, "Natural Resources Management and Services" (EVK2-2002-00165, Ecosystem Vulnerability).

References

- Collier M.D., Sheppard L.J., Crossley A. and Hanke, D.E. (2003) *Plant, Cell and Environment* 26, 1929-1939.
Quarrie S.A., Whitford P.N. and Appleford N.E.J. (1988) *Planta* 173, 330-339

Chromosomal aberrations as a tool to follow the development patterns in barley plants

I. Kreft*

Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1001 Ljubljana, Slovenia

*corresponding author (ivan.kreft@guest.arnes.si)

Introduction

Morphological and gene expression research indicate the distinct group of cells in the seed embryo apex that define the boundaries among apex cells (Aida and Tasaka, 2006). Epigenetic development of plants depends on the status of genetic material (Schuebeler and Elgin, 2005). Mutations are a possible tool to follow the development patterns in barley (Frydenberg and Jacobsen, 1966). Chromosomal aberrations are relatively frequent and may cause the reduced spike fertility (Kreft, 1969). The purpose of this investigation was to provide information on the chimaerism of barley (*Hordeum vulgare*) spikes. Differences in seed fertility between both lateral halves, and between basal and apical halves of spikes were studied.

Material and methods

Dry barley seeds cv. Union were irradiated with fast neutrons in nuclear reactor of Josef Stefan Institute, Ljubljana, speed 166 rad/h of fast neutrons (energy about 2 meV, to obtain fast neutron dose 620 rad), 8 rad/h thermal neutrons and 17 rad/h of gamma radiation. Plants from treated seeds were grown at the experimental field of Biotechnical Faculty, Ljubljana. During the harvest, spikes were sorted (spikes on main or lateral shoots) and differences in seed fertility between both lateral halves, and between basal and apical halves of spikes were studied.

Results and discussion

Fertility differences between both lateral spike halves and between basal and apical spike halves were scored (Table 1.).

Table 1: Frequencies of spike types

Treatment	Type of spikes	Spike types, number (see explanation)				Total
		A	B	C	D	
Untreated	Main	263	17	11	9	563
Untreated	Lateral	311	27	8	7	353
Irradiated	Main	136	18	57	19	230
Irradiated	Lateral	162	24	13	2	201

A – number of spikes with the difference in fertility between lateral halves of spike 30% or less, and with the difference in fertility between the basal and apical halves of spike 30% or less (in comparison to full fertility – 100 %).

B – number of spikes with the difference in fertility between lateral halves of spike 30% or less, and with the difference in fertility between the basal and apical halves of spike more than 30%.

C – number of spikes with the difference in fertility between lateral halves of spike more than 30%, and with the difference in fertility between the basal and apical halves of spike 30% or less (in comparison to full fertility – 100 %).

D – number of spikes with the difference in fertility between lateral halves of spike more than 30%, and with the difference in fertility between the basal and apical halves of spike more than 30%.

Differences in fertility between lateral spike halves were observed more frequently than between basal and apical halves in irradiated plants, in comparison to the control. In lateral spikes from irradiated plants it was observed less chimaerism in comparison to main spikes of irradiated plants. So, it appeared a possibility of appearance of chimaeric spikes with parts with different chromosomal genotypes, some of them with the reduced fertility, due to chromosomal aberrations. The embryo apex meristems do not take part in the development of embryo itself, but are activated only during seed germination and further development of the plant (Bhalla and Singh, 2006). In the seed embryo apex, there appeared chromosomal aberrations, which are inherited by progenitor cells. According to the results, in the seed embryo apex, there are at least four individual cells, which are responsible for the formation of the spike on the main shoot of the barley plant.

Acknowledgment

Members of the Nuclear Reactor Department, Josef Stefan Institute, Ljubljana, kindly provided radiation treatment of barley seed samples and performed the measurement of treatment doses.

References

- Aida M. and Tasaka M. (2006). *Plant Molecular Biology* 60, 915-928.
Bhalla P.L. and Singh M. B. (2006). *Plant Cell Rep.* 25, 249-256.
Frydenberg O. and Jacobsen P. (1966). *Hereditas* 55, 227-248.
Kreft I. (1969). *Hereditas* 62, 14-24.
Schuebeler D. and Elgin S.C.R. (2005). *Nature Genetics* 37, 917-918.

Intracellular localization of glutathione and its precursors in plant cells

M. Müller*, G. Zellnig and B. Zechmann

Institute of Plant Sciences, University of Graz, Schubertstraße 51, 8010 Graz, Austria

*corresponding author (maria.mueller@uni-graz.at)

Introduction

Glutathione is a tripeptide thiol and has numerous important functions in plants. It is involved in the transport and storage of reduced sulfur and in the detoxification of reactive oxygen species, xenobiotics, heavy metals, etc., which are able to damage cellular components (Noctor et al. 1998, Tausz et al. 2004). Glutathione is synthesized out of its constituents cysteine, glutamate and glycine in two ATP-dependent steps. In the first step cysteine and glutamate are linked together to form γ -glutamyl-cysteine, catalyzed by γ -glutamylcysteine synthetase, which takes place either exclusively in plastids (Wachter et al. 2005) or in both plastids and the cytosol (Gomez et al. 2004) depending on the plant species. In the second step glycine is added to γ -glutamylcysteine catalyzed by glutathione synthetase to form the final product glutathione. This reaction takes place in plastids and in the cytosol, which are therefore considered as the main centers of glutathione synthesis (Gomez et al. 2004, Wachter et al. 2005). Degradation of glutathione is thought to take place at the plasmalemma, at the tonoplast or within vacuoles.

The aim of the present study was to visualize the subcellular distribution of glutathione and its precursors cysteine, glutamate and glycine for the first time in plant cells. Additionally, we investigated how the artificial elevation of cysteine, which is usually the rate limiting factor of glutathione synthesis (Kopriva and Rennenberg 2004) affects subcellular glutathione contents. These investigations were aimed to gain a deeper insight into glutathione synthesis and degradation in plant cells.

Materials and Methods

Seeds of *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB. (Styrian oil pumpkin) were germinated on a humid Perlite cloth. Plant material was kept in growth chambers with a photoperiod of 12 hours. Day and night temperatures were 22°C and 18°C, respectively; the relative humidity was 70%. One part of two week old seedlings, were treated for 48 hours with 1 mM of L-2-oxothiazolidine-4-carboxylic acid (OTC) by immersing the roots into the solution. Roots, cotyledons and first true leaves from selected samples were harvested and prepared for transmission electron microscopical (TEM)-investigations as previously described (Müller et al. 2004). Immunogold labeling of glutathione and its precursors was performed with ultrathin sections on nickel grids. Sections were treated with the primary antibodies (either anti glutathione rabbit polyclonal IgG, anti cysteine rabbit polyclonal IgG, anti glutamate rabbit polyclonal IgG, or anti glycine rabbit polyclonal IgG) diluted 1:50 (glutathione), 1:300 (cysteine), 1:300 (glutamate) and 1:50 (glycine), in phosphate buffered saline (PBS) containing 1% goat serum for 2 hours. After a short wash in buffer the samples were incubated with a 10 nm gold-conjugated secondary antibody (goat anti rabbit IgG) diluted 1:50 for glutathione and cysteine and 1:100 for glutamate and glycine in PBS for 90 minutes. After a short wash in distilled water labeled grids were observed in a Philips CM10 TEM.

Results and Discussion

OTC is a cysteine precursor and led to increased labeling of cysteine in all investigated cell compartments of roots (up to 113% in plastids) and cotyledons (up to 68% in peroxisomes). Subsequently glutathione contents were strongly elevated in these organs (up to 145.5% in peroxisomes of cotyledons) demonstrating that cysteine is the limiting factor of glutathione synthesis in plants. OTC-treated roots showed a strong increase in glutamate levels (up to 120% in mitochondria), whereas in cotyledons glutamate levels

decreased (up to 26% in nuclei). The above described results can also be applied for plastids and the cytosol, which are supposed to be the main centers of glutathione synthesis (Sugiyama et al. 2004, Wachter et al. 2005, Zechmann et al. 2005). Glycine was strongly decreased in roots and cotyledons (up to 59% in mitochondria of cotyledons). Therefore it can be concluded that if cysteine, which is usually together with γ -glutamylcysteine the rate limiting factor during glutathione synthesis in plants (Kopriva and Rennenberg 2004) occurs in excess glycine becomes the limiting factor of glutathione synthesis in roots and both glycine and glutamate limit glutathione production in cotyledons. Within first true leaves glutathione levels were only elevated in the cytosol (93%) and plastids (24.5%) demonstrating that glutathione synthesis takes exclusively place in these cell compartments (Noctor and Foyer 1998; Foyer et al. 2001; Sugiyama et al. 2004). After its production glutathione could then be relocated into other organelles like mitochondria, nuclei, or peroxisomes, which depend on the import of cytosolic GSH (Wachter et al. 2005).

The present experiments also demonstrate that glutathione precursors can be found in vacuoles but not in the apoplast. This indicates that glutathione degradation takes place in vacuoles or that degradation products are rapidly transported back from the apoplast into the cells. Vacuoles of cells from cotyledons showed a strong increase in cysteine contents after OTC-treatment indicating that vacuoles can act as a sink for cysteine if it occurs in excess and can not be cooperated into organic components within leaves. During the present study only cysteine and glycine were found in vacuoles whereas glutathione and glutamate were absent. These results are similar to the current opinion that the distribution of glutathione within plant cells is limited to the cytosol and to organelles since numerous methods aimed in the detection of glutathione within vacuoles failed (Rennenberg, 2001). The lack of glutathione within vacuoles and the fact that we only detected cysteine and glycine within vacuoles demonstrates that glutathione is either degraded in the tonoplast or rapidly within vacuoles by γ -GT which transfers glutamate from reduced glutathione to other dipeptides and leaves the dipeptide Cys-Gly. Glutamate is thereby transferred to dipeptides, which will not be detected by the glutamate antibody used in these investigations since they do only bind to free glutamate, but not to glutamate bound in peptides. The remaining dipeptide Cys-Gly is then metabolized by a dipeptidase to the component amino acids. Such a pathway within vacuoles has been suggested by Foyer et al., 2001, but the first step of this degradation pathway still needs to be clearly demonstrated in vacuoles.

Summing up the present study allowed investigating the subcellular distribution of glutathione and its precursors cysteine, glutamate and glycine in one plant sample simultaneously. Since changes of these components can be visualized in single cell compartments of cells from one sample this method can now be used to investigate changes in glutathione and its precursor levels during various biotic and abiotic stress situations, which will give a deeper insight into glutathione synthesis and degradation.

Acknowledgement: This work was supported by the Austrian Science Fund (FWF P16273 and P18976)

References

- Foyer C. H., Theodoulou F. L. and Delrot S. (2001). Trends in Plant Science 6 (10), 486–492.
- Gomez L. D., Vanacker H., Buchner P., Noctor G. and Foyer C. H. (2004). Plant Physiology 134, 1662–1671.
- Kopriva S. and Rennenberg H. (2004). Journal of Experimental Botany 55, 1831–1842.
- Kuźniak E. and Skłodowska A. (2005). Journal of Experimental Botany 56, 921–933.
- Müller M., Zechmann B. and Zellnig G. (2004). Protoplasma 223, 213–219.
- Müller M., Zellnig G., Urbanek A. and Zechmann B. (2005). Phytion (Horn) Austria 45, 45–55.
- Noctor G. and Foyer C. H. (1998). Annu Review of Plant Physiology and Plant Molecular Biology 49, 229–279.
- Noctor G., Arisi A. C. M., Jouanin L., Kunert K. J., Rennenberg H. and Foyer C. H. (1998). Journal of Experimental Botany 49 (1), 623–647.
- Rennenberg H. (2001). Glutathione – an ancient metabolite with modern tasks. In: Grill D, Tausz M, De Kok LJ (Eds.) *Significance of glutathione to plant adaptation to the environment*. Kluwer Academic Publishers, Dordrecht, Boston, London: pp 1–11.
- Sugiyama A., Nishimura J., Mochizuki Y., Inagaki K. and Sekiya J. (2004). Plant Biotechnology 21. 79–83.
- Tausz M., Šircelj H. and Grill D. (2004). Journal of Experimental Botany 55, 1955–1962.
- Wachter A., Wolf S., Steininger H., Bogs J. and Rausch T. (2005). Plant Journal 41, 15–30.
- Zechmann B., Zellnig G. and Müller M. (2005). Plant Biology 7, 49–57.

Formation of surface callus in wounded stems of Norway maple (*Acer platanoides* L.)

L. Marion^{1,2}, P. Oven^{1,*}

¹University of Ljubljana, Biotechnical Faculty, Dep. of Wood Science and Technology, Rožna dolina, cesta VIII/34, SI-1000 Ljubljana, Slovenia,

²Tisa d.o.o., Izanska cesta 213, SI-1000 Ljubljana, Slovenia

* corresponding author (primoz.oven@bf.uni-lj.si)

Introduction

Harsh growing conditions of urban environment with frequent mechanical wounding influence tree vitality and its growth. Tree response to mechanical wounding is usually interpreted in the sense of compartmentalization (Shigo 1986). One of the responses of xylem being exposed after removal of bark due to traffic accidents or careless logging in forests is formation of surface callus (Stobbe et al. 2002a; Stobbe et al. 2002b; Balder 2003; Stobbe and Dujesiefken 2004). This phenomenon has been observed in various tree species (Dujesiefken et al. 2001; Stobbe et al. 2002b), but information on anatomical changes associated with callus formation is only fragmentary. The information on relationship between rate of surface callus formation, time of wounding and the vitality of the tree is practically missing. The aim of this preliminary report was to assess the chronology of anatomical response and closure of wounded surface in connection with tree vitality in Norway maple trees (*Acer platanoides* L.).

Materials and methods

Two groups of Norway maple trees (*Acer platanoides* L.) were selected in the City of Ljubljana: a control group of six unaffected trees and a group of six trees with affected crowns due to polluted soil with de-icing salts. Two trees from each group were wounded by debarking in April, two trees in June and two in August in year 2005. Bark was cut with sharp knife and chisel and then removed without touching the exposed surface of stem wood. Eight to nine square shaped wounds with surface of 9 to 25 cm² were made per tree at stem heights between 80 and 170 cm and were covered with black plastic foil. Sampling of surface callus was performed with sharp knife and chisel twice a week during two months period. A photograph of each wound was taken at each sampling date. Tissues were immediately fixed in FAA (formaldehyde-ethanol-acetic acid solution) and thereafter dehydrated in a graded series of ethanol and embedded in paraffin. Cross-sections were prepared with a rotary microtome, stained with safranin and astra blue and mounted in Euparal for light microscopy. Area of surface callus was measured with image analysis system Lucia G. Wounds induced in April (June, August) will be referred to as April (June, August) wounds in this report.

Results and discussion

The surface of exposed xylem appeared whitish and moist immediately after removal of the bark. Residuals of brownish fibrous tissue were seen on the surface of xylem in affected trees. Microscopy revealed that the bark was usually separated from a tree either within the zone of differentiating xylem or differentiating phloem cells or, within the youngest secondary phloem increment in affected trees. Superficial cells were collapsed and became necrotic by day 7 after wounding. Under necrotic layer, individual ray parenchyma enlarged. New callus developed by hypertrophy and hyperplasia of undifferentiated radial and axial xylem cells having only primary wall under necrotic cells in 7 to 14 days after wounding. By day 18, three tangential layers differentiated within surface callus. Outermost layer was composed of dead thick-walled lignified cells with reddish deposits in lumina. In the middle layer, radially flattened cells arranged in relatively regular radial files indicated initial stages of new cambium differentiation. Inner layer of callus, adjacent to xylem cylinder, exhibited differentiation of solitary vessels. During the further course of the experiment only increase in mass of outer and inner layer was recorded.

Occurrence of new callus tissue on the surface of xylem was distinguishable by naked eye in 7 to 14 days after wounding. Regardless of tree vitality and time of wounding, surface callus was formed in 96 % of wounds. It covered in average 22,7% of area of in April wounds, June wounds were covered more than twice more (52,6%) and August wounds were covered almost 4 times more (85,3%). The average area of surface callus was significantly larger (31,1% and 91,1%) in April and August in affected trees comparing to healthy ones (14,2% and 79,5%). In June wounds, the area of surface callus (43,3%) was smaller for a third in affected trees when compared to healthy ones (61,9%).

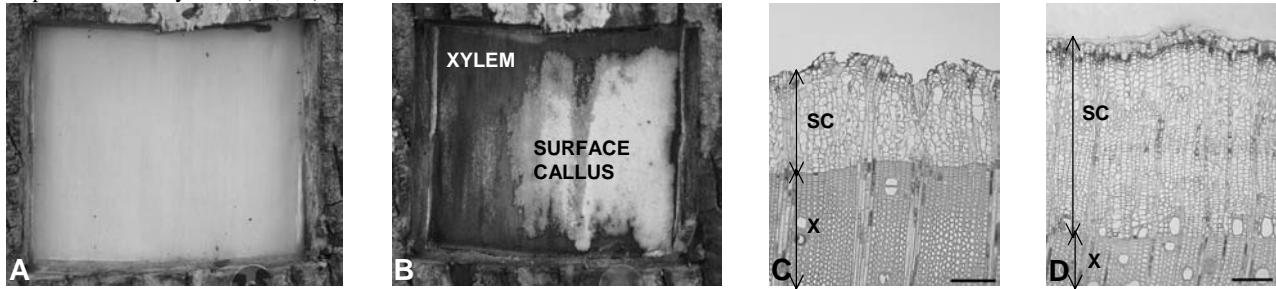


Figure 1: Norway maple (*Acer platanoides* L.). (A) Exposed xylem immediately after debarking. (B) Surface callus did not develop on the entire area of the exposed xylem by day 14 after wounding in June. (C) Initial stage in development of surface callus (SC); day 7 after wounding in April. (D) Anatomy of surface callus on day 47 after wounding in April. X – xylem. Scale bar = 100µm.

Conclusion

Formation of surface callus is relatively quick response in unaffected and affected trees, regardless of time of wounding in the growing season. It is assumed, that early formation of outer thick-walled lignified layer prevents desiccation of underlying tissue and hence enables formation of new cambium inside the callus. New comprehensions about protection and wound tissue formation would contribute to solution in protecting many mechanical wounding caused to trees by traffic or vandalism in urban area or by logging in the forest.

Acknowledgements

This study was supported by the Ministry of Higher Education, Science, and Technology of the Republic of Slovenia, the Slovenian Research Agency and the Municipality of Ljubljana. We thank Martin Zupančič and Peter Cunder for technical assistance.

References

- Balder, H. (2003). *AFZ-Der Wald* (6), 276-277.
 Dujesiefken, D., Stobbe, H., Kowol, T. (2001). *Forstw. Cbl.* 120, 80-89.
 Shigo, A. L. (1986) A new tree biology. Durham, Shigo and Trees Associates, 595.
 Stobbe, H., Dujesiefken, D. (2004). *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 56 (10), 260-263.
 Stobbe, H., Dujesiefken, D., Eckstein, D., Schmitt, U. (2002a). *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 54 (12), 312-318.
 Stobbe, H., Schmitt, U., Eckstein, D., Dujesiefken, D. (2002b). *Annals of Botany* 89, 773-782.

Effect of experimentally controlled heating and cooling on xylem- and phloemogenesis in stem portion of Norway spruce

J. Gričar^{1*}, K. Čufar¹ and P. Oven¹

¹Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Rožna dolina, C. VIII/34, SI-1001 Ljubljana, Slovenia,

*corresponding author (jozica.gricar@bf.uni-lj.si)

Introduction

Environmental factors provide physical conditions for growth and development of trees. Effect of individual factors on wood and phloem formation could be demonstrated in experiments with shoots, stem cuttings or intact stem portions growing under controlled conditions. It was already shown in the previous studies that application of temperature can cause alterations in regular cambial activity. However, experiments in the controlled conditions have been mainly performed over shorter periods of time. Moreover, the effect of elevated temperatures on cambial activity has been mostly studied during the dormancy (Little/Bonga 1974, Little 1981, Riding/Little 1981, 1984, Savidge/Wareing, 1981, Barnett/Miller 1994, Oribe/Kubo 1997, Oribe et al. 2001, 2003, Gričar et al. 2006).

The scope of this research was to evaluate the response of active cambium of Norway spruce (*Picea abies*) to experimentally increased (23-25°C) and decreased (9-11°C) temperatures during the entire growing season 2005. The response of the cambium and the formation of xylem and phloem to both treatments were investigated every 21 days by means of light microscopy. In order to reveal the effect of treatments on the general anatomy of xylem and phloem growth rings, samples were taken at the end of the vegetation period 2004. In addition, the temperature of the cambial region in the isolated stem portion, below and above the system was recorded at each sampling date.

Material and methods

For the experiment, three 70-year old Norway spruce test trees (DBH = 35 cm) were selected in an urban Norway spruce/beech forest Rožnik in Ljubljana, Slovenia. One adult tree was used for the heating experiment and one for the cool treatment. The third tree was not treated and served as a control. We started with both experiments on 26 April 2005. We finished cool treatment on 20 September and heat-treatment on 11 October 2005. Temperature of heating was set to 23-25°C and cooling to 9-11°C. Blocks of intact tissue were taken at the beginning of each of the treatments and thereafter at 21-day intervals. Sampling was performed at breast height) from treated trees and the control tree. At the same time, we measured the temperature of the cambial tissue in the isolated stem portion, and below and above it using thermo-cables. After sampling, blocks of tissue were immediately fixed in FAA and, after one week, dehydrated in a graded series of ethanol and acetone and embedded in paraffin. For light microscopy, permanent cross sections of 13 µm in thickness were prepared on a Leica RM 2245 rotary microtome, stained with safranin and astra blue and mounted in Euparal. A Nikon Eclipse 800E light microscope and Lucia G 4.8 image analysis system were used for anatomical observations.

Results and discussion

We demonstrated that application of high or low temperatures caused alterations in regular cambial activity of Norway spruce. Experimental heating of the stem portion of Norway spruce during the entire growing season 2005 did not cause drastic changes in regular cambial activity or in the process of differentiation. Regular cambial activity was not considerably prolonged despite continuously elevated temperatures in the second part of the growing season. Divisions in the cambium stopped in the first part of August, a few days later than in the control tree. However, the rate of the cell divisions was increased at the beginning of the growing

season comparing to the control tree. It seems that higher temperatures were favourable for cambial cell production at the very beginning of the growing season. On the other hand, no effect of the elevated temperatures on cambial activity in the second part of the growing season indicates that higher temperatures do not affect cambial activity or increase the rate of cell production. Number of the cells in the accomplished xylem growth ring was slightly higher in the heated tree (35 layers of cells) than in the control tree (27 layers of cells). Cooling experiment during the entire growth season 2005 shortened regular cambial activity in comparison with the control tree. The xylem increment was reduced for about one third and contained only 10 layers of cells. In addition, the rate of the divisions in the cambium was slowed down.

Measurements of the temperature in the cambial region of the isolated stem portion, and 10 cm below and above it in the year 2005 showed only negligible transfer of the temperature along the stem from the site of its application. The temperature above and below the treated stem portion deviated on average $\pm 2^{\circ}\text{C}$. The temperature in the cambial region oscillated proportionally to the air temperatures with certain delay in regard to oscillations of the air temperatures. In general, the temperature in the cambium was affected by long-term rise or drop in air temperatures.

Long-term experiments in the year 2005 affected the structure and the widths of the phloem growth increments. The structure of early phloem was comparable in cooled, heated and control samples. Early phloem was in all trees formed in comparable period of time (by mid-May). It was composed of 3 to 4 layers of early phloem sieve cells with wide radial dimensions and thin cell walls. However, the tangential band of axial parenchyma cells separating early and late phloem was not continuous in cooled sample which is typical for trees growing under stress conditions. In addition, the temperature affected the number of late phloem cells. The number of cells was in cool-treated sample reduced (consisted of 2 to 3 layers) and in heat-treated sample increased (7 to 8 layers) comparing to the control sample (4 to 5 layers). As for our observations, the temperature affected the phloem production mainly in the second part of the growing season. It appears that the rate of the phloem production stayed the same, yet the distinctions in the number of cells arose from different time of cessation of the cambial activity among samples. Our experiments demonstrated that heating and cooling influenced cambial activity in Norway spruce and confirmed the importance of external factors for cambial activity and corresponding cell differentiation. However, internal factors, such as phytohormones, sugars etc., should be taken into consideration, as well.

Acknowledgements

We are grateful to our colleagues Martin Zupančič, Peter Cunder, Lena Marion and Maks Merela for helpful field assistance. We are indebted to the Slovenian Forestry Institute for enabling experimental work in the field. We thank Dr. Tom Levanič from the Slovenian Forestry Institute for providing the meteorological data. The work was funded by the Slovenian Research Agency (ARRS), the Ministry of Higher Education, Science and Technology (MVŠZT) of the Republic of Slovenia and by the WFS (World Federation of Scientists) and SZF (Slovenian Science Foundation).

References

- Barnett J.R. and Miller H. (1994). *Journal of Experimental Botany* 45, 135-143.
- Gričar J., Zupančič M., Čufar K., Koch G., Schmitt U. and Oven P. (2006). *Annals of Botany*, (in press).
- Little C.H.A. and Bonga JM. (1974). *Canadian Journal of Botany* 52, 1723-1730.
- Little C.H.A. (1981). *Canadian Journal of Botany* 59, 342-348.
- Oribe Y. and T. Kubo. (1997). *Tree Physiology* 17, 81-87.
- Oribe Y., Funada R., Shibagaki M. and Kubo T. (2001). *Planta* 212, 684-691.
- Oribe Y., Funada R. and Kubo T. (2003). *Trees* 17, 185-192.
- Riding R.T. and Little C.H.A. (1984). *Canadian Journal of Botany* 62, 2570-2579.
- Riding R.T. and Little C.H.A. (1986). *Canadian Journal of Botany* 64, 2082-2087.
- Savidge R.A. and Wareing P.F. (1981). *Planta* 153, 395-404.

Sugar induced changes in cellular and extracellular protein and glycoprotein patterns of sugarbeet cell lines

D. Pavokovic^{1*}, I. Sola¹, D. Hagege² and M. Krsnik-Rasol¹

¹Department of Molecular Biology, Division of Biology, Faculty of Science, University of Zagreb, Horvatovac 102A, HR-10000 Zagreb, Croatia,

²Département de Biologie, UFR Faculté des Sciences, Université d'Orléans, Rue de Chartres, BP 6759, 45067 Orléans Cedex 2, France

*corresponding author (dubravko@zg.biol.pmf.hr)

Introduction

Plant development is characterized by extreme plasticity and open form of growth. It is influenced by a complex interaction of internal and external stimuli. Carbohydrates as signal molecules by changing gene expression affect developmental programs. High intracellular sucrose concentration is related to cell growth and maturation while high glucose concentration is related to cell division and dedifferentiation (Weber *et al.*, 1996). In our study we used three *in vitro* cultivated sugar beet cell lines. The lines originate from the same mother plant but differ in their developmental state: N cell line is differentiated but not organogenic, HO is differentiated and organogenic, and HNO is dedifferentiated (Causevic *et al.*, 2005). The N accumulates preferentially sucrose, the HO accumulates sucrose and hexoses, while the HNO preferentially glucose and fructose (Omarzad, unpublished). We wanted to test the influence of exogenously supplied carbohydrates on protein expression patterns and to establish whether this response depends on developmental state of cells. We have electrophoretically separated cellular and extracellular proteins of sugarbeet cell lines fed with different carbohydrates. Different types of glycoproteins have been revealed by lectin hybridization.

Material and methods

Sugar beet cell lines were grown in axenic and controlled conditions in the liquid PG (Negrutiu *et al.*, 1975) nutrient medium supplied with 88 mM sucrose, glucose or 3-O-methylglucose (3-OMG). Sucrose served as a control. After three days, cellular proteins were extracted and extracellular proteins were collected from the liquid medium. Equal amounts of proteins per each sample were separated electrophoretically in 12% SDS polyacrylamide gels and silver stained. Glycoproteins with different glycan components were revealed by cross-reaction with various lectins after protein electrotransfer onto a nitrocellulose membrane.

Results and discussion

Extracellular proteins of three days old sugar beet cell suspensions have been electrophoretically separated and silver stained (Figure 1A). Application of glucose and 3-OMG provoked upregulation of 66, 39 and 26 kDa protein in the N line. In the HO line glucose upregulated proteins of 69 and 36 kDa and 3-OMG the ones of 38 and 34 kDa in the HNO line. Differences in glycoprotein pattern were also visible (Figure 1B): in the N line where the 63 kDa glycoprotein was upregulated, by 3-OMG in the HO a 23 kDa glycoprotein was missing. In the HNO line no differences in glycoprotein pattern were observed. Under the influence of carbohydrates cellular protein profiles showed less differences (Figure 1C) than the profiles of glycoproteins (Figure 1D), where a 100 kDa glycoprotein was missing in the 3-OMG fed N cells. In the HNO cells a 67 kDa glycoprotein was upregulated by 3-OMG while glycoproteins of 45, 22, 21, 18, 16, 15, 14 and 13 kDa were downregulated. In the HO line, no differences in glycoprotein pattern were observed.

The protein expression profiles depended on developmental state of sugar beet cells. Upon addition of glucose or 3-O-methylglucose, each cell line responded by changing protein expression in a specific manner. Differences in response were also visible on glycoprotein pattern. Similar differences in extracellular protein expression patterns between cell lines were reported also in cactus

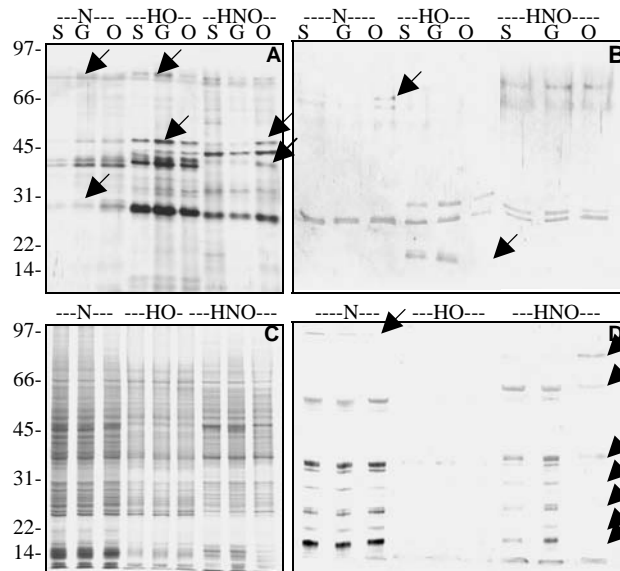


Figure 1: The effect of carbohydrates on expression profiles of sugar beet proteins. Proteins were extracted 3 days after subculturing and separated in 12% SDS polyacrylamide gels. (A) Silver stained extracellular proteins, (B) Nitrocellulose membrane with mannose-containing glycoproteins detected with the GNA lectine, (C) Silver stained cellular proteins, (D) Nitrocellulose membrane with mannose-containing glycoproteins detected with the GNA lectine. N – normal, HO – habituated organogenic, HNO habituated nonorganogenic cell line. G – glucose, S – sucrose, O – 3-O-methylglucose.

Mammillaria gracillis (Balen *et al.*, 2002). The glycosylation profile of proteins can be influenced by plant development and growth conditions (Stevens *et al.*, 2000). The structure of extracellular N-linked glycans varies with different developmental stages and organisation level (Elbers *et al.*, 2001). This confirms our results that suggest that developmental state of the cell affects not only protein expression but also response to carbohydrates. Identification of carbohydrate-induced proteins should be the next step that could provide insights in their specific function.

References

- Balen B., Milošević J. and Krsnik-Rasol M. (2002) *Food Technology and Biotechnology* 40 (4), 275-280.
- Causevic A., Delaunay A., Ounnar S., Righezza M., Delmotte F., Brignolas F., Hagège D. and Maury S. (2005) *Plant Physiology and Biochemistry* 43 (7), 681-691.
- De Greef W. and Jacobs M. (1979) *Plant Science Letters* 17, 55.
- Elbers I. J. W., Stoopan G. M., Bakker H., Stevens L. H., Bardorff M., Molthoff J. W., Jordi W. J. R. M., Bosch D. and Lommen A. (2001) *Plant Physiology* 126, 1314-1322.
- Negrutiu I., Beeftinck, F. and Jacobs, M. (1975) *Plant Science Letters* 5, 293-304.
- Stevens L. H., Stoopan G. M., Elbers I. J. W., Molthoff J. W., Bakker H. A. C., Lommen A., Bosch D. and Jordi W. (2000) *Plant Physiology* 124, 173-182.
- Weber H., Borisjuk L.J. and Wobus U. (1996) *The Plant Journal* 10 (5), 823-834.

Gibberellin biosynthesis in developing fruit of the Christmas rose (*Helleborus niger* L.)

V. Magnus^{1*}, B. Ayele², S. Mihaljević¹, D. M. Reinecke², J. A. Ozga² and B. Salopek-Sondi¹

¹Department of Molecular Biology, Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia, ²Department of Agricultural, Food and Nutritional Science, University of Alberta, T6G 2P5, Canada

*corresponding author (magnus@irb.hr)

Introduction

The perianth of the Christmas rose (*Helleborus niger* L.), which is white or rose at anthesis, turns increasingly green as the seeds start developing. This process stops when the fruit are removed, but can be reinitiated by treatment with cytokinins and gibberellins (Salopek-Sondi et al. 2002). We have already shown that endogenous cytokinin levels in the fruit rise enormously, during early seed development and then remain nearly constant throughout the maturation phase (Tarkowski et al. 2006), suggesting a role for cytokinins in the induction and maintenance of the photosynthetic apparatus in the perianth. Here we address the question of whether the endogenous gibberellins show similar concentration dynamics.

Materials and methods

Carpels of (proterogynous) flowers in their female and male phases (0.17 and 0.25 g FW) and three stages of developing fruit (0.52, 0.77 and 2.95 g FW) were collected in the 'Gorski kotar' region of Croatia. The freeze-dried plant material was extracted with 80% aqueous methanol containing butylated hydroxytoluene as an antioxidant and defined amounts of [²H₂]GA₁, [²H₂]GA₃, [²H₂]GA₄ and [²H₂]GA₇ as internal standards. Non-gibberellin plant constituents were removed by a series of purification steps including solvent partition, solid-phase extraction, and high-performance liquid chromatography (HPLC). For separation and identification, the gibberellins were converted to volatile methyl ester trimethylsilyl ethers and subjected to gas chromatography-mass spectrometry-selected ion monitoring. The criteria for identification were (1) the retention times relative to a series of alkane standards (Kováts retention indices), as observed in the final gas chromatographic step, and (2) the presence of the respective molecular ions and two (per GA) characteristic fragment ions, as revealed by selected ion monitoring. Quantification was accomplished by comparing the peak areas in ion chromatograms for the molecular ions of the endogenous gibberellins (*i. e.* their volatile derivatives) and the corresponding deuterated internal standards.

To obtain *H. niger* GA20ox cDNA, RNA was extracted according to Southerton et al. (1998) from different tissues of Christmas rose flowers, followed by amplification by RT-PCR using SuperScript™ II RNase H⁻ Reverse Transcriptase and Taq Polymerase Platinum (Invitrogen) according to the manufacturer's protocols. Degenerate primers for putative GA 20-oxidase were designed at highly conserved motifs, based on nucleotide sequences available in the NCBI database. The RT-PCR products were gel-purified and ligated into the pGEM-T-Easy vector (Promega). Sequencing of the cDNA clones was performed at the DNA sequencing facility at the Ruđer Bošković Institute.

Results and discussion

To gain first insight into gibberellin physiology, in the Christmas rose, we screened pistils and developing fruit for GA₁, GA₃, GA₄, and GA₇, which are the major bioactive GAs in other plant species (Sponsel and Hedden 2004). All four gibberellins were detected, but with widely different concentration dynamics (Table 1). Their *total quantities* per flower (*i.e.* per fruit cluster) increased about ten-fold, from late anthesis to the most advanced stages of fruit development examined. While GA₃ and GA₇ remained minor gibberellins throughout development, GA₄ started to accumulate markedly during initial perianth greening; GA₁ accumulation

followed when perianth greening entered the stationary phase. It is thus tempting to infer that a fraction of the gibberellins produced in the fruit is exported to the sepals to participate in the induction and maintenance of the photosynthetic apparatus. Gibberellins also stimulated chlorophyll formation in other experimental systems, as first noted for regreening orange fruit (Coggins et al. 1960).

Table 1: GA₁, GA₃, GA₄ and GA₇ in pistils and developing fruit of *Helleborus niger*.

Developmental stage	GA ₁		GA ₃		GA ₄		GA ₇	
	ng/gFW	ng/flower	ng/gFW	ng/flower	ng/gFW	ng/flower	ng/gFW	ng/flower
Anthesis, female phase	0.5	0.1	0.4	0.1	40.3	6.8	0.8	0.1
Anthesis, male phase	17.4	4.3	1.4	0.3	21.7	5.3	0.4	0.1
Initial perianth greening	0.4	0.2	0.5	0.2	34.5	16.2	0.7	0.3
Perianth greening complete	7.4	5.9	0.7	0.5	24.1	19.0	0.4	0.3
2 weeks before seed ripening	18.2	48.0	0.4	1.0	24.6	64.8	0.1	0.3

The concentration of GA₁ had a sharp concentration maximum during the male phase of anthesis and, after a minimum during initial perianth greening, increased again towards the end of seed ripening. The GA₄ concentration peaked during the female phase of anthesis, passed through a second, less explicit, maximum during initial perianth greening, to level off towards seed ripening. A similar pattern was, for instance, observed in orange ovaries, in which GA₄ levels were high before bud opening and during anthesis to decline after petal drop, while GA₁ passed through a maximum at anthesis (Talon et al. 1992). The difference between the two plant systems is: orange flowers self-pollinate before bud opening – in *Helleborus* flowers, the pistils are receptive (female phase) before the anthers shed their pollen (male phase). Thus, the period 'before bud opening' in orange, with its GA₄-maximum, corresponds to the 'female phase' in *Helleborus*. The period of 'anthesis' in orange flowers, when GA₁ levels peak, thus follows pollination, as does the 'male phase' in Christmas rose flowers. During advanced seed development, GA-levels declined rapidly in commonly studied species (Pharis and King 1985). They remained high in *Helleborus*, apparently due to the fact that embryo development is extremely slow, barely reaching the early cotyledonary stage when the seed is released from the ripe fruit.

To explore the possible role of tissue-specific GA biosynthesis in seed development and perianth greening, a partial GA 20-oxidase cDNA clone was isolated from developing *Helleborus* seeds by RT-PCR using a pair of degenerate primers. Sequence analysis identified a clone designated *HnGA20ox* (ca. 640-bp long) as a partial sequence of a GA 20-oxidase encoding a 212 amino acid polypeptide that is closely related to highly conserved regions of GA 20-oxidases previously isolated from *Citrus cv.* (accession no. AJ250187, 78 % identity), potato (accession no. AJ291453, 76 % identity), *Populus sp.* (accession no. AJ001326, 74 % identity), bean (accession no. U70530, 74 % identity), pumpkin (accession no. 308480, 73 % identity) and pea (accession no. PSU58830, 69 % identity).

References

- Coggins C.V. jr., Hield H.Z., Garber M.J. (1960). *Journal of the American Society of Horticultural Science* 76, 193-198
- Pharis R.P. and King R.W. (1985) *Annual Review of Plant Physiology* 36, 517-568.
- Salopek-Sondi B., Kovač M., Prebeg T. and Magnus V. (2002). *Journal of Experimental Botany* 53, 1949-1957.
- Southerton S.G., Marshall H., Mouradov A., Teasdale R.D. (1998). *Plant Physiology* 118, 365-372.
- Sponsel V. and Hedden P. (2004) In: Davies P.J. (Ed.) *Plant hormones. Biosynthesis, signal transduction, action*. Kluwer, Dordrecht: 63-94.
- Talon M., Zacarias L., Primo-Millo E. (1992) *Plant Physiology* 99, 1575-1581.
- Tarkowski P., Tarkowska D., Novák O., Mihaljević S., Magnus V., Strnad M. and Salopek-Sondi B. (2006). *Journal of Experimental Botany* in press.

Cytokinin levels in developing maize caryopsis

T. Rijavec¹, M. Kovač², M. Dermastia^{1*}

¹Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1001 Ljubljana, Slovenia, ²National Institute of Biology, Večna pot 111, SI-1001 Ljubljana, Slovenia

*corresponding author (marina.dermastia@bf.uni-lj.si)

Introduction

Cytokinins, derivatives of adenine, are an important group of plant hormones. They affect the progression of the plant cell cycle and promote cell division. They are involved in lateral bud development, leaf expansion, chloroplast development and stomatal opening. They also delay leaf senescence. In combination with auxins they affect morphogenesis in cell cultures (Davies, 2004) Also important, but not well characterized, is their role in maize kernel development. Because maize is an important crop, a deeper understanding of kernel developmental processes is required.

In the current study, we tried to assess the cytokinin levels in developing kernels of the maize cultivar W22. We compared the normally developed wild type (*Mn1*) maize kernels to abnormally developed mutant type (*miniature1*; *mn1*) kernels twelve days after pollination (12 DAP). The *mn1* seed mutation is one of the most drastic non-lethal single gene seed mutations in maize. The *mn1* seeds are deficient for cell wall invertase2, which is specific for developing endosperm (Carlson, 2000). Histological studies show that the endosperm development of *mn1* kernels is normal up-to nine DAP, but it is almost arrested at ~ 14 DAP, which resulted in loss of seed weight of nearly 80 % (Chourey, 2006). Recently we have shown the seed weight loss is associated with reduced mitotic activity and inhibited cell expansion (Vilhar et al., 2002). We assumed that the morphological difference between the wild type and mutant kernels in maize could also be detected on the cytokinin level.

Materials and methods

Wild type (*Mn1*) and mutant (*mn1*) kernels of cultivar W22 were collected twelve days after pollination and immediately lyophilized. The kernels were dissected into upper (endospermal) and lower (pedicelar) part. Cytokinins were extracted from each kernel part with 80 % cold methanol. The extracts were cleaned with polyvinylpyrrolidone (PVPP) and isoprenoid cytokinin immunocolumns (OlchemIm, Czech Republic). The identity of the extracted cytokinins was determined by retention time and characteristic absorption spectra using high performance liquid chromatography (HPLC) and photodiode array detector (Salopek-Sondi et al. 2002).

Results and discussion

The overall concentration of the most abundant cytokinins detected in kernels, zeatin and zeatin riboside, was four times higher in *mn1* kernels in comparison with the wild type at 12 DAP. Regardless of the genotype, the identified cytokinins were zeatin-9-glucoside, zeatin, zeatin riboside, dihydrozeatin, and dihydrozeatin riboside. Additionally, traces of isopentenyl adenosine were also detected. In both genotypes the cytokinin concentrations were higher in the pedicelar part of the kernels than in endospermal part. The exception was zeatin riboside with the similar level in upper and lower part of the kernels. While the obtained concentrations of the detected cytokinins were similar in different isolation procedure from the wild type kernels, they were more variable in *mn1* seeds, presumably due to their less uniform development.

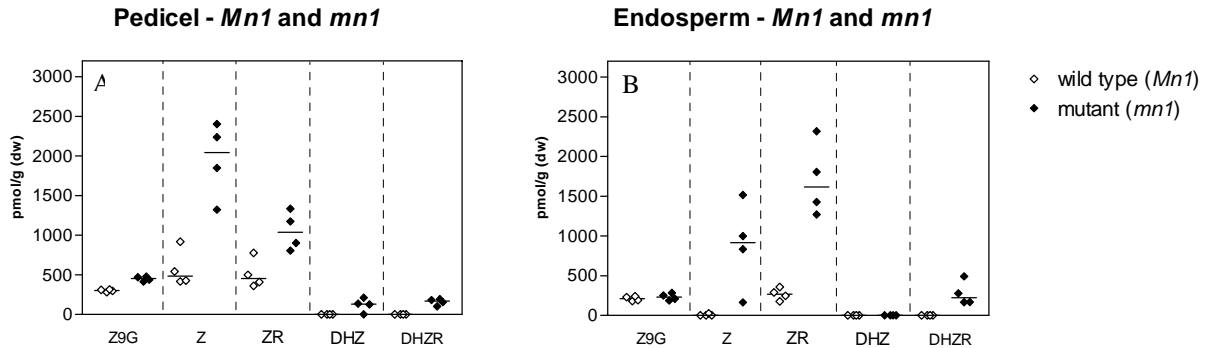


Figure 1: Cytokinin content in wild type (*Mn1*) and mutant (*mn1*) kernels. Comparison between both types in the pedicel part (A) and the endospermic part (B). Mutant (*mn1*) genotype shows higher amounts of all cytokinin species in both parts of the kernel. zeatin-9-glucosyde (Z9G); zeatin (Z); zeatin riboside (ZR); dihydrozeatin (DHZ); dihydrozeatin (DHZR).

Acknowledgements

This work was supported by the Slovenian Research Agency.

References

- Chourey P. S., Jain M., Li Q. B. (2006). *Planta* 223, 159-167.
- Carlson S. J., Shanker S., Chourey P. S. (2000). *Molecular and General Genetics* 263, 367-373.
- Davies P. J. (2004). The plant hormones: Their nature, occurrence and function. In: Davies P. J. (Ed.) *Plant hormones: Biosynthesis, signal transduction, action!* Kluwer, Dordrecht, Boston, London, 1-15
- Salopek-Sondi B, Kovač M., Preberg T., Magnus V. (2002). *Journal of Experimental Botany* 53, 1949-1957.
- Vilhar B., Kladnik A., Blejec A., Chourey P. S., Dermastia M. (2002) Cytometrical evidence that the loss of seed weight in the *miniature1* seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. *Plant Physiology*, 129: 23-30