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Fotosyntetické charakteristiky ve šlechtění rostlin: cíle, možnosti a omezení

Photosynthetic characteristics in plant breeding: targets, options and limitations

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Introduction

The utilisation of photosynthesis in plant breeding: motivation, targets, options and limitations

During the next 35 years, human population is expected to increase by about 35% from its current status. This means that a proportionally greater food supply will have to be provided for all these people. Moreover, the desire for a better-quality food together with the shift to the consummation of meat and milk products which require much greater primary crop production will even more intensify demands for agriculture (Hall and Richards 2013, McKersie 2015). The current estimates of the required increase in crop production range from 185 to 220% relative to that in 2013 (Ray *et al.* 2013, Long *et al.* 2015, Ort *et al.* 2015, Kromdijk and Long 2016). At the same time, global climate changes that are occurring even now and that are expected to be more prominent in the near future will undoubtedly negatively affect crop yields in most regions. Problems caused by water shortage (which is currently the most limiting factor for agricultural production) will be even more amplified by the predicted global temperature increase and changes in rainfall patterns. Elevated temperatures will probably also lead to an increased amount of biological pests and will support growth of more weeds. Enhanced salinisation and soil erosion is expected to result from more frequent occurrence of extreme weather conditions (McKersie 2015). Thus, the challenges we are facing are substantial and the prediction of Kromdijk and Long (2016) that we are "one crop breeding cycle from starvation" has to be taken quite seriously.

How to deal with this demand for increased agricultural crop production under conditions of climate change? Further expansion of cultivated land area (*e.g.*, by deforestation in tropical regions) is strongly advised against as it not only contributes to greenhouse gas emissions but significantly decreases Earth biodiversity (and, besides, cannot be infinite). New agricultural technologies and management practices that enable improved water conservation, the avoidance of stress periods by manipulation of plant sowing/harvest dates, the use of the same cultivation area for double cropping, *etc*., can be employed; however, their long-term effectivity is doubtful (McKersie 2015). The most promising possibility for the necessary enhancement of crop yields is the development of new cultivars with increased yield potential as well as improved yield stability across various environments. The so-called Green Revolution, which begun in the 1950s and continued (more slowly) almost to the end of the 20th century, resulted in more than doubling grain yields of wheat and rice. This was achieved mostly by breeding for short plant stature, leaf erectness, an improved seed size/set and some phenological changes (Xu and Shen 2002, de Ribou *et al.* 2013, McKersie 2015). However, this success equally depended on the high input of nitrogen in fertilizers and sufficient irrigation, had the most impact in Latin America and Asia but almost none in Africa, and did not pertain to other crops (Pingali 2012, McKersie 2015). What is more important, such spectacular improvement is at its end. The average global relative rates of the yield increase for the four major crop species (wheat, rice, maize and soybean) are currently calculated to be 0.9% (wheat), 1.0% (rice), 1.3% (soybean) and 1.6% (maize) – but to achieve the yields predicted to be necessary for the year 2050, they would have to be more than doubled. Unfortunately, recent data clearly show that yields of these four topmost crops have already reached their plateaus in most agricultural regions worldwide (Hall and Richards 2013, Ray *et al.* 2013, Long *et al.* 2015). In order to further increase yield potentials as required, the breeding objectives will thus have to be completely reassessed.

Yield potential is defined as "yield (mass of the harvested material per the unit of land area) that the respective crop can attain under optimum management practices (when grown in environments to which it is adapted) and in the absence of biotic and abiotic stresses" (Long *et al.* 2006, 2015, de Ribou *et al.* 2013). It is usually described using the following formula:

$$Y_p = 0.487 \times S_t \times \varepsilon_i \times \varepsilon_c \times \varepsilon_p$$

S_t (sometimes designated as Q) is the total incident solar radiation received during the duration of growing season per the unit of land area (the coefficient 0.487 represents limitation of this total radiation to photosynthetically active radiation). ε_i is the efficiency of the interception of this radiation by the respective crop (this depends on various factors including the speed with which the canopy develops and closes, the canopy size, architecture, length of canopy duration and the absorbance of leaves). ε_c is the efficiency of the conversion of intercepted radiation energy into

biomass (*i.e.*, the combined photosynthetic efficiency of all leaves within the canopy minus plant respiratory losses). Finally, ε_p is the proportion of plant biomass (the amount of total biomass energy) partitioned into the harvested product (also called the partitioning efficiency or the harvest index) (Long *et al.* 2006, 2015, Zhu *et al.* 2010, de Ribou *et al.* 2013). S_t parameter cannot be greatly changed by human efforts and both ε_p and ε_i have most probably achieved their biological limits and cannot be further substantially improved (Long *et al.* 2006, 2015, Kromdijk and Long 2016). However, the remaining component of yield potential, *i.e.*, the photosynthetic efficiency, has been mostly overlooked by most breeders in the past and is currently at about one third of its potential value (Zhu *et al.* 2008, 2010, Long *et al.* 2015, Yin and Struik 2015). Clearly, there are some serious gaps in this component of yield potential that need to be filled in case we want to further improve crop yields. With the large amount of data currently available from the experiments made with plants subjected to elevated CO_2 concentration (FACE studies), the evidence that photosynthesis can indeed be improved and that an increase in yield accompanies this improvement is now very compelling (Long *et al.* 2006, Kromdijk and Long 2016).

The omission of targeted breeding for greater ε_c until now has probably been caused by several factors. First, plant breeders and scientists have long believed (and were supported in this view by various studies) that there is no positive relationship between leaf photosynthetic rates and plant productivity (Evans 1993, 1998). Scientists are now realising that this negative correlation or the absence of any correlation is in fact false due to *i)* the inverse association of the photosynthetic rate on the leaf level and the total leaf area in the germplasm used for such screening (this would counterbalance any advantage the higher photosynthetic rate could bring to crop production on the canopy level); *ii)* the fact that the photosynthetic measurements were (and still mostly are) made in definite time points and on selected leaves under light-saturated conditions instead of across a whole plant, under natural light conditions and during a whole growing/yield producing period (Xu and Shen 2002, Long *et al.* 2015). Actually, many studies demonstrating a positive relationship between various photosynthetic traits and yield do indeed exist (Xu and Shen 2002, Driever *et al.* 2014). Second, most plant breeders reasoned thus: provided that photosynthesis plays a key role in the determination of yield potential, we should see a significant improvement in photosynthetic efficiency accompanying the development of high-yielding cultivars – but no such improvement has been originally observed. However, the increase of yield potential after the successes of the Green Revolution was achieved by increases in ε_p and ε_i which means that the ε_c component of the equation was left almost untouched and the above-stated presumption thus does not apply (Kromdijk and Long 2016). Indeed, it seems that breeding programmes for wheat have unintentionally selected for cultivars with low photosynthetic capacities, again mostly for reasons related to targeted selection for parameters associated with greater ε_p and ε_i (Driever *et al.* 2014). Recent studies that compared original and modern germplasm of soybean across the 20th century have demonstrated that yield gains have indeed been accompanied by some improvements in the photosynthetic energy conversion into biomass (Koester *et al.* 2014, 2016). Third, there is an ongoing debate whether the source (*i.e.*, photosynthesis) or the sink (*i.e.*, the development of harvestable organs) is in fact *the* limiting factor for plant production; evidence for one or the other has been presented by various scientists. Fortunately, we are coming to a more reasonable view that *both* source and sink are important, thus, concentrating only on the sink and completely leave out the source in order to achieve improved crop yields is not acceptable any more (Richards 2000, Long *et al.* 2006, Evans 2013, Kromdijk and Long 2016). Thus, in order to meet the demands for increased crop production, the photosynthetic efficiency of plants as a means of yield increase should be seriously targeted by plant breeders.

How can breeding for greater photosynthetic efficiency be incorporated into the current and future breeding programmes? What do we have to be careful of in order to achieve success? How to best utilise the instruments for measurements of photosynthetic characteristics that are currently available? Which new technologies should be further developed? Which parts of photosynthesis and the associated metabolic pathways and structures should we concentrate on? Several excellent reviews recently summarised main options (as well as limitations) for the improvement of photosynthesis with a view to increase crop yields (Zhu *et al.* 2010, Parry *et al.* 2011, Singh *et al.* 2014, Lawson *et al.* 2012, Evans 2013, Carmo-Silva *et al.* 2015, Furbank *et al.* 2015, Long *et al.* 2015, Ort *et al.* 2015, Erb and Zarzycki 2016, Flexas 2016, Kromdijk and Long 2016, Nunes-Nesi *et al.* 2016, Schuler *et al.* 2016, von Caemmerer and Furbank 2016, Yamori *et al.* 2016). Five major objectives that plant breeders should focus on (according to these authors) are *i)* the

improvement of plant CO_2 uptake/concentrating mechanisms; *ii)* the improvement of photosynthetic carbon conversion; *iii)* the optimisation of photosynthetic light harvesting systems and the chloroplast electron transport; *iv)* the modification of plant canopy architecture and *v)* the increase in the export of carbohydrates to sink organs. Two main approaches to accomplish these objectives are possible: targeted genetic manipulation in order to either improve the existing photosynthetic mechanisms or to introduce completely new ones, and utilisation of natural genetic variability in specific photosynthetic traits for selection/breeding of genotypes with high photosynthetic efficiency under required environmental conditions.

Plant CO_2 uptake and difussion in leaves depends mainly on stomatal (g_s) and mesophyll (g_m) conductances. Natural genetic variability for g_s does exist in various crop species and positive relationships between g_s and the CO_2 assimilation rate, as well as between g_s and yield, have been demonstrated (Nunes-Nesi *et al.* 2016). However, these relationships change with some stress conditions (high temperature, the interaction with other plants, *etc.*) and the targeted manipulation of g_s, *e.g.*, by an increase in stomatal aperture is usually associated with greater water loss, throwing serious constraints on the potential improvement of photosynthesis under future climatic conditions by the manipulation of g_s (Flexas 2016). On the other hand, g_m appears to be a much better target for plant breeders (particularly in C3 plants; in C4 crops such as maize, sorghum or sugarcane, light-saturated photosynthesis under optimum conditions is not constrained by CO_2 availability, thus, improvement of g_m would not be of great help) (Zhu *et al.* 2010, Flexas 2016, Yamori *et al.* 2016). Unfortunately, the current methods for g_m measurements suffer from some interpretation problems and our knowledge on the regulation of g_m by leaf anatomical properties (mesophyll structure, cell wall thickness, distribution of chloroplasts in cells) and biochemical processes (carbonic anhydrases, aquaporins as CO_2 channels, relationship with photorespiration and respiration) is still very limited (Sharkey 2012, Flexas *et al.* 2012, 2013, Evans 2013, Flexas 2016). Carbonic anhydrases are necessary for the conversion of HCO_3^- (*i.e.*, the form in which CO_2 is transported into chloroplasts) and the maintenance of high CO_2 availability at the sites of photosynthetic carbon fixation (*i.e.*, near the ribulose-1-5-bisphosphate carboxylase/oxygenase – Rubisco – enzyme). Their concentration in chloroplast stroma in leaves of current crop germplasm is well below that which would ensure the maximum carbon flux. Thus, some possibilities for improvement by changes in the amounts of these enzymes exist; however, no definite results have yet been obtained (Singh *et al.* 2014). Regarding aquaporins, although the enhancement of photosynthesis and increased g_m have been observed in transgenic plants with genetically manipulated aquaporins, this phenomenon was most likely caused by pleiotropic effects and was generally associated with a decreased water use efficiency, which is not a very desirable trait (Evans 2013, Singh *et al.* 2014, Flexas 2016).

An interesting option how to increase CO_2 concentration at the site of Rubisco and thus improve the photosynthetic carbon fixation efficiency of C3 plants is the utilisation of carbon concentrating mechanisms totally foreign to this plant type. Cyanobacterial and algal systems have been proposed as good candidates for such manipulations and their introduction into crop plants is one of the main objectives of the international RIPE (Realizing Increased Photosynthetic Efficiency) consortium (*http://ripe.illinois.edu/*). However, the attempts to create transgenic tobacco plants expressing such cyanobacterial bicarbonate pumps have not yet resulted in any significant enhancement of photosynthesis (Pengelly *et al.* 2014). The IctB protein, the original cyanobacterial candidate for a bicarbonate transporter, the expression of which led to very promising results regarding the increase in photosynthesis in soybean, tobacco and *Arabidopsis* (Singh *et al.* 2014), has since been discovered to have in fact nothing to do with the transport of bicarbonates (Price *et al.* 2013). It is also probable that in order to achieve good results of such genetic manipulations, Rubisco would have to be retargeted into specialised chloroplast structures such as carboxysomes or pyrenoids (associated with CO_2-concentrating mechanisms in cyanobacteria and algae, respectively) and that the genes necessary for formation of these structures, as well as those coding for various regulatory proteins, would also have to be successfully introduced into crop plants. Some work in this direction is now being conducted on plant models but the physiological assessment of such transgenics is still missing and the way from plant models to crops is very long (McGrath and Long 2014, Furbank *et al.* 2015, Erb and Zarzycki 2016, Rolland *et al.* 2016, Yamori *et al.* 2016).

By far the greatest effort (and considerable time) on the enhancement of the efficiency of CO_2-concentrating mechanisms has been spent on the possible introduction of another naturally

evolved carbon concentrating system, *i.e.*, C4 photosynthesis, into C3 crop plants (with a particular emphasis on rice, see *http://c4rice.irri.org/* and *https://c4rice.com/*). Most C4 plants (although not all) are characterised by the existence of two distinct types of photosynthetic cells (mesophyll and bundle sheath) with functionally different chloroplasts ("Kranz" anatomy), and they utilise the phospho*enol*pyruvate carboxylase (PEPC) as the primary CO_2-fixing enzyme. Production of four-carbon compound in mesophyll cells and its transport into bundle sheath cells, where it is decarboxylated and CO_2 released in the vicinity of Rubisco, ensures greatly improved efficiency of photosynthesis compared to the C3 mechanism. Although an additional energy is necessary for C4 metabolism, the elimination of energy loss by photorespiration is a great advantage particularly under high-light, high-temperature conditions. Consequently, engineering of the C4 mechanism into C3 crops should substantially increase the ε_c component of yield potential (especially in a tropical species such as rice). Moreover, it should be also accompanied by a significant positive impact on the nitrogen- and water-use efficiency. Unfortunately, both biochemical and anatomical aspects of C4 photosynthesis are very complex. One obstacle against the introduction of the C4 machinery into C3 plants is a much greater distance between bundle sheath and mesophyll cells in C3 species. Another one is a considerably smaller number and volume of chloroplasts in C3 bundle sheath cells. The third challenge for C4→C3 manipulation is an almost complete absence of any knowledge on regulatory elements and mechanisms necessary to ensure cell-specific gene expression, and the fourth major problem is associated with a need to introduce a rather large number of genes together (complicated even more by the existence of gene copies). However, the main C4 enzymes are already present in C3 plants, although they fulfill other functions there, and various C3-C4 intermediate plants do exist in the nature, so it *should be* possible to make such transition artificially. The goal of increasing photosynthetic rates in C3 plants either by te direct gene manipulation or by the utilisation of selection for C4-type traits (*e.g.*, pre-Kranz anatomy) in breeding programmes seems to be worth the effort in the opinion of most scientists working in this field (Long *et al.* 2006, von Caemmerer *et al.* 2012, Furbank *et al.* 2015, Schuler *et al.* 2016).

The C4 photosynthetic metabolism *per se* could possibly be also improved in order to enhance photosynthesis in existing C4 crops. The affinity of PEPC for CO_2/HCO_3^- and its catalytic efficiency is regulated by its phosphorylation as well as by metabolite levels. Some potential changes here could lead to positive results at least under stress conditions (although it currently seems unlikely). Regeneration of phospho*enol*pyruvate also limits the photosynthetic rate in C4 plants, and the main enzyme participating in this process, *i.e.*, the pyruvate,*ortho*phosphate dikinase, could perhaps be genetically engineered to become more effective (von Caemmerer and Furbank 2016).

The Rubisco enzyme in its present form that occurs in most plants is one of the major impediments for more efficient photosynthesis due to two main reasons. Rubisco functions both as a carboxylase and an oxygenase, leading to a great loss of assimilated carbon, considerable consumption of energetically rich compounds and an ammonia release in the photorespiration cycle. Moreover, its catalytic rate for carboxylation reaction is remarkably slow, resulting in large demands for its amounts. To increase the Rubisco content in plants even more could theoretically increase photosynthesis but it would demand utilisation of great amounts of nitrogen for its synthesis. Nitrogen would have to be supplied in fertilisers, so it is clearly not a good way to improve photosynthetic efficiency. To increase the Rubisco affinity for CO_2, thus improving its catalytic properties (and, incidentally, decrease nitrogen requirement as a reduced amount of enzyme would be sufficient), would be better. Various efforts have been put into overcoming this challenge. Although the crystal structure of Rubisco is very well-resolved and the catalytic mechanism of this enzyme is thoroughly understood, all targeted genetic approaches to manipulate Rubisco catalytic properties up to now have failed (indeed, a less-efficient enzyme resulted from these attempts), maybe due to their focus on the Rubisco large subunit. Replacing Rubisco in some crop by Rubisco with a higher catalytic rate from another species (*e.g.*, C4 plants, plants adapted to dry environments, carnivorous plants, evergreen species, algae or even bacteria or archaea with completely different Rubisco) is another possibility. Variations in the affinity of Rubisco to CO_2 and Rubisco catalytic properties exist in the nature and could be utilised in plant breeding (in case of related species) or genetic manipulations. However, the current eukaryotic Rubisco holoenzyme has a rather complex structure of 8 large and 8 small subunits, which are coded for in different cell compartments, thus necessitating the proper coordination of their export and assembly as well as various posttranslational modifications. Furthemore, genes for the small subunit comprise a

multigene family, which means that their silencing (that should accompany the introduction of a new type of Rubisco) would be rather difficult (Zhu *et al.* 2010, Singh *et al.* 2014, Carmo-Silva *et al.* 2015, Furbank *et al.* 2015, Ort *et al.* 2015, Erb and Zarzycki 2016, Yamori *et al.* 2016).

To function properly, Rubisco has to be activated by another specialised protein, the Rubisco activase. A good correlation between the amount of this catalytic chaperone and crop yield was observed. Moreover, the Rubisco activase also seems to regulate the amounts of Photosystem (PS) II proteins or the assembly of this complex and chloroplast grana formation, which affects the efficiency of the photosynthetic electron transport, its function under low-light conditions and photoprotection against high light. However, the Rubisco activase is rather labile at high temperatures, greatly limiting the potential photosynthetic capacity of plants under such conditions. Efforts toward engineering or selecting for greater thermostability of this protein (or its reduced ability to be degraded, *e.g.*, by stabilising its chaperones) should be also made (Singh *et al.* 2014, Carmo-Silva *et al.* 2015, Yamori *et al.* 2016).

Prevention or at least significant diminishion of the photorespiration process is also a major goal of scientists seeking to improve plant photosynthetic efficiency. In addition to steering the Rubisco enzyme in favour of more carboxylation and less oxygenation, and the introduction of the C4 metabolism into C3 plants, additional possibilities have been proposed to solve this problem by completely bypassing the Rubisco part of this process. This could be performed using genetic modifications that would enable various alternative metabolic pathways called by Erb and Zarzycki (2016) the chloroplastic glycerate bypass, the chloroplastic glycolate oxidation bypass, the peroxisomal glycerate bypass and the 3-hydroxypropionate bypass. Some work on these bypasses has been performed in model transgenic plants but scientists are still far from success (Singh *et al.* 2014, Furbank *et al.* 2015, Long *et al.* 2015, Ort *et al.* 2015, Xin *et al.* 2015, Erb and Zarzycki 2016, Yamori *et al.* 2016). Even more wild proposition aims to design a whole completely new CO_2-fixing pathway that would not utilise Rubisco at all, but this is still more of a theoretical approach then a practically tried alternative (Bar-Even *et al.* 2010, 2012, Erb and Zarzycki 2016).

While the photosynthetic rate under low CO_2 concentrations is limited primarily by the Rubisco carboxylation efficiency, this limiting factor has now ceased to be the most important one under CO_2 concentration conditions that currently exist on the Earth. It is being replaced in its limiting role by the efficiency of ribulose-1-5-bisphosphate (RuBP) regeneration. In the near future, this will be even more significant as a gradual increase of atmospheric CO_2 concentration will further continue. The RuBP regeneration rate is modulated by two main processes: the reactions catalysed by enzymes of the "regenerative" phase of the Calvin cycle and the electron transport in chloroplast thylakoid membranes. Changing the amounts of the sedoheptulose-1,7-bisphosphatase, the fructose-1,6-bisphosphatase, the chloroplast aldolase or the transketolase has been proposed as a way to achieve improvements in photosynthesis and, consequently, yield potential. Transgenic plants overexpressing genes for these enzymes have been already created not only for model species but also for crops (soybean, rice). However, the observed increases in photosynthesis were either none or only small, were detectable only under stress conditions or were probably caused by a pleiotropic effect associated with an increased Rubisco activation (Long *et al.* 2006, Singh *et al.* 2014, Furbank *et al.* 2015, Flexas 2016, Kromdijk and Long 2016, Yamori *et al.* 2016). Whether the natural variability in the amounts/activities of these enzymes could be used for traditional selection and breeding remains to be seen because its existence has not yet been examined.

The rate of RuBP regeneration and the overall photosynthetic electron transport capacity is proposed to be strongly affected by the cytochrome b_6f complex that functions in the linear electron transport between PS II and PS I, is equally important for the cyclic electron transport around PS I and creates the protonmotive force necessary for ATP synthesis in chloroplasts. An increase in the amounts of the cytochrome b_6f complex would possibly be one way for improvement of the electron transport in thylakoid membranes and enhancement of photosynthetic efficiency of plants. However, because this complex is composed of both nuclear- and chloroplast-encoded subunits, gene manipulations necessary for ensuring a stable increase in the amounts of the fully functional complex are challenging and have as yet been mostly unsuccessful (Evans 2013, Kromdijk and Long 2016). On the other hand, the overexpression of plastocyanin (a protein that transfers electrons between the cytochrome b_6f complex and PS I) or its replacement by the cytochrome c_6 protein which fulfills the same role in algae led to increased photosynthesis and biomass production in *Arabidopsis* (Singh *et al.* 2014, Yamori *et al.* 2016). The thylakoid ATP synthase is another possible target for genetic manipulations vith a view to improve plant productivity and some work

on this has already been conducted (Kramer and Evans 2011, Evans 2013). By contrast, the proposal to replace the current plant PS I reaction centre with the reaction centre from purple photosynthetic bacteria, which would function together with the cytochrome b_6f complex to promote protonmotive force and thus ATP synthesis, and at the same time to replace chlorophyll *a* in the PS II reaction centre with chlorophyll *d* (enabling the electron transfer from the PS II quinones directly to the NADH dehydrogenase to support greater NADPH synthesis), thus completely redesigning the current electron transport chain in thylakoid membranes, remains completely in the field of wishful speculations (Ort *et al.* 2015).

The potential efficiency of the photosynthetic electron transport can be further reduced by various protective mechanisms for the dissipation of an excess excitation energy. However, when these protective mechanisms are insufficient, the damage to PS II and PS I proteins (as well as other chloroplast components), caused by the increased production of reactive oxygen species (ROS), occurs. During evolution, plants have created several mechanisms for the disposal of the portion of the captured light energy that cannot be utilised by the primary photosynthetic processes and for its dissipation as heat. Increasing the capacity of some of these photoprotective systems, *e.g.*, the xanthophyll cycle or the PsbS protein, has been suggested as a further means for improvement of photosynthesis. Recently, transgenic tobacco plants overexpressing both PsbS protein and the enzymes of the xanthophyll cycle have been shown to recover more rapidly from high light conditions (Kromdijk *et al.* 2016). Another possibility is the reduction of the size of light-harvesting antennae, thus ensuring that the imbalance between the energy capture and its utilisation in the photosynthetic electron transport would not be as large. This could be best achieved by the reduction in the chlorophyll content. Both approaches for the optimisation of plant photoprotection should be possible either by targeted gene engineering or by the utilisation of natural variability in these parameters (which can be very easily measured using rapid and non-destructive methods, see below) in physiological selection and breeding (Zhu *et al.* 2010, Ort *et al.* 2011, 2015, Furbank *et al.* 2015, Yin and Struik 2015, Nunes-Nesi *et al.* 2016). However, crop plants have to be able not only to cope with high-light conditions but also with low-light ones. The upper part of their canopy shades the lower leaves and the light environment during the day and even more during the vegetation season strongly fluctuates (*i.e.*, clear/cloudy days, sunflecks). This means that the ability of photosynthetic processes to rapidly shift from high-light to low-light conditions and *vice versa* is as much important as the ability to manage excess light. In this respect, the smaller antenna size in shaded leaves would bring disadvantages; thus, very fine tuning of photosynthetic energy capture and the ability to more rapidly relax the photoprotective mechanisms is necessary (Long *et al.* 2006, Zhu *et al.* 2010, Lawson *et al.* 2012, Evans 2013, Singh *et al.* 2014, Furbank *et al.* 2015, Ort *et al.* 2015). The potential usefulness for the improvement of photosynthesis and crop production using this approach has been clearly demonstrated by Kromdijk *et al.* (2016) in their transgenic tobacco plants. These plants were not only able to better recover from high light but also to better adapt to fluctuating light conditions, which resulted in the 14-20% increase in their biomass production compared with wild-type plants.

The „smart canopy“ concept goes even further. It suggests that plants should be selected/bred for or genetically manipulated in such a way that leaves on different canopy levels should have very different types of the photosynthetic apparatus. Vertically oriented leaves containing reduced chlorophyll amounts and Rubisco with a high catalytic rate of carboxylation should be on upper canopy levels. More horizontal leaves with a greater content of chlorophyll (or even some other types of photosynthetic pigments, such as chlorophylls *d* or *f*, which are able to absorb radiation in the near-infrared region) and Rubisco with greater specificity for CO_2 should be on lower canopy levels. This should facilitate significant improvement of canopy photosynthesis and overall yield potential. However, these different properties should not be rigid but should be able to change according to plant development (*i.e.*, gradual canopy closure) as well as according to specific environmental conditions which plants momentarily encounter (Long *et al.* 2006, 2015, Evans 2013, Singh *et al.* 2014, Furbank *et al.* 2015, Ort *et al.* 2015, Yin and Struik 2015, Yamori *et al.* 2016). Dynamic models taking into an account various biophysical, biochemical, anatomical and other properties of photosynthetic complexes, organelles and organs together with their response to environmental conditions, and incorporating cell, leaf and canopy levels, are necessary for the correct estimation of the effects such changes in individual properties would have in the context of final crop photosynthetic and yield production (Singh *et al.* 2014, Furbank *et al.* 2015, Ort *et al.* 2015).

The selection for the faster canopy closure (*i.e.*, the "early vigour" trait) and the delayed leaf senescence (*i.e.*, the "stay-green" trait) falls also in the category of canopy modifications aimed at the improvement of ε_c and has already led to significant increases of yield in several important crop species, *e.g.*, wheat or sorghum (Richards 2000, Thomas and Ougham 2014, Yin and Struik 2015).

Finally, photosynthesis as the source of carbohydrates and their accumulation in the sink organs (seeds, roots, stem) are closely interconnected. Reduced sink formation leads to a decrease in photosynthetic processes, increased utilisation of carbohydrates by the sink requires more efficient photosynthesis. Understandably, the transport of carbohydrates plays an important role in this relationship and the changes in the efficiency of phloem loading and sucrose transport could elevate plant photosynthetic rates. Some work with transgenic plants has been conducted in this field but significant improvements of photosynthetic CO_2 assimilation or yield has not been yet achieved (Singh *et al.* 2014, Flexas 2016, Yamori *et al.* 2016).

Various options for the enhancement of photosynthetic efficiency of crop plants are thus theoretically possible and could be introduced into breeding and selection programmes. Breeding process *per se* consists of three main steps. The first is to determine which trait(s) should be selected/manipulated/breeded for in order to achieve desired results. Regarding photosynthesis, the potential targets for improvement have been thoroughly discussed in previous paragraphs. As mentioned, gene/genome engineering (*i.e.*, the creation of transgenic crops) could be utilised to achieve the required changes in some of these target traits. However, in many cases this approach is likely to meet with some constraints. Many of them are caused by our still incomplete knowledge on various aspects of photosynthetic processes from the molecular to canopy level. Some purely technical limitations also exist. Fortunately, modern technologies of recombination-mediated gene engineering using Zn-finger nucleases, TALENs or CRISPR-Cas systems are now being rapidly introduced into plant GM technologies. They should enable directed insertions of mutated genes, thus overcoming problems of the positional effect and unpredictable random insertions associated with the more conventional transformation methods (Long *et al.* 2015, Barabaschi *et al.* 2016). However, the existence of homologous gene copies in most crop plants, as well as the imperfections in the accuracy of currently existing genome sequences and our insufficient knowledge on the details of various regulatory elements somewhat diminish the potential of these modern tools of plant genetic engineering. Moreover, plastid transformation (which would be necessary for many of the proposed genetic manipulations aimed at the enhancement of photosynthesis) is still not available for any major crop species. Most of the required changes in nuclear-encoded genes would involve transforming the whole gene complexes, which would neccessitate the utilisation of technologies that would enable the introduction of very large DNA fragments; unfortunately, such technologies are still missing (Long *et al.* 2015, Ort *et al.* 2015). The cost of the development of genetically engineered crops must be also carefully compared to the cost of gaining the required trait by some more conventional breeding method, otherwise their transfer into field would not be effective (Brennan and Martin 2007, Furbank *et al.* 2015). The rather rigid regulations for GM plants currently existing in many countries would have to be overcome, which unfortunately depends on general public opinion and politics.

An alternative approach to photosynthesis improvement is based on the utilisation of the natural variability in photosynthetic characteristics in breeding programmes based on slightly more traditional proceedings. Moreover, there is an additional advantage for including the selection for improved photosynthetic efficiency (on various levels) into breeding programmes under current conditions of changing climate and more stressful environments. The photosynthetic apparatus strongly and usually very rapidly responds to all abiotic and biotic stress factors (Ashraf and Haris 2013, Nishiyama and Murata 2014) and various photosynthetic traits have been suggested as potentially good markers of crop resistance to unfavourable external conditions such as drought (Monneveux *et al.* 2012, 2013), heat (Cossani and Reynolds 2012, Bita and Gerats 2013), cold (Crosatti *et al.* 2013), waterlogging (Herzog *et al.* 2016) or others. Thus, the evaluation of photosynthesis is now widely recommended as an important part of so-called physiological breeding which aims at the integration of physiological analyses made on various levels together with genetic/genomic analyses in order to develop crops with improved yield over a range of environments, *i.e.*, a better general adaptability (Ghanem *et al.* 2015, Reynolds and Landgridge 2016).

For any physiological trait to be worthwile of breeders´ attention, a good correlation to yield in target environment(s) as well as an adequate genetic variability in the evaluated population/genotype collection, together with a good heritability and repeatability are necessary criteria (Araus 2008). Thus, the second step of a breeding process is to assess the genetic variability in the targeted trait in order to select the best potential genetic resources (parents) for further breeding. Such natural intraspecific variability in photosynthesis can be found in currently available breeding germplasm of both major and minor crop species (including landraces and wild species related to crop plants that could be used for interspecific hybridisation; Reynolds *et al.* 2009, Reynolds and Landgridge 2016). It has been observed not only for the general photosynthetic capacity but also for some of its component traits: photosynthetic characteristics associated with the light reactions (particularly the photoprotective systems), the chlorophyll content, the kinetic properties and expression levels of some Calvin cycle enzymes and their regulatory factors, the stomata-related traits, *etc.* (Driever *et al.* 2014, Nunes-Nesi *et al.* 2016). Modelling approach based on the utilisation of the genetic variability in photosynthetic characteristics in rice calculated that mining of this variability should increase rice productivity by about 22-29% in a surprisingly short time (Gu *et al.* 2014). This is a sufficiently large number to strongly advocate for the purposeful exploitation of such a rich resource of potential improvement of plant biomass production in breeding programmes.

The third step of the breeding process is to transfer the desired characteristics (or, more precisely, alleles for them) from the selected germplasm into existing cultivars that already have other desirable properties, or to combine potentially complementary traits (alleles) of parents by strategic crossing (Reynolds *et al.* 2009, Ghanem *et al.* 2015, Reynolds and Landgridge 2016). The knowledge of the genetic basis and (perhaps even more importantly) the degree of heritability and the assessment of the precise nature of genetic mechanisms participating in the inheritance of such traits and their manifestation in progeny is thus a necessary and integral part of the breeding process. Most photosynthetic characteristics have to be viewed as quantitative traits and have to be analysed using the methods of quantitative genetics. Statistical approaches based on models including different types of genetic effects and using various designs of evaluated populations, together with diverse methods of estimation and evaluation of the relative importance of additive and non-additive genetic effects have mostly been used for the assessment of the inheritance of traits associated with photosynthesis in the second half of the 20th century. This topic is more thoroughly covered in *Part 1* of this thesis, because it has been one of the areas my own early research was focused on. From the beginning of the 21st century, the quantitative genetics of photosynthesis mostly (although not entirely) switched to another approach. Advances in molecular genetics and the establishment of large numbers of DNA markers in various crops have enabled the identification of several quantitative trait loci (QTLs) for some photosynthetic traits (Yamori *et al.* 2016). Such QTLs could be utilised, *e.g.*, in the QTL-pyramiding approach for improvement of crop photosynthetic efficiency; first attempts for this have already been made (Adachi *et al.* 2014). The genomic selection (GS), which works by calculating the genomic estimated breeding values based on a whole genome rather than a relatively small number of genes, is also proposed to be a good candidate for modern "next generation" breeding (Barabaschi *et al.* 2016). However, it is not yet much widely used, particularly in physiological breeding (Ghanem *et al.* 2015).

The inclusion of physiological traits into breeding and selection programmes should result in one or more of the following gains: general acceleration of the breeding process, reduction of its time and spatial demands, the possibility of the assessment of a greater number of genotypes, facilitation of a more thorough and reliable testing and identification of genetic material, and, finaly, reduction of labour inputs and overall costs of the whole process. This can be achieved, *e.g.*, by utilisation of relatively simple (cheap, labour-inexpensive) measurement instruments, permitting fast measurements of many samples (preferably of a non-destructive character for plants), providing required information on the desired objective in earlier generations (thus shortening the breeding cycle) of phenotypical testing of younger plants (requiring less space in the field). The cultivation and testing of large numbers of plants in controlled-environment facilities, so that several generations could be grown each year and the environmental variation inevitably associated with field studies could be reduced, might be also useful. However, this approach has both advantages and disadvantages, because there can be problems with extrapolation of the data obtained in controlled-environment conditions to a real situation in the field (Brennan and Martin 2007, Ghanem *et al.* 2015).

The facilitation of selection by DNA markers based on strong phenotype/genotype association is another option how to achieve these objectives. DNA markers associated with QTLs for targeted traits could be employed in marker-assisted selection (MAS) to speed up creation of new cultivars (Mackay *et al.* 2009, Mackay 2014). The next generation sequencing technologies now facilitate very high-throughput and genome-wide identification of DNA polymorphisms that can be potentially used as molecular markers in plant genotyping necessary for MAS, GS and genome-wide association studies. However, their utilisation in such processes is currently limited by an insufficient level of phenotyping, essential for creating good marker-trait relationship. This is another area where the analysis of photosynthetic characteristics offers exciting possibilities. Rapid, simple, labour-inexpensive as well as non-invasive measurement technologies, enabling the evaluation of large amounts of plants/genotypes, exist for several types of these traits. Two major classes of photosynthetic parameters suitable for high-throughput phenotyping and introduction into breeding programmes are recommended: the contents/ratios of photosynthetic pigments and the efficiency of primary photosynthetic processes (Lopes *et al.* 2012, Mullan 2012, Grosskinski *et al.* 2015, Walter *et al.* 2015). The measurements of the net photosynthetic rate and associated parameters are sometimes also included in this category. However, they are more time-consuming, require a more precise control of the instruments used for such purposes and cannot be as yet properly applied for the assessment of whole canopies (Long and Bernacchi 2003, Lopes *et al.* 2012). Neither the enzymatic activities associated with photosynthetic carbon fixation cycle nor the structural/anatomical parameters related to photosynthesis are recommended for such purpose because their analysis is both time- and labour-expensive.

The content of photosynthetic pigments in plants can be evaluated by destructive methods, *i.e.*, high-performance liquid chromatography (which facilitates very precise determination of the amounts of photosynthetic pigments including individual carotenoids but is totally unsuitable for phenotyping purposes) or spectrophotometry (less expensive, possible for smaller sets of genotypes; chlorophyll *a*, *b* and total carotenoid contents can be determined using this method). However, nondestructive approaches based on the measurement of spectral absorbance (transmittance) or spectral reflectance are much better for the purposes of phenotyping large sets of plants. This can be done by multispectral or hyperspectral imaging which informs about discrete, resp. continuous, spectral properties of leaves/whole canopies in the near-infrared and visible ranges of the solar spectrum. The comparison of the transmittance or the reflectance at specific wavelengths yields various spectral indices, some of which inform (more-or-less precisely) on the contents or ratios of photosynthetic pigments. Various handheld devices and remote-sensing systems for these measurements have been developed and are very popular for phenotyping and physiological breeding purposes (Mullan 2012, Dale *et al.* 2013, Grosskinski *et al.* 2015).

Similarly to photosynthetic pigments, both destructive and nondestructive approaches exist for the measurements of the efficiency of primary photosynthetic processes. Isolated chloroplasts or thylakoid membranes can be used for the assessment of various parts of the photosynthetic electron transport chain based either on polarography or spectrophotometry. These approaches utilise various combinations of artificial electron acceptors, donors and inhibitors of the electron transport in order to measure the activities of PS II, PS I (including individual parts of electron transport *within* these complexes) or the whole electron transport chain. They are based either on the determination of natural oxygen production or oxygen consumption induced by the addition of various artificial compounds (polarography), or on the changes in the absorption spectrum of artificial electron acceptors due to the changes in their reduction state caused by photosynthetic processes (Izawa 1980, Trebst 2007). Another possibility for the measurement of the PS II activity is the use of thermoluminiscence; however, this requires some very special equipment and has further disadvantages (Ducruet and Vass 2009). With the advent of the chlorophyll fluorescence analysis, the above-mentioned methods rather grew out of fashion, although they still have some utilisation value for highly specific purposes.

The basic principle of the chlorophyll fluorescence analysis is very simple: the light energy absorbed by photosynthetic pigments bound to light-harvesting antennae can be either utilised for the excitation of electrons and their transport in photosynthetic thylakoid complexes, dissipated as heat in one of the photoprotective processes, or emitted as chlorophyll fluorescence. Any change in one of these processes is directly reflected in the changes in the other two; thus, the relative efficiency of primary photosynthetic reactions as well as the dissipation of the excess excitation energy in form of heat can be measured as the photochemical, resp. nonphotochemical quenching

of chlorophyll fluorescence (Baker and Rosenqvist 2004, Baker 2008). Diverse instrumentation for such measurements has been developed in the past and can be applied both to individual plants (handheld devices) and whole canopies (remote-sensing systems based either on laser-induced fluorescence imaging or measurements of solar-induced fluorescence). All are very suitable for large-scale phenotyping and selection for physiological traits (Furbank and Tester 2011, Fernandez-Jaramillo *et al.* 2012, Fiorrani and Schurr 2013, Porcar-Castell *et al.* 2014, Grosskinski *et al.* 2015, Guo and Tan 2015, Walter *et al.* 2015).

Currently, most chlorophyll fluorescence analyses are based on the dissection of so-called Kautsky effect, *i.e.*, the kinetics of chlorophyll fluorescence induction. Upon the transition of plant from darkness to light, chlorophyll fluorescence first rapidly rises (over a time period of 1 s) and then more slowly decreases until it reaches a steady-state level (in a time range of minutes). Several inflexion points can be observed on the fluorescence kinetics curve during this period; these represent various states of the photosynthetic electron transfer and reflect also subsequent processes of photosynthetic carbon fixation and energy dissipation as heat. The pulse-amplitude-modulated (PAM) fluorescence analysis of the slow phase of chlorophyll fluorescence kinetics is a usual method of choice for such measurements; however, the analysis of the rapid (so-called OJIP) part of the fluorescence transient is also very popular among plant breeders. Many parameters can be derived from both types of these analyses; some of them have already found their use in breeding programmes aimed at screening crop plants for an improved photosynthetic performance, examining the natural genetic variability existing within the currently used germplasm or phenotyping for the purposes of the QTL analysis (Baker and Rosenqvist 2004, Baker 2008, Lopes *et al.* 2012, Brestič and Živcák 2013, Murchie and Lawson 2013, Guo and Tan 2015).

Regarding the rate of photosynthetic carbon fixation (usually measured as the net photosynthetic rate), various methods such as gravimetry, manometry, polarography and others were used for its determination in the past (Hunt 2003). Today, portable infrared gas analysis systems are usually employed for measurements of the net photosynthetic rate (Long *et al.* 1996, Long and Bernacchi 2003, Lopes *et al.* 2012). These systems usually also enable an evaluation of the transpiration rate, g_s, the internal concentration of CO_2 and the water use efficiency. Thus, these parameters are often being assessed together with the net photosynthetic rate in studies/breeding programmes making use of this type of instrumentation. Both simple and more sofisticated equipment is currently available for these measurements; the more sofisticated instruments offer also the possibility to analyse the response of the photosynthetic apparatus to various concentrations of CO_2 or various light intensities. Such analyses provide estimates of the Rubisco activity, limitation of photosynthesis by the electron transport and/or triosephosphate utilisation, quantum yield of photosynthetic carbon fixation, *etc.* In combination with the measurements of dark respiration and chlorophyll fluorescence they also offer an estimate of g_m (Long and Bernacchi 2003, Pons *et al.* 2009, Lopes *et al.* 2012). The disadvantage of these analyses lies mostly in their more time-consuming character. Additionaly, no remote-sensing systems for such measurements have yet been created (and probably will not be, provided the whole approach for the determination of the net photosynthetic rate will not undergo radical changes).

To conlude: photosynthesis undoubtedly shows a great promise for its utilisation in crop improvement efforts. Many opportunities for its incorporation into modern breeding programmes are currently available and they will probably further expand with the expected development of new technologies and the improvement of existing ones in the future. However, various factors still limit the widespread use of photosynthetic traits in plant breeding and prevent the achievement of their full potential. Despite many long-term and significant efforts of plant physiologists, molecular biologists and practical breeders, our understanding of many aspects of this vital process is still incomplete. Information gaps concerning mechanisms of the regulation of photosynthesis by various internal (regulatory proteins, phytohormones, *etc.*) and external (abiotic and biotic stressors) factors still exist and need to be filled by both fundamental and applied research. Breeders lack a more detailed knowledge on the precise genetic factors affecting the inheritance of photosynthetic characteristics from parents to their progeny. The role of photosynthesis in the formation and manifestation of hybrid vigour, a phenomenon that is frequently utilised in crop breeding, is mostly unexplored. These all are subjects my own research in the Laboratory of Plant Genetics of the Faculty of Science, Charles University, has focused on. Various papers I have authored or co-authored, together with brief introductions into each of these topics, comprise the following three parts of this thesis.

Part 1

Inheritance of photosynthetic characteristics in plants grown under optimum conditions

Phenotypic variation of any quantitative trait (σ^2_P) can be expressed as a sum of its genetic component (σ^2_G), environmental component (σ^2_E) and the interaction between these two ($\sigma^2_{G\times E}$). This fundamental concept was developed almost 100 years ago by Fisher (1918) and remains unchanged even in this age of molecular/omics biology. Naturally, breeders are mainly interested in the σ^2_G part of this equation, which, divided by σ^2_P, gives an estimation of heritability that can be used for many purposes in breeding and selection programmes. However, σ^2_G can be further subdivided into the additive (σ^2_A), dominant (σ^2_D) and interaction/epistatic (σ^2_I) variation. Other genetic effects (*e.g.*, the maternal, paternal, cytoplasmic ones, the linkage influence, *etc.*) can be added depending on their relevance for studied trait/organism. Obviously, these diverse types of genetic effects strongly influence the inheritance of the respective trait and determine how it will be manifested in the progeny. Thus, breeders always have to take into an account the relative importance of the individual genetic effects in order to design an efficient breeding programme for some crop species. The additive part of the genetic variation is particularly utilisable in the selection process, because it is the only component of σ^2_G that is certain to be transferred into a next generation. Dominance and interaction effects play an important role in the formation of heterosis which is employed in breeding of many crop plants. If a significant proportion of σ^2_G is constituted of the maternal and like genetic factors, it can influence a breeder´s decision whether to use a particular genotype as a male of female parent (Kearsey and Pooni 1996, Lynch and Walsh 1998). A thorough quantitative genetic analysis aimed at the detection and estimation of individual genetic effects constituting σ^2_G in the respective germplasm (and considering the main purpose of the respective breeding programme and target environments) is thus always necessary.

One approach for the detailed dissection of σ^2_G in crop plants deals with the phenotypic evaluation of genotypes organised in specific mating designs such as the North Carolina designs I, II or III, the triple test cross, different types of diallels, multiple generation sets, *etc*. Various scientists developed many genetic/biometrical models which incorporate statistical analyses based on principles of correlation between relatives, usually utilizing many different second-degree statistics (Singh *et al.* 2004). Numerous such studies exist for yield and various yield components in many crop species. Unfortunately, only a limited number of papers presenting such analysis is available for photosynthetic characteristics. *Table 1* summarises the results of these studies made in plants grown under optimum, non-stress conditions (the quantitative genetic analysis of photosynthesis under stress conditions is dealt with in *Part 2* of this thesis). Most studies have been performed on field-grown plants, although some authors cultivated their experimental material also in growth chambers or greenhouses. Unfortunately, their authors mostly used relatively simple genetic models that enabled them to determine only the general and specific combining abilities of their genotypes (*i.e.*, additivity and non-additivity without further specifications). Some focused mostly on the effects of reciprocal crosses (without further differentiation between cytoplasmic or other maternal effects) and did not attempt a true quantitative genetic analysis (Diethelm *et al.* 1989, Krasichkova *et al.* 1989, Kidambi *et al.* 1990). More detailed genetic models that were employed for the assessment of the non-additive genetic effects have varied from those enabling only the detection of dominance (Mencáková 1967, Waly and Johnston 1974, Gaziants 1983, Krebs *et al.* 1996), other non-specified genetic effects (Crosbie *et al.* 1978, Mehta *et al.* 1992, Chohan *et al.* 2012) or the separation of various types of intergenic epistatic interactions (Fousová and Avratovščuková 1973, Avratovščuková and Fousová 1975, Simón 1994). Complete or incomplete diallels or multiple generation sets were usually the designs of choice for these more complex analyses.

QTL-mapping experiments can also provide the information on the possible additive, dominant or epistatic character of any identified QTL using specific mapping populations and methods (Lynch and Walch 1998, Würschum 2012, Mackay 2014). Unfortunately, although a considerable number of papers identifying QTLs for various photosynthetic traits in several crop species has now been published, most authors settled for the simple localisation of QTLs or their characterisation as major or minor ones but did not evaluate a precise nature of their genetic effects. However, several studies that enabled the analysis of a potential dominant or epistatic character of QTLs for some photosynthetic traits do exist. Their results (again only for plants

grown under optimum conditions) are summarised in *Table 2*. With some exceptions, these studies were conducted mostly in rice, wheat or maize and usually dealt only with the chlorophyll content, although some papers analysing QTLs for other photosynthetic traits are also available.

Table 1. Genetic effects participating in the inheritance of photosynthetic traits in various crop plants grown under optimum conditions as evaluated by "classic" quantitative genetics studies that enabled the detection of the non-additive genetic effects.

Plant species	Analysed traits	Detected genetic effects	Reference
Maize	Chlorophyll content	Additivity + non-additivity (non-specified)	Oelke and Andrew 1966
Maize	Net photosynthetic rate	Additivity + non-additivity (non-specified)	Fousová and Avratovščuková 1967
French bean	Net photosynthetic rate	Additivity, no cytoplasmic effects	Izhar and Wallace 1967
Tobacco	Chlorophyll content	Dominance (partial), no additivity	Mencáková 1967
Perennial ryegrass	Net photosynthetic rate, chlorophyll content	Additivity + non-additivity (non-specified)	Wilson and Cooper 1969
Maize	Net photosynthetic rate	Additivity + dominance (complete, overdominance) + epistasis	Fousová and Avratovščuková 1973
Tall fescue	Net photosynthetic rate	Additivity + non-additivity (non-specified)	Asay *et al.* 1974
Marrow-stem kale	Net photosynthetic rate, chlorophyll content	Additivity + dominance (almost complete)	Waly and Johnston 1974
Maize	Net photosynthetic rate	Additivity + dominance (complete) + epistasis	Avratovščuková and Fousová 1975
Maize	Net photosynthetic rate	Additivity + dominance (partial, complete, overdominance), no maternal effects or other effects of reciprocal crosses	Crosbie *et al.* 1978
Soybean	Net photosynthetic rate	Additivity, no other types of genetic effects	Wiebold *et al.* 1981
Maize	Net photosynthetic rate	Additivity, no other types of genetic effects	Albergoni *et al.* 1983
Cotton	Net photosynthetic rate, photophosphorylation activity	Additivity + dominance (overdominance)	Gaziants 1983
Maize	Rubisco activity and content, chlorophyll content	Additivity + maternal effects for Rubisco activity; additivity + non-additivity (non-specified), no effects of reciprocal crosses for Rubisco and chlorophyll content	Baer and Schrader 1985
Pea	Chlorophyll content, Rubisco activity	Additivity + non-additivity (non-specified), no cytoplasmic effects	Hobbs and Mahon 1985
Soybean	Net photosynthetic rate, Rubisco activity	Dominance (partial), no cytoplasmic effects	Diethelm *et al.* 1989
Cotton	Hill reaction activity	Maternal effects + other non-additivity (non-specified)	Krasichkova *et al.* 1989
Sorghum	Net photosynthetic rate	Additivity + non-additivity (non-specified) + maternal effects + other effects of reciprocal crosses	Kidambi *et al.* 1990
Maize	Net photosynthetic rate, chlorophyll (*a, b*, total) content, chlorophyll *a/b* ratio	Additivity + dominance (complete, overdominance) + epistasis for net photosynthetic rate and chlorophyll contents; additivity + dominance (complete) for chlorophyll *a/b* ratio	Mehta *et al.* 1992
Wheat	Net photosynthetic rate	Additivity + dominance + epistasis	Simón 1994
Maize	Chlorophyll and carotenoids content and their ratio, chlorophyll fluorescence parameters (F_v/F_m, F_v'/F_m', Rfd, q_P, q_N)	Additivity, no effects of reciprocal crosses for chlorophyll and carotenoids content and chlorophyll fluorescence parameters (q_N also non-additivity)	Krebs *et al.* 1996
Barley	Chlorophyll fluorescence parameters (Φ_{PSII}, Rfd, F_{vm}', $1/F_m'$, q_P, NPQ)	Additivity + non-additivity (not for all parameters but further non-specified), no effects of reciprocal crosses	Marcial and Saraffi 1996
Sugarcane	Chlorophyll fluorescence parameters (F_0, F_m, F_v/F_m, $T_{1/2}$, Rfd)	Additivity + non-additivity (non-specified)	Zhang *et al.* 2000
Maize	Net photosynthetic rate	Additivity, no other genetic effects	Ahmadzadeh *et al.* 2004
Maize	Chlorophyll content	Additivity, no other genetic effects	Lee *et al.* 2005
Maize	Net photosynthetic rate	Additivity + dominance (partial), no other genetic effects	Chohan *et al.* 2012

Table 2. Genetic effects participating in the inheritance of photosynthetic traits in various crop plants grown under optimum conditions as evaluated by "molecular" (QTL-mapping) quantitative genetics studies that enabled the detection of the non-additive genetic effects.

Plant species	Analysed traits	Detected genetic effects	Reference
Sorghum	Chlorophyll content	Additivity + epistasis	Subudhi *et al.* 2000
Rice	Chlorophyll content	Additivity + epistasis	Yang *et al.* 2003
Maize	Net photosynthetic rate, chlorophyll content, chlorophyll fluorescence parameters (F_0, F_v/F_m, F_v'/F_m', Φ_{PSII}, q_P)	Additivity + dominance (partial, complete or overdominance) for net photosynthetic rate and chlorophyll fluorescence parameters; additivity, minor role of dominance for chlorophyll content	Fracheboud *et al.* 2004
Rice	Chlorophyll content	Additivity + epistasis	Jiang *et al.* 2004
Rice	Chlorophyll content	Additivity + epistasis	Shen *et al.* 2007
Wheat	Chlorophyll content, chlorophyll fluorescence parameters (F_0, F_v, F_m, F_v/F_0, F_v/F_m)	Additivity, no epistasis for chlorophyll content, F_0 and F_v/F_0; additivity + epistasis for F_v, F_m and F_v/F_m	Yang *et al.* 2007
Rice	Chlorophyll content	Additivity + epistasis	Yoo *et al.* 2007
Rice	Net photosynthetic rate, chlorophyll content	Additivity + epistasis for chlorophyll content; less importance of epistasis for net photosynthetic rate	Hu *et al.* 2009
Rice	Chlorophyll content	Additivity, dominance is much less important	Takai *et al.* 2009
Rice	Rubisco content	Additivity + epistasis	Kanbe *et al.* 2009
Wheat	Chlorophyll content	Additivity + epistasis	Zhang *et al.* 2009a
Wheat	Chlorophyll content	Additivity + epistasis	Zhang *et al.* 2009b
Wheat	Chlorophyll fluorescence parameters (F_v/F_m)	Additivity, no epistasis	Liang *et al.* 2010
Wheat	Chlorophyll content, chlorophyll fluorescence parameters (F_0, F_v, F_m, F_v/F_m, T_m)	Additivity + epistasis	Zhang *et al.* 2010
Maize	Chlorophyll content	Additivity, minor role of epistasis	Messmer *et al.* 2011
Chinese cabbage	Chlorophyll content	Additivity + dominance (partial, complete, overdominance)	Ge *et al.* 2012
Rice	Net photosynthetic rate, chlorophyll fluorescence parameters (Φ_{PSII}, F_v'/F_m', q_P)	Additivity, no epistasis	Gu *et al.* 2012
Lettuce	Chlorophyll content	Additivity + epistasis	Hayashi *et al.* 2012
Maize	Chlorophyll (*a*, *b*, total) content	Additivity + dominance + epistasis	Irfan *et al.* 2014
Rice	Chlorophyll content	Additivity + dominance (overdominance, not much partial dominance) + epistasis	Jiang *et al.* 2014
Maize	Chlorophyll fluorescence parameters (F_v/F_m)	Additivity, minor role of dominance	Rodríguez *et al.* 2014
Wheat	Chlorophyll fluorescence parameters (F_0, F_v, F_m, F_v/F_m)	Additivity + epistasis	Azam *et al.* 2015
Rice	Chlorophyll content	Additivity + epistasis	Huang *et al.* 2015

The results of the "classic" genetic analyses made in the past have clearly indicated that the situations in which no other than the additive component of σ^2_G is present are rather rare (Izhar and Wallace 1967, Wiebold *et al.* 1981, Albergoni *et al.* 1983, Ahmadzadeh *et al.* 2004, Lee *et al.* 2005). In most studies, the dominant genetic effects (ranging from the partial dominance to the overdominance) were detected regardless of the mating design and the genetic model used for the evaluation. Some authors also found epistatic interactions (Fousová and Avratovščuková 1973, Avratovščuková and Fousová 1975, Mehta *et al.* 1992, Simón 1994) or even maternal genetic effects (Baer and Schrader 1985, Krasichkova *et al.* 1989, Kidambi *et al.* 1990) or other effects associated with the differences between reciprocal crosses (Kidambi *et al.* 1990) to be very important components of the genetic variability in photosynthetic characteristics. The more recent QTL-mapping analyses also support these findings; they particularly emphasise the role of epistatic interactions between both major and minor QTLs for photosynthetic traits and some authors point out that the dominance can be also very important (Fracheboud *et al.* 2004, Ge *et al.* 2012, Irfan *et al.* 2014, Jiang *et al.* 2014).

However, various discrepancies between results of different studies exist for both "classic" and "molecular" analyses. Even if the same parameter is measured in the same crop species grown under similar growth conditions and evaluated in a similar developmental stage, situations in which some authors found, *e.g.*, an important role of dominance whereas others did not, frequently occur and the same applies for epistatic or maternal effects. Any new study which analyses the character of genetic effects participating in the inheritance of some photosynthetic trait is thus valuable and can refine our understanding of this phenomenon. When I started my scientific career, such analyses (made mostly with field-grown, non-stressed crop plants) were the main research subject of my Laboratory and my first papers thus focused on this topic. In the following paragraphs I will present their brief overview (their full texts follow) and will finish this part of my thesis with some outlooks for my eventual future work in this area.

The first paper I published (**Synková *et al.* 1997**) dealt with the quantitative genetics analysis of some parameters characterising the primary photosynthetic processes together with the content of photosynthetic pigments in tomato. We wanted to ascertain whether the genetic variability in these parameters exists in this plant species, and to assess the relative importance of the additive and non-additive genetic effects in their inheritance. We worked with the complete diallel cross based on five parental lines and F_1 hybrids (including reciprocal crosses) and the individual components of genetic variation were estimated using two different genetic models. We found significant genotypic differences for the contents of photosynthetic pigments (including the contents of individual carotenoids; their evaluation in order to assess the mode of their inheritance had not been, to my knowledge, previously – or since – conducted). These differences were due to both additive and non-additive genetic effects (including the effects of reciprocal crosses suggesting either important cytoplasmic or other – probably maternal – participation in the inheritance of these characteristics). However, the parameters describing the efficiency of primary photosynthetic processes (measured using either slow chlorophyll fluorescence kinetics or the photochemical activities of isolated chloroplasts) showed much less prominent variability which was mostly due to the non-genetic effects (leaf development). This suggested that this type of parameters (unlike the content of photosynthetic pigments) is not much suitable, *e.g.*, as a physiological marker for the possible utilisation in tomato breeding (at least under non-stress conditions).

My attention then transferred to another, more agronomically important crop species, *i.e.*, maize. It became my favourite model object for many following years. I firstly worked with the field-grown plants, which neccessitated multiple-year evaluations in order to obtain results that would be scientifically sound and reliable. During my Ph.D. study years I have analysed the efficiency of primary photosynthetic processes and the content of light-harvesting proteins in several diallel sets of maize inbred lines and their F_1 hybrids as well as in multiple generation sets (parents, F_1, F_2, backcrosses). I have used various approaches for the quantitative genetic analysis based on these mating designs and particularly the multiple generation sets enabled me to separate not only the additivity from the non-additivity but also the dominant genetic effects from three types of epistatic interactions. I have found that, contrary to tomato, maize shows significant genetic variability in the efficiency of primary photosynthetic processes (this time assessed as the activities of PS I and PS II in isolated photochemically active chloroplasts). This variability depended on the way these parameters were expressed (it was greater for the expressions per leaf area or dry matter units than per the chlorophyll content unit), which was interesting from a methodical point of view. Besides additive effects, highly prominent and positive dominant effects also participated in the inheritance of these two photosynthetic parameters, leading to significant positive heterosis (see also *Part 2* of this thesis). No effects of reciprocal crosses were present in this case. These findings were published in the paper **Holá *et al.* (1999)**.

Regarding the genotypic differences in the content of light-harvesting proteins, they also existed but were much less pronounced than in case of the photochemical activities of chloroplasts. Moreover, they depended on plant age / the developmental stage. Positive dominance together with additivity was also observed but no epistatic interactions were present. The F_1 hybrid generation again showed positive heterosis which decreased (as expected) in the F_2 generation. I published my results in the paper **Holá (1999)**. This was the first case the content of some proteins of the primary photosynthetic processes was examined in order to evaluate the genetic mechanisms of their inheritance using the quantitative genetic analysis.

The proteins of the external component of photosynthetic light-harvesting antennae play an important role in re-distribution of light energy between PS II and PS I in so-called state transition processes and, consequently, in structural changes of the chloroplast inner membrane system, *i.e.*, the degree of thylakoid stacking, with PS II located predominantly in the thylakoid grana and PS I in the intergranal thylakoids. I was therefore interested in ascertaining whether the differences between parents and their hybrids, observed for the efficiency of primary photosynthetic processes and the content of light-harvesting antennae proteins (particularly the positive heterotic effect in the F_1 generation), could be similarly found for the chloroplast ultrastructure. We thus started to cooperate with Dr. Jaromír Kutík from the Department of Plant Physiology (currently the Department of Experimental Plant Biology) of our Faculty and compared various quantitative parameters of chloroplast ultrastructure (accompanied by the functional measurements of chloroplast photochemical activities) in maize inbred lines and their F_1 hybrids. In our first joint study, these parameters were determined in leaves of two maize inbreds, which were in different stages of development (young, mature or senescing). We found that the genotypic differences in the activity of the photosynthetic electron transport chain (the Hill reaction activity measured in mesophyll chloroplasts) mostly corresponded to the differences in the thylakoid volume density in these chloroplasts. However, they also strongly depended on the developmental stage of leaves. Curiously enough, the thylakoid volume density in chloroplasts from senescing leaves of one parental genotype increased (whereas the activity of the Hill reaction decreased as expected with senescence), which opened some questions on the quality of these thylakoids. We also evaluated chloroplast ultrastructure in the mature leaves of F_1 hybrids of these two parental lines and found positive heterosis for the thylakoid volume density (both granal and intergranal thylakoids) and starch inclusions. These results were published in the paper **Kutík *et al.* (1999)**.

We then continued to examine the relative importance of the development of mesophyll chloroplasts on manifestation of the parent/hybrid differences in structural and functional parameters of thylakoid membranes making another study of maize leaves (**Kutík *et al.* 2001**). This time, only one maize inbred line and its F_1 hybrid were evaluated and the analysis was again conducted using either mature or senescing leaves. However, in addition to these two different developmental stages of leaf, we also examined different parts of the leaf blade, because maize as a grass species shows a typical gradient of chloroplast development from the leaf base to its apex. Both analysed genotypes again differed in various aspects of mesophyll chloroplast ultrastructure (particularly regarding starch inclusions and plastoglobuli, but also thylakoids and peripheral reticulum) and function (the activities of the Hill reaction and – to a smaller degree – PS I). They also differred in the contents of photosynthetic pigments (differences in these parameters were probably the most distinctive ones). The F_1 hybrid displayed slightly faster development of mesophyll chloroplasts compared to its parental inbred line but the general trend of chloroplast heterogeneity across the leaf blade was the same as in its parent.

To further examine this heterogeneity in chloroplast development and the parent/hybrid relationship, we expanded our analyses to include not only mesophyll chloroplasts but also the bundle sheath cell chloroplasts (for various reasons, the results of this analysis were published much later than it was actually performed, in the paper **Vičánková *et al.* 2007**). Surprisingly, we found that while the course of the mesophyll chloroplast development correlates well in both genotypes during the onset of leaf senescence, the situation for the bundle sheath cell chloroplasts was greatly different. In this case, the respective hybrid and parent were characterised by their own paterns of chloroplast development. This was particularly evident in the absence of any significant correlation in the heterogeneity of the bundle sheath cell chloroplast shape and size and was caused mainly by the different patterns of starch accumulation. Clearly, although some characteristics of chloroplast development can be inherited directly in the maternal mode from a parent to its progeny, this does not apply universally and is cell-type specific.

As far as I know, no one had conducted such evaluation of chloroplast ultrastructure in parents and hybrids of some plant species prior to these three published papers (and very few papers on this subject have appeared since). Thus, our results in this field can be regarded as priority ones. It would be perhaps interesting to perform a more detailed examination of genetic effects participating in the inheritance of characteristics of chloroplast ultrastructure using larger sets of genotypes, however, this is not feasible. Even in this age of computerised image analyses, the quantitative assessment of chloroplast ultrastructure is an extremely labourious and time-consuming procedure and only a small number of samples can be evaluated on a realistic time

scale. While perhaps interesting from a purely scientific point of view, the evaluation of chloroplast ultrastructure (or, similarly, leaf anatomy) for the practical purposes of plant breeding for improved photosynthetic efficiency is impossible. I do not believe that a continuation of this direction of my original research would be worthwile.

In the years following the publication of papers described in the previous paragraphs, my scientific interests shifted from the quantitative genetic analysis of mechanisms participating in the inheritance of photosynthetic characteristics in plants grown under optimum conditions to the examination of intraspecific differences in plant resistance/sensitivity to various abiotic stressors (again mainly in relation to photosynthesis and the parent/hybrid differences; this is described in a more detail in *Part 2* of this thesis) and, later, to the role of plant steroids in the regulation of photosynthesis (*Part 3*). However, I have recently returned to this research topic in a slightly different context: forestry and tree breeding.

As good adaptability to changing environment will probably soon become one of the major objectives of forest tree breeding, forestry scientists and breeders are becoming more and more interested in utilisation of various physiological measurements for the evaluation of their breeding populations. Many studies conducted in crop plants have demonstrated that photosynthesis is well associated with plant adaptability to various stressors. The measurements of photosynthetic parameters (particularly chlorophyll fluorescence or spectral reflectance-associated ones) offer an easy and dependable method of phenotyping necessary for identification of QTLs associated with desired traits, MAS and other approaches of quantitative genetics utilised in modern tree breeding. Unfortunately, studies dealing with the genetic variability in photosynthetic parameters in large populations of forest trees (particularly conifers), which would enable the estimation of the degree of their heritability and genetic effects participating in their inheritance, are still rather rare.

My Laboratory recently started to cooperate with the team of Professor Milan Lstibůrek from the Department of Genetics and Physiology of Wood Trees, Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague. Our first joint project was aimed at the evaluation of the genetic variability in various parameters derived from the analysis of fast chlorophyll fluorescence kinetics in two Czech populations of Scots pine. Based on this evaluation, the quantitative genetic analysis was performed using a mixed-linear model originaly developed for animal studies. Its results are described in the paper **Čepl *et al.* (2016)**. We found that the genetic variability indeed exists for most of these parameters (interestingly, the parameter F_v/F_m, which is very popular for phenotyping studies in crop plants, showed no apparent genetic variation) and that the heritability of these parameters ranges from 0.15 to 0.23. Their inheritance was primarily associated with the additive genetic effects, which could be advantageous for breeding purposes.

Our first experiments on the potential utilisation of chlorophyll fluorescence measurements for the assessment of genetic variability in Scots pine populations encourage us to further pursuit this direction of research. An analysis of the correlation between genetic variability in primary photosynthetic processes, which was already identified in populations examined in the study mentioned in the previous paragraph, with that in the original production populations (seed orchards), together with a more detailed quantitative genetic analysis will probably be our next step. As the data on SSR marker polymorphisms are also available for the studied populations of Scots pine, a possibility for the identification of QTLs associated with the primary photosynthetic processes in this species opens up. Another set of photosynthetic parameters dealing with the content of photosynthetic pigments and based on the measurements of spectral reflectance has also been already evaluated in these populations and now awaits its detailed quantitative dissection and publication of the results. An assessment of the suitability of such analyses as a means for the evaluation of genetic variability associated with the response of selected Czech conifer populations to unfavourable environment will probably be also made in the future. All this could potentially help with the selection of elite tree genotypes in conifer breeding programmes.

I mentioned in the *Introduction* chapter of this thesis that the efficiency of primary photosynthetic processes can be studied either using the measurements of chlorophyll fluorescence or the measurements of photochemical activities of the individual thylakoid complexes in suspensions of isolated chloroplasts. The disadvantage of the majority of routinely performed chlorophyll fluorescence assessments is that they provide information only on the functional state of PS II. Other parts of the photosynthetic electron-transport chain (particularly those related to the PS I efficiency) are usually neglected. The second mentioned approach, *i.e.*, the work with isolated chloroplasts, offers interesting possibilities how to overcome this shortcoming. Unfortunately, the

conventional methods suitable for the isolation of photochemically active chloroplasts from herbaceous plants (which I have routinely utilised in the past) are not applicable to conifers. After a comprehensive survey of the literature on this topic we developed an optimised method for the isolation of chloroplasts from spruce needles and we also adjusted the polarographic measurements of PS I and PS II activities in chloroplasts isolated by this method. When we compared our approach with all 20 methods previously described in various papers, we established that when both PS I and PS II activities are considered, our procedure yielded the best results. We also demonstrated that it can be applied to a wide variety of conifer species with needle-like leaves. This was published in the paper **Holá *et al.* (2012)**.

Since then, we adapted this methodics for the measurement of the whole photosynthetic electron-transport chain activity and it was further expanded by one of my students to include a spectrophotometric assessment of the activity of PS II with an inactive oxygen-evolving complex (OEC). This measurement, by comparison with the activity measured in PS II with an active OEC, could, *e.g.*, provide information on the role this component of the

PS II complex plays in the overall efficiency of primary photosynthetic processes in chloroplasts isolated from conifers subjected to various stressors or other environmental factors. We would also like to further develop our original metodical approach to enable the polarographic determination of the effectivity of additional components of the photosynthetic electron-transport chain, particularly the part related to the cytochrome b_6f complex. Such analysis could be utilised in various studies which require the detailed assessment of primary photosynthetic processes in conifer trees. The information obtained from such measurements could nicely complement the information from chlorophyll fluorescence analyses and bring more details, *e.g.*, on what is actually occurring in the thylakoid membranes of needles exposed to various external factors associated with ongoing climatic changes.

Part 2

Better acclimation of the photosynthetic apparatus to stress conditions as a cause of heterosis?

Although scientific studies analysing the genetic effects participating in the inheritance of photosynthetic traits in plants grown under normal (optimum) conditions do not precisely abound (see *Part 1* of this thesis), even less frequent are those that attempt to dissect these effects in plants subjected to some abiotic or biotic stressor. However, some evidence that both intra- (dominance) and intergenic (epistasis) non-additive allelic relationships can significantly influence genetic variation in photosynthetic traits in plants exposed to unfavourable environment has been presented by both "classic" and "molecular" types of studies. Van de Dijk (1987) analysed the net photosynthetic rate in a partial diallel cross of tomato grown under low energy (low irradiance, low temperature) conditions and reported that both additive and non-additive (further non-specified) genetic effects are present; however, the presence of cytoplasmic effects was not confirmed. Malik *et al.* (1999), who studied the inheritance of the same trait in multiple generation sets of wheat exposed to drought, found a significant dominance effect, although it was less important than the additive effects. Farshadfar *et al.* (2013) used a diallel mating design for the genetic analysis of wheat drought resistance and concluded that the non-additive genetic effects (dominance and epistasis) have a significant role in controlling the inheritance of the stomatal conductance, but that only additivity is important for the genetic variability in the chlorophyll fluorescence parameter F_v/F_m. Chohan *et al.* (2012) conducted a similar analysis for the net photosynthetic rate in maize and reported the existence of additive genetic variation together with a partial dominance. This dominance was more positive under water-stress conditions compared with the control ones.

Regarding QTL studies, all analyses have been performed in plants grown either under low temperature or drought conditions. Fracheboud *et al.* (2002) searched for QTLs determining various photosynthetic traits (the net photosynthetic rate, the content of photosynthetic pigments, some chlorophyll fluorescence parameters) in cold-stressed maize and found that at least some QTLs for the parameter Φ_{PSII} are in epistatic interactions. Later, they expanded this study and detected also dominant genetic effects (from partial dominance to overdominance) for another chlorophyll fluorescence parameter, F_v'/F_m' (Fracheboud *et al.* 2004). They also demonstrated that most QTLs discovered for various photosynthetic parameters in plants stressed by low temperature were specific for this environmental condition (by comparing them with QTLs detected in control plants). The QTLs for the resistance to chilling-dependent photoinhibition also displayed a dominant character in maize (Pimentel *et al.* 2005). Another analysis conducted by Rodríguez *et al.* (2014) in cold-stressed maize revealed that under cold conditions, the dominant effects of QTLs for the Φ_{PSII} parameter were much greater than the additive effects and were also more important than under control, non-stress conditions. Significant epistatic interactions were described for some chlorophyll fluorescence-associated QTLs in cold-stressed barley by Tyrka *et al.* (2015).

Some epistatic interactions between QTLs controlling the "stay-green" trait associated with the chlorophyll content were also observed in drought-stressed sorghum by Subudhi *et al.* (2000). On the other hand, Yang *et al.* (2007) did not confirm any epistatic interactions between QTLs for the chlorophyll content in drought-stressed wheat. However, the epistasis was significant for some chlorophyll fluorescence parameters and its importance for the phenotypic variability was greater in the stressed than in the non-stressed plants in this study. Hu *et al.* (2009) analysed QTLs for the net photosynthetic rate and the chlorophyll content in rice subjected to water deficiency and detected digenic interactions that explained more than 18% of the total phenotypic variation in the first trait. However, according to these authors, these interactions played a smaller role in the stressed plants than in the control ones, and for the chlorophyll content, no epistatic interactions were detected at all. Gu *et al.* (2012), who also analysed drought-stressed rice, mapped QTLs for the net photosynthetic rate, Φ_{PSII} and F_v'/F_m' parameters and evaluated them for epistatic interactions, but found none. Similar situation was reported by Messmer *et al.* (2011) for the chlorophyll content in drought-stressed maize: the epistatic interactions were rare and had a relatively low effect on the variability in this trait.

It is interesting to note that those few authors who compared the genetic effects participating in the inheritance of photosynthetic traits in the stressed plants *as well as* in the non-stressed ones often (with the exception of Hu *et al.*, 2009) observed a significant increase of the

relative importance of the non-additive genetic effects with a positive character (either dominance or epistasis) in the stressed plants compared with the non-stressed ones (Yang *et al.* 2007, Chohan *et al.* 2012, Rodríguez *et al.* 2014). This was usually manifested as an increase in hybrid superiority under stressful conditions. The superiority of hybrids over their parents for various yield, morphological and physiological traits is termed "heterosis" or "hybrid vigour" (although these two terms are not completely interchangeable). Scientific investigation of this phenomenon in plants dates back to the 18th and 19th centuries and the first attempts to explain heterosis from a genetic point of view appeared soon after the true beginnings of genetics as a scientific discipline. However, although heterosis has been known and analysed for a long time, it is still very poorly understood both on the genetic/molecular level and on the physiological/whole plant level (Chen 2013).

The original, "classic" genetic models tried to explain heterosis by two hypotheses called the "dominance hypothesis" or the "overdominance hypothesis". The first one was based on the presumption that the dominant allelles are preferred in the nature over the recessive ones during plant growth and development; thus, hybrids accumulate/complement the dominant alleles from their parents, which results in their superiority. Contrary to this, the "overdominance hypothesis" simply stated that the heterozygosity is more advantageous than the homozygosity and that the expression of both different alleles in a heterozygous locus may lead to existence of protein variants which would offer better adaptability to the heterozygous hybrid compared with its homozygous parents. Later, these two hypotheses were joined by the "pseudo-overdominance hypothesis" which suggested a positive effect of the tight linkage between loci with dominant alleles, and also by the "epistasis hypothesis" which took into an account functional interactions between different loci, which could not occur in the parents but could be newly formed in the hybrids (Chen 2010, Goff 2011, Veitia and Vaiman 2011, Kaeppler 2012, Schnable and Springer 2013, Fu *et al.* 2015). Various evidence supporting or challenging these hypotheses accumulated over the years but most scientists dealing with this topic currently tend to view heterosis as an extremely complex phenomenon originating from the combination of all of the above mentioned genetic mechanisms (the "wholistic hypothesis") (Fu *et al.* 2015). A debate whether the polygenic of monogenic inheritance is the basis for heterosis is also going on. The examples of both can be found in the nature and again, we can do nothing else than accept that both cases can be true and that the precise nature of heterosis always depends on a particular trait and an organism it is studied in (Birchler *et al.* 2010, Goff 2011, Chen 2013).

The advent of molecular biology and particularly the development of high-throughput "omics" technologies during recent years brought an interesting information on the possible molecular basis of heterosis. The hybrids are often characterised by different levels of transcripts compared with their parents and many genes have been shown to have an allele-specific type of expression which could certainly result in heterosis. This can be associated with the allele-specific regulation of gene expression by epigenetic marks on chromatin (DNA methylation, histone modifications), small RNAs or the allele-specific binding of transcriptional factors. Some authors have also observed non-additive patterns of expression not only on the transcriptional but also on the translational level, *i.e.*, the hybrid/parent differences in protein amounts. The qualitative differences and the protein isoform variation between hybrids and their respective parents have also been found in some cases. Together, these studies suggest that the genes/proteins that show heterotic patterns participate mainly in the following processes: photosynthesis, carbon and energy metabolism, amino acid and protein metabolism, secondary metabolism, cell division and growth, cell detoxification, stress response, defence and disease pathways, signal transduction and regulation of gene expression (Chen 2010, 2013, Groszmann *et al.* 2011, Baranwal *et al.* 2012, Kaeppler 2012, Goff and Zhang 2013, He *et al.* 2013, Schnable and Springer 2013, Ryder *et al.* 2014, Fu *et al.* 2015, Feng *et al.* 2015, Xing *et al.* 2016).

The results of these "omics" studies together with the data obtained by examination of various biochemical and physiological parameters in heterotic hybrids led some scientists to propose several models explaining how could changes in the expression of specific functional categories of genes (both on RNA and protein levels) induce changes in cell metabolism that would in the end result in the manifestation of heterosis on a whole plant level (Baranwal *et al.* 2012). The role of the altered response of various defence and stress-associated genes to some plant hormones in heterotic hybrids was proposed by Groszmann *et al.* (2015). These authors have argued that the reduced expression of defence genes, displayed by hybrids and associated with the reduced levels

of salicylic acid and increased levels of auxin, enables greater growth of hybrids because the processes of plant immunity and growth are mutually antagonistic. However, even if the hybrids displayed reduction in the basal levels of defence gene expression, they still maintained a good ability to withstand at least some biotic stressors, which means that some additional mechanisms other than simple reduction in defence pathways signalling clearly have to be operating here. Goff (2011) offered another model based on the hypothesis that hybrids contain more stable proteins and are additionally characterised by reduction in various processes associated with general protein metabolism. This would mean that the hybrid genotypes could use their available sources of energy more efficiently compared with their inbred parents, because they would not have to spend so much on the synthesis of proteins. This feature could also provide them with an advantage over a larger scale of environmental conditions (Goff and Zhang 2013). Another interesting possibility for explaining heterosis on the physiological level would be an increased energy gain in the hybrids caused by their greater photosynthetic efficiency (Baranwal *et al.* 2012, Blum 2013, Fu *et al.* 2015, Offermann and Peterhansel 2014). Heterosis for various photosynthetic traits (*e.g.*, the net photosynthetic rate, the chlorophyll content, the efficiency of primary photosynthetic processes, the Rubisco activity or content, *etc.*) has been described by many scientists. Recently, it has been proposed to be associated with altered regulation of the circadian clock genes (Ni *et al.* 2009**,** Miller *et al.* 2015, Shen *et al.* 2015, Ko *et al.* 2016). Such changes in circadian clock regulation could again affect not only photosynthesis but also general plant response to various stressors (Chen 2010, 2013).

Considering that the research on the inheritance of photosynthetic traits in my Laboratory gradually focused more and more on maize, *i.e.*, the plant species in which a positive heterotic effect is perhaps the best manifested, heterosis and its possible genetic and physiological basis (together with some possibilities of its prediction) has naturally become an object of my scientific interest. The idea that heterosis is somehow related to a better ability of hybrids to acclimate to unfavourable environmental conditions and at the same time to photosynthesis was for me an atractive one and deserved to be further explored. Together with my colleagues I have started to analyse whether the photosynthetic apparatus of maize hybrids could really be better adapted to stress conditions than that of their inbred parents, and whether/how this could be associated with possible changes in the relative importance of various genetic effects participating in the inheritance of photosynthetic traits. Our studies at first focused on low temperature because maize plants cultivated in the temperate climatic zone of our country can be unfavourably exposed to this stressor particularly during spring. During the first decade of the 21st century I have published a series of papers dealing with this topic. Some of them brought (in my opinion) several interesting findings and they are briefly described in the next paragraphs, again with their full texts following.

My first paper dealing with the topic of the changes in parent/hybrid relationship under conditions of chilling stress (**Körnerová and Holá 1999**) was based on the evaluation of three maize inbred lines and their reciprocal F_1 hybrids. This evaluation (as well as all subsequent ones) was performed in young, greenhouse-grown plants and the efficiency of primary photosynthetic processes together with the content of photosynthetic pigments was analysed. While mid-parent heterosis in the PS I activity changed only slightly under low temperature conditions, a dramatic increase in heterosis for the Hill reaction activity was observed in our cold-stressed plants compared with the control ones. This suggested that the PS II complex in leaves of F_1 hybrids is better able to acclimate to low temperature conditions than in their inbred parents but that this improved adaptability does not extend to the PS I complex. The analysis of the genetic effects participating in the inheritance of these parameters showed that positive dominance plays a very important role in the formation of positive heterosis in the PS II activity in the F_1 generation of stressed plants but not in the non-stressed ones. Similar situation was found for the content of chlorophylls and carotenoids, for which the heterotic effect also increased in plants subjected to low temperature (although not as dramatically as for the Hill reaction activity). Again, the positive dominance was clearly expressed here, often replacing the slightly negative dominant effects that characterised non-stressed plants. Maternal effects of an additive type were also rather important in the inheritance of the content of photosynthetic pigments.

Some years later, I have decided to return to the problem of the relative importance of different genetic effects in relation to the response of the photosynthetic apparatus to cold stress using a more detailed quantitative genetic analysis. I evaluated the efficiency of primary photosynthetic processes in a multiple generation set (inbred parents, F_1 and F_2 hybrids,

backcrosses) of maize exposed to low temperature and compared the significance of various genetic effects under these conditions with that under optimum conditions. Additionally, the activities of some antioxidant enzymes were also included in this analysis. Our experimental plants again displayed a considerable increase in the F_1 heterotic effect for the PS II activity (associated mostly with positive dominance). In this case, the increase in heterosis for the PS I activity was also found, which could be due to the slightly different set of genotypes used in this study. However, our finding that, contrary to our expectations, heterosis in the F_2 generation did not diminish was of a more interest. The quantitative genetic analysis revealed that this phenomenon was caused by the strong influence of maternal-dominant effects which compensated for the reduction in the positive dominance associated with changes in genotypic composition of the second filial generation. Thus, surprisingly, even the second generation of hybrid plants could retain improved adaptability of their photosynthetic apparatus to low temperatures that was primarily induced in the first hybrid generation. Regarding the antioxidant enzymes, some intriguing differences between the four evaluated enzymes (the superoxide dismutase – SOD, the ascorbate peroxidase – APX, the glutathione reductase – GR and the catalase – CAT) were also observed. While heterosis for the activities of APX and GR was mostly negative in our chilling-stressed plants (this was associated with negative dominant genetic effects), for SOD and CAT, positive heterosis in F_1 hybrids (which only slightly diminished in the F_2 generation) was found. The full text of the paper **Kočová *et al.* (2009)** presents these findings in a more detail.

In the meantime, we have decided to experiment with different intensities of chilling stress and to evaluate also the ability of the photosynthetic apparatus of maize inbred parents and their F_1 hybrids to recover from the cold-induced damage. We found that the rapidity of the onset of low temperature strongly affects how the photosynthetic apparatus in leaves of individual genotypes responds to this type of stress. Generally, when the external temperature decreased slowly and gradually, our experimental plants were often able to completely acclimate their PS II to changing temperature and did not show any decrease in the activity of this complex. The dramatic increase in the heterotic effect for the PS II activity in plants stressed by cold, observed in our original study as well as here, was retained (and even further increased) after the return of plants to more optimum conditions. However, one of the two examined hybrid combinations displayed this phenomenon only under the conditions of the rapid onset of chilling stress while the F_1 hybrid of the second hybrid combination (which was originally characterised by very low heterosis in the PS II activity) displayed this only under gradual temperature changes and to a much smaller extent. We concluded that the superiority of hybrids over their inbred parental lines in the ability of their PS II complex to better withstand low temperatures and in the ability to acclimate back to optimum conditions are independent processes which strongly depend on the exact type of chilling stress and also on the particular genetic combination (**Holá *et al.* 2003**).

We further continued with this direction of research and made some experiments that evaluated the response of maize inbred parents and their F_1 hybrids to chilling periods of various duration. We used one of the hybrid combinations from the previous study (the one with the hybrids that showed both good ability to withstand low temperature stress *per se* and to recover from it). The analysis of photosynthetic characteristics (the activities of the electron transport chain in mesophyll chloroplasts, the contents of photosynthetic pigments) was again supplemented here by the evaluation of the activities of antioxidant enzymes. The results of this study were published in the paper **Holá *et al.* (2007)**. Interesting differences between the behaviour of both inbred parents and their F_1 hybrids were observed. One parental line was characterised by the sensitivity of its PS II to low temperatures (which depended of the length of the stress period) and this was accompanied by its increased need for ROS detoxification as demonstrated by its increased APX and GR activities. This genotype could not much recover from chilling stress, either. The second parental line was also sensitive to low temperatures; however, it was able to return to optimum conditions rather well and to completely repair chilling-induced damage to its PS II complex. As expected, the F_1 hybrids showed the best acclimation ability of their photosynthetic apparatus to both stress and recovery periods (more-or-less independently of the length of these periods) and a reduced need for ROS detoxification systems (APX, GR; however, for CAT and partially also for SOD activities, positive heterosis in F_1 hybrids was again usually manifested). Another interesting phenomenon observed in these experiments was the dependance of cold stress-induced response of the examined antioxidant enzymes in the hybrids on the maternal genotype of their parents.

Our evaluation of the effects of low temperature on manifestation of the heterotic effect in the functionality of the photosynthetic apparatus of chloroplast thylakoid membranes in maize F_1 hybrids was also further expanded by the evaluation of the associated changes in chloroplast ultrastructure. Two papers dealing with this topic were published. The first one, **Kutík *et al.* (2004)**, quantitatively characterised the ultrastructure of mesophyll and bundle sheath cell chloroplasts in leaves of two maize inbreds and their hybrids used in our previous studies and subjected to moderate (gradually occurring) chilling stress. We found that the F_1 hybrids displayed a smaller decrease in the thylakoid volume density (particularly regarding the granal thylakoids) under low temperature conditions than their inbred parents. This nicely complemented our previous findings on the functionality of PS II in hybrid *versus* inbred plants subjected to this type of stress. The mesophyll chloroplasts in leaves of F_1 hybrids also displayed other symptoms of improved resistance to low temperature, including only a minor increase in the volume and number of plastoglobuli and smaller changes in chloroplast shape.

The second paper regarding the ultrastructure of maize mesophyll chloroplasts under low temperature conditions (**Holá *et al.* 2008**) again focused on the differences between chilling patterns/intensities and their influence on the parent/hybrid relationship. Significant interactions between the genetic variability and the chilling pattern were found for all quantitative parameters of chloroplast ultrastructure and shape that were evaluated (*i.e.*, the volume densities of granal and intergranal thylakoids, plastoglobuli, peripheral reticulum, the chloroplast volume and the ratio of cross-section length to width). This indicated that the response of individual genotypes to one type of chilling stress cannot be predicted based on their response to a second type of chilling stress. The relationships between hybrids and their parents were influenced by this and no clearly-defined parent/hybrid correlations (or more general trends in the behaviour of individual genotypes) were evident. This led us to advise an extreme caution in the interpretation of results of any analysis of chloroplast ultrastructure conducted in plants subjected to some unfavourable stress factor, because these assessments are usually based on the evaluation of only one genotype under some very specific type of conditions.

Taken together, the results of all experiments with chilling-stressed young maize plants, which were performed by me and my colleagues, suggested that (at least in this species) the F_1 hybrids really possess an improved ability of their photosynthetic apparatus (particularly the PS II complex) to acclimate to this stress factor. This ability could also be transferred into the next generation (F_2) and was associated with strong dominant and maternal-dominant genetic effects. This supports the hypothesis explaining the physiological basis of heterosis by the existence of not only better energy gains in hybrids caused by their generally greater photosynthetic efficiency, but particularly by the fact that after the exposure to low temperature, the energy gain does not diminish to such an extent as in the parental genotypes. As suggested by Professor Abraham Blum in his review paper published in 2013 (who cited also our studies as examples), homeostatic photosynthesis with respect to temperature could be a general mechanism causing a superior behaviour of hybrids in various plant species.

The occurrence of reduced damage to the photosynthetic apparatus in the leaves of hybrid plants compared with their inbred parents could be further supported by our results related to the changes in antioxidant systems. The increased activities of antioxidant enzymes that are usually observed in relation to plant exposure to some stress factor can be viewed in two ways. First possibility is that they enable plants to better detoxify increased amounts of ROS that start to appear in plant cells with the occurrence of the respective stressor, thus conveying an improved ability to acclimate to stress conditions. Alternatively, they are a symptom of the "last attempt" to counter the dramatic overproduction of ROS that already damages various parts of plant cells (particularly chloroplasts and their photosynthetic apparatus) and thus represent a worse ability of the respective plant genotype for stress acclimation. Because plants contain various antioxidant systems that are interconnected on several levels and connected also with many other processes taking place in plant cells, the results of any analysis of the activities or contents of antioxidant enzymes are always rather difficult to interpret. Our experiments suggested at least a partial independence of the antioxidant systems based on the ascorbate-glutathione cycle and those that directly detoxify superoxide or hydrogen peroxide by other mechanisms. It is possible that our maize hybrids could produce reduced amounts of superoxide from the PS I and PS II-associated reactions (because their photosynthetic apparatus was able to function better than in their parents even under stress conditions) and contained well-functioning superoxide dismutases able to deal

with this type of ROS, as well as highly active CAT systems detoxifying the resulting hydrogen peroxide. They would then have no need for an increased activity of the ascorbate-glutathione system, unlike their parents where the overproduction of ROS would be such that it would also necessitate the greater involvement of this system. It would be certainly interesting to analyse the situation with various enzymatic and non-enzymatic antioxidants and the contents of various ROS in maize hybrids and inbreds stressed by cold in a more detail; perhaps sometime in the future I will return to this subject again and will make a study directly focused on this topic.

However, given the increasing importance of water shortage as a major factor limiting plant production on a global scale, in the later years my attention switched from low temperature to drought and to the more general examination of plant drought resistance mechanisms on various physiogical and molecular levels. As our results obtained from the chilling-stressed plants strongly suggested the better adaptability of the PS II complex in hybrids to low temperature, I wanted to know whether the same applies for the conditions of water deficiency. Should this be the case, our conclusions based on the behaviour of hybrids under cold stress could perhaps be considered to be of an even more general value. I continued to work with maize as the optimum model for heterotic evaluations as well as the species that is susceptible to drought-induced stress.

The first published paper of my Laboratory dealing with this type of stress (**Holá *et al.* 2010**) was based on the examination of the set of five inbred lines and ten F_1 hybrids. We measured some photosynthetic parameters suggested by various scientists as good secondary physiological markers in plant breeding for improved drought resistance in order to ascertain whether the parents showing good photosynthetic efficiency under conditions of water deficiency will transfer this feature to their progeny. We found that this was not the case. The non-additive (dominant, maternal) genetic effects were again at least as important for the inheritance of the evaluated parameters as the additive ones. Interestingly, this time the dominance detected by the quantitative genetic analysis had quite often a negative character, making the F_1 hybrids less photosynthetically effective than their inbred parents. Similarly to the chilling-stressed maize, the relative importance of individual genetic effects changed between control and stressed plants, clearly indicating that the relationship between parents and progeny, assessed using photosynthetic measurements under optimum conditions, cannot be approximated to the conditions of water deficiency. Moreover, there was no significant correlation between the response of hybrids and their respective maternal/paternal parents to this type of stress, which suggested that the practical utilisation of photosynthetic parameters as secondary (physiological) markers in breeding programmes aimed at the improvement of maize resistance to drought is rather limited. In this, the situation was similar to that observed for low-temperature stress: the non-additive genetic effects can indeed greatly complicate the prediction of the progeny behaviour based on the evaluation of their parents. The changes in genetic effects participating in the inheritance of photosynthetic parameters with plant exposure to different environments almost completely devaluate the potential usability of these parameters for such purposes. Although discouraging, this view should be taken into an account when proposing photosynthesis as a valuable physiological marker in plant breeding. In my opinion, the employment of photosynthetic parameters to simply assess drought resistance of some genotype is indeed useful, but to expect that a genotype selected by such method will always be able to transfer this ability into its progeny is very unrealistic.

The examination of 15 genotypes mentioned in the previous paragraph (as well as later examination of 25 maize inbred lines, the results of which are as yet unpublished other than as a part of the Ph.D. thesis of one of my students) exposed another interesting phenomenon. In general, plants responding to drought stress are expected to rapidly initiate a closure of their stomata in order to diminish water loss, and this ability is usually being correlated to improved drought resistance. However, one of our inbred lines showed very good resistance to this stressor (based on the evaluation of various drought-resistance indices associated with biomass production) but did not close its stomata not only under mild drought but also under more severe conditions of water deficiency. This was intriguing and we undertook to examine its behaviour in more detail. We conducted a comparative analysis of various parameters describing plant morphology, water status, photosynthesis, antioxidant systems and a leaf proteome in this inbred line as well as in another one that showed a more "typical" behaviour under drought conditions (*i.e.*, an early stomatal closure). The results of this analysis enabled us to propose a hypothesis that the early stomatal closure can in fact diminish the ability of plant to cope with drought, because it leads to early inhibition of photosynthesis and, consequently, to less efficient synthesis of various proteins (particularly those

of various detoxification systems). On the other hand, the ability to maintain stomata open even under an insufficient supply of water leads to elevated proteosynthesis (enabled by the fully functional photosynthetic processes and concerning again mainly the protective proteins) and thus confers better resistance to drought. This was published as the paper **Benešová *et al.* (2012)**.

We continued with this direction of research and made a similar, even more complex analysis of the behaviour of these two inbred lines of maize as well as their reciprocal F_1 hybrids under conditions of more severe drought stress. This analysis included many parameters of plant physiology not examined in the previous studyand again evaluated also the changes in a leaf proteome using the iTRAQ method (**Holá *et al. submitted 2017***). The strategies for the response to water deficiency in both parents remained more-or-less similar to the strategies we observed under less severe drought conditions (described in the previous paragraph). The ability to keep its stomata open and to maintain normal photosynthetic efficiency and active proteosynthesis despite water limitation was the main cause for drought resistance of the same parental line as in the previous study. This was probably caused by its smaller ratio of shoot to root biomass and smaller leaves, resulting in reduced loss of water. Another features of this genotype that were advantageous for its good resistance to drought were associated with its PS I complex and the activity of APX. On the other hand, the parental line sensitive to drought was genetically predisposed towards a greater shoot size. This feature necessitated its early stomatal closure which led to the disadvantage under drought conditions associated with an insufficient supply of energy by photosynthesis and a decrease in protein amounts, evident particularly for the proteins of the photosynthetic carbon fixation cycle. Moreover, this inbred line was further handicapped by reduced amounts of the PS I electron acceptors and destabilisation of its PS II complex. Even its elevated accumulation of osmoprotectants was not sufficient to overcome all these negative attributes. However, the most interesting patterns of drought response were found in the F_1 hybrids. The hybrid that had the sensitive inbred line as the maternal parent showed even more pronounced sensitivity to drought and strongly reduced proteosynthesis associated with an early stomatal closure than this line. Chloroplast ribosomal proteins were particularly negatively affected in this hybrid. Some other negative features of the photosynthetic apparatus (*e.g.*, high damage to its OEC, reduced excitonic connectivity between individual PS II complexes) also contributed to the inability of this genotype to acclimate to drought, which was really outstanding. Again, its exceptionally large biomass was probably the original cause for this phenomenon. The second F_1 hybrid had a slightly smaller leaf area and lost less water from its leaves. Although this hybrid contained greater amounts of protective dehydrins and displayed almost no protein degradation, drought still caused negative effects on its morphology and physiology. This was due to various negative aspects of photosynthetic processes clearly inherited from its paternal parent and associated with the PS I complex, the proteins of carbon fixation or the instability of PS II. Some features of its light-harvesting antennae perhaps also contributed to its inferior performance under drought conditions.

We concluded that in case of water deficiency stress, the genetic predisposition of F_1 hybrids towards a larger size is a disadvantage. The greater reduction in their photosynthetic efficiency, induced by an early closure of stomata (caused by a need to diminish water loss from a large shoot) is always in the background of this disadvantage. This is in strong contrast to our observations made in cold-stressed maize plants; evidently, a particular stress type has to be taken into an account when proposing some hypothesis on the physiological/molecular basis of heterosis in plants. Moreover, the precise consequences (and causes) of the original predisposition towards increased biomass accumulation can differ even between closely related genotypes (incidentally, this shows that both reciprocal F_1 hybrids should always be analysed in experiments dealing with various aspects of heterosis). In some hybrids the negative effects of drought can be associated with reduction in proteosynthesis (which would contradict the proposal of Goff and his colleagues, mentioned in the introduction to this chapter), in others they are not and are associated with other attributes.

Thus, our experiments with plants stressed by low temperatures or water deficiency clearly demonstrated that these two types of stressors can influence parent/hybrid relationships (and, consequently, manifestation of the heterotic effect in the F_1 generation) very differently. In other words: what applies for one type of stress, does not necessarily apply for another one. In my opinion, it would be very interesting to find out what is the situation in plants subjected to other stressors, *e.g.*, salinity, the excess of water, exposition to some toxic elements/molecules, *etc*. This will probably be one of the areas my research will be focused on in the future. Currently, my

Laboratory is evaluating the relationship between maize inbred lines and their F_1 hybrids in plants experiencing hypoxia due to waterlogging and the first publication on this subject can hopefully be expected soon. Combinations of various stress factors and their effects on the intraspecific variability (the parent/hybrid relationship) in photosynthetic or other physiological traits, together with an analysis performed on the molecular level could also bring new interesting data related to possible causes of heterosis. In each case, I would always like to make the most complex analysis possible, *i.e.*, to examine our experimental plants on various levels of their morphology, physiology and cell/molecular biology, because only such thorough evaluation can, in my opinion, give an accurate picture of the whole subject.

Another problem worth attending is the examination of the ability of hybrids (as compared with their parents) to withstand not only stress *per se*, but also to recover from stress. The ability of plants to recover rapidly from the damage caused by some stressor is equally (or even more) important for the agricultural practice as the plant efficiency in dealing with stress itself. The susceptibility of individual genotypes to stress factors and the subsequent recovery can be at least partially independent (as demonstrated in case of our chilling-stressed maize), suggesting that the selection for improved genotypes should require the independent selection for both stress resistance and the capacity to recover. As already stated, photosynthesis can provide an excellent information on plant performance under stress and post-stress conditions, although its utilisation for the prediction of the behaviour in subsequent progeny generations is rather limited. The detailed analysis of the changes in genetic effects participating in the inheritance of photosynthetic traits in plants grown under control, stressed *and* recovery conditions has not yet been performed and would be perhaps worth further attention.

Thirdly, we are now starting to examine what happens in plants that are subjected to a repeated exposure to the same stressor. The consecutive cycles of repeated stress and recovery occur under natural conditions more often than not. An interesting phenomenon called "priming" has been observed in connection with this stress/recovery cycling. According to the "priming" hypothesis, the first exposure of plant to stress conditions can somehow leave an imprint which then causes the different response (usually better acclimation) during following stress period(s). However, the nature of this imprint is unknown and the possible mechanisms by which such "priming" could occur are currently being only speculated on (*e.g.*, accumulation of various protective compounds – proline, soluble sugars, *etc*. – that would persist till and during the second exposure to stressful conditions, persisting changes in cell Ca^{2+} concentration, accumulation of specific signalling proteins or transcription factors, generation of oxylipins, up-regulation of antioxidant enzymes or some epigenetic changes associated with histone/DNA modifications or small RNAs). Does experiencing the first stress period really "prime" plants so that they will be better prepared for the second one, as is postulated by some scientists? Should this be the case, do genotypes that are sensitive or resistant to the respective stressor differ in their "priming" ability? Which changes occurring on molecular/cell/organ/whole plant levels are responsible for such differences? Does "priming! of plants for some stressor help them also when they encounter another type of stressor? These are questions that my Laboratory will probably be trying to answer in the following years, because there is currently a very little reliable information on this subject and the existing studies usually present ambiguous results. The answers (if obtained) could be interesting not only for fundamental plant science but also for practical plant breeding, particularly in the near future with its expected climatic changes and the more frequent occurrence of alternating stress periods that is predicted.

Part 3

Plant steroids in the regulation of photosynthesis: the intraspecific and interspecific variability

Photosynthesis is a complex process regulated by many extrinsic and intrinsic factors. Many intricate relationships between photosynthesis and various cell signalling and regulatory pathways still await their complete deciphering. One example is the relationship between the photosynthetic processes and sterol compounds that are present in plant organism. The best examined group of these sterols is called brassinosteroids after the plant species they were first isolated from (Grove *et al.* 1979). Now, almost 40 years after their initial discovery, brassinosteroids are postulated to be involved in various important processes occurring in plants. They regulate plant growth (both cell elongation and cell division), particularly in young vegetative tissues such as epicotyls, hypocotyls or coleoptiles but their growth-promoting effect applies also to more developed stems, leaves, roots and reproductive organs. Other plant processes they are involved in include gravitropic response, differentiation of vascular system (xylogenesis), seed germination, circadian rhythm signalling, photosynthesis and nitrogen metabolism, plant response to various biotic and abiotic stress factors, regulation of senescence, *etc.* (Gudesblat and Russinova 2011, Hao *et al.* 2013, Fridman and Savaldi-Goldstein 2013, Fariduddin *et al.* 2014, Oklešťková *et al.* 2015, Singh and Savaldi-Goldstein 2015, Vardhini and Anjum 2015, Wei and Li 2016).

The evidence supporting these statements seems to be more than ample. Studies conducted in plant model species, usually utilising various mutants in brassinosteroid synthesis/perception, have significantly contributed to our current knowledge on the molecular mechanisms and signalling processes involved in brassinosteroid-regulated plant morphogenesis (Choudhary *et al.* 2012, Gruszka 2013, Guo *et al.* 2013, Zhu *et al.* 2013, Belkhadir and Jaillais 2015, Clouse 2015, Lozano-Durán and Zipfel 2015, Singh and Savadi-Goldstein 2015, Jaillais and Vert 2016). Other analyses have been performed with plants treated with exogenously applied brassinosteroids or (less frequently) with inhibitors of brassinosteroid synthesis (*e.g.*, brassinazole). These provided the majority of data on the participation of brassinosteroids in various physiological and metabolic processes, that are currently available. Such studies have usually been carried out in various agronomically important plant species and the potential target of their authors has been the practical utilisation of the acquired knowledge to improve total plant yield, stress resistance or other target traits. Indeed, brassinosteroids seem to be destined for the agricultural use, because they are nontoxic, nonmutagenic and environmentaly friendly. Moreover, they are effective in extremely low concentrations, can be easily applied (by either soaking of seeds in their solutions or foliar spraying of more mature plants) and now it is even possible to artificially synthesise them on a commercial scale (Kang and Guo 2011, Bhardwaj *et al.* 2012, Zhang *et al.* 2014).

The exogenous application of brassinosteroids usually (although not always) results in the improvement of plant photosynthetic efficiency. It is possible that these compounds regulate the CO_2-concentrating mechanisms, change the development of chloroplasts, affect the expression of various photosynthetic genes, regulate the activities of enzymes of photosynthetic carbon fixation, influence the synthesis/degradation of light-harvesting pigments or the efficiency of primary photosynthetic processes. Some time ago, I was invited to contribute a chapter to a book on brassinosteroids and decided to gather information then available on the functional relationship between these compounds and the photosynthetic processes. The copy of this chapter (**Holá 2011**) follows after the introduction to this part of my thesis and offers more details on this topic. Of course, after the date of its publication various new studies appeared, but they mostly did not bring any particularly revolutionary information that would change my previous view of the role of brassinosteroids in the regulation of photosynthesis. Three exceptions from this statement exist. The regulation of redox signalling by brassinosteroids has since been shown to significantly participate in the effect these compounds have on plant photosynthetic efficiency (Jiang *et al.* 2012a, b, 2013, Cheng *et al.* 2014). The molecular mechanisms of brassinosteroid-induced regulation of the opening/closure of stomata have been partially resolved and have been shown to strongly depend on a particular developmental stage or a type of plant organ (Kim *et al.* 2012, Kong *et al.* 2012, Serna 2013, Xia *et al.* 2014, Wang *et al.* 2015, Shang *et al.* 2016). Finally, some new studies presenting a more detailed information on the changes in thylakoid membrane organisation caused by brassinosteroids were also published since (Krumova *et al.* 2013,

Dobrikova *et al.* 2014). However, many aspects of the relationship between brassinosteroids and photosynthesis remain unresolved and there is still much to do in this area of research.

The scientific papers that present some data on the efficiency of photosynthetic processes in brassinosteroid-treated plants or mutants usually greatly vary regarding their overall design, cultivation conditions, the examined plant species, the developmental stage, *etc*. Moreover, although many papers dealing with this topic have been published, their authors usually analysed only one genotype/variety/cultivar of the respective plant species (in fact, individual scientific groups usually have some favourite cultivar which they use for all analyses). Studies examining the genotypic differences in the response of the plant photosynthetic apparatus to brassinosteroids are not precisely common and some of them are rather problematic (and published in dubious journals). *Table 3* summarises the main aspects of scientific papers that presented data on brassinosteroid action on some photosynthetic trait, and in doing so, have evaluated more than one genotype (mutants in brassinosteroid synthesis or perception are not included in this table; however, such studies concerning photosynthesis are extremely rare). The overwhelming majority of these studies has dealt with plants subjected to some unfavourable environmental condition (drought, salinity, high or low temperature, heavy metals, low irradiance, bacterial infection) and usually only two (tolerant/sensitive) cultivars were compared. With some exceptions, 24-epibrassinolide or 28-homobrassinolide were mostly used for the respective treatments, in concentrations ranging from nanomolar to milimolar, and they were applied mostly by spraying of plants with their aqueous solutions. The important conclusion that can be made from these studies is this: in almost all cases, the authors found that the different genotypes differ in their response to brassinosteroid application. Any analysis that is made on a single genotype (or, indeed, a single plant species) in any study on brassinosteroid action thus significantly narrows our understanding of this phenomenon and could also limit potential practical application of these compounds.

Table 3. Major features of studies evaluating the effects of brassinosteroid application on photosynthetic traits in different cultivars/varieties/genotypes of various plant species. EBL = 24-epibrassinolide, HBL = 28 homobrassinolide.

Plant species	Analysed genotypes	Analysed traits	Brassinosteroid application	Plant growth conditions	Reference
Wheat	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll content	Foliar or seed, 20 to 100 μM HBL	Drought stress	Sairam *et al.* 1994
Wheat	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll *a* content, chlorophyll fluorescence parameters (F_v/F_m), intercellular CO_2 concentration	Seed, 50 nM to 0.15 μM EBL	Salt stress	Ali *et al.* 2008
Wheat	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll *a, b* content, chlorophyll fluorescence parameters (F_v/F_m), intercellular CO_2 concentration	Foliar, 25 to 75 nM EBL	Salt stress	Shahbaz *et al.* 2008
Tomato	2 (non-specified)	Net photosynthetic rate, chlorophyll content, carbonic anhydrase activity, intercellular CO_2 concentration	Foliar, 10 nM EBL or HBL	Cd stress	Hayat *et al.* 2010
Tomato	2 (tolerant/ sensitive)	Net photosynthetic rate, maximum photosynthetic rate, light saturation point, chlorophyll *a, b* content, chlorophyll fluorescence parameters (F_v/F_m)	Foliar, 2 nM to 0.2 μM EBL	Low irradiance stress	Wang *et al.* 2010
Tomato	2 (normal/ abscisic acid-deficient)	Net photosynthetic rate, intercellular CO_2 concentration	Foliar, 1 μM EBL	Drought stress	Yuan *et al.* 2010
Tomato	2 (non-specified)	Net photosynthetic rate, chlorophyll content, carbonic anhydrase activity, intercellular CO_2 concentration	Foliar, 10 nM EBL or HBL	Cd stress	Hasan *et al.* 2011
Soybean	2 (non-specified)	Net photosynthetic rate, chlorophyll fluorescence parameters (OJIP)	Foliar or seed, 2 μM or 0.5 μM EBL	Drought stress	Janeczko *et al.* 2011
Oilseed rape	2 (non-specified)	Chlorophyll fluorescence parameters (F_v/F_m, OJIP)	Foliar, 20 μM EBL	Bacterial infection	Skoczowski *et al.* 2011
Wheat	5 (non-specified)	Net photosynthetic rate, chlorophyll content, chlorophyll fluorescence parameters (F_v/F_m), carbonic anhydrase activity, intercellular CO_2 concentration	Foliar, 10 nM HBL	Ni stress	Yusuf *et al.* 2011

Table 3 continued

Plant species	Analysed genotypes	Analysed traits	Brassinosteroid application	Plant growth conditions	Reference
Wheat	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll content	Foliar, 0.5 to 2 µM brassinolide	Drought stress	Dhayal *et al.* 2012
Tomato	2 (non-specified)	Net photosynthetic rate, chlorophyll content, carbonic anhydrase activity, intercellular CO_2 concentration	Foliar, 10 nM EBL or HBL	Cd stress	Hayat *et al.* 2012
Groundnut	2 (spreading/ semispreading type)	Chlorophyll content	Into medium, brassinolide (concentration non-specified)	Normal	Verma *et al.* 2012
Mungbean	2 (tolerant/ sensitive)	Chlorophyll content, carbonic anhydrase activity	Foliar, 0.1 nM to 1 µM EBL	Ni stress	Yusuf *et al.* 2012
Papaya	2 (non-specified)	Chlorophyll content, chlorophyll fluorescence parameters (F_v/F_m)	Foliar, 0.2 µM spirostane analogue	Drought stress	De Assis Gomes *et al.* 2013
Cucumber	2 (slicing/ pickling type)	Net photosynthetic rate, chlorophyll content, chlorophyll fluorescence parameters (F_v/F_m), carbonic anhydrase activity, intercellular CO_2 concentration	Foliar, 10 nM EBL	Salt and Cu stress	Fariduddin *et al.* 2013
Sunflower	6 (non-specified)	Net photosynthetic rate, chlorophyll content	Foliar, 0.1 mM EBL	Cu stress	Filová *et al.* 2013
Tomato	2 (non-specified)	Net photosynthetic rate, chlorophyll content, carbonic anhydrase activity, intercellular CO_2 concentration	Root, 10 nM EBL or HBL	Cd stress	Hasan *et al.* 2013
Mungbean	2 (tolerant/ sensitive)	Net photosynthetic rate	Foliar, 0.2 to 2 mM brassinolide	Drought stress	Lal *et al.* 2013
Melon	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll content, chlorophyll fluorescence parameters (F_v/F_m, Φ_{PSII}, F_v'/F_m', q_P, NPQ), intercellular CO_2 concentration	Foliar, 1 to 3 µM EBL	Heat stress	Zhang *et al.* 2013
Sunflower	3 (non-specified)	Chlorophyll content	Foliar, 10 nM or 1 µM HBL	Drought stress	Filová 2014
Wheat	4 (2 tolerant/ 2 sensitive)	Chlorophyll content, chlorophyll fluorescence parameters (F_0, F_v/F_m, Φ_{PSII}, ETR, q_P, Φ_{NPQ}, $\Phi_{f,d}$, F_0 *vs* temperature curve), gene expression (*RbcL, RbcS, OEC, PsbP, PsbO*)	Foliar or seed, 50 nM or 100 to 1 µM brassinosteroid (non-specified)	Heat stress	Hairat and Khurana 2015
Mungbean	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll content, chlorophyll fluorescence parameters (F_v/F_m), carbonic anhydrase activity, intercellular CO_2 concentration	Foliar, 10 nM or 1 µM HBL	Ni stress	Yusuf *et al.* 2014
Pistachio	5 (non-specified)	Chlorophyll *a, b* content, chlorophyll fluorescence parameters (F_0, F_m, F_v, F_v/F_m)	Foliar, 0.1 nM to 1 µM HBL	Normal	Farazi *et al.* 2015
Mungbean	2 (tolerant/ sensitive)	Net photosynthetic rate, chlorophyll content, intercellular CO_2 concentration	Foliar, 0.1 nM to 1 µM HBL	Normal	Alyemeni and Al-Quwaiz 2016
Tobacco	3 (different tolerance to Cr)	Net photosynthetic rate, chlorophyll (total, *a, b*) and carotenoids content, chlorophyll fluorescence parameters (F_v/F_m), intercellular CO_2 concentration, chloroplast development/ultrastructure	Foliar, 0.1 µM EBL	Cr stress	Bukhari *et al.* 2016
Rye	2 (tolerant/ less tolerant)	Chlorophyll fluorescence parameters (OJIP), Rubisco activity	Foliar, 0.5 µM EBL	Cold acclimation	Pociecha *et al.* 2016
Maize	2 (non-specified)	Rubisco activity	Foliar, 0.2 µM EBL	Drought stress	Talaat and Shawky 2016
Safflower	3 (non-specified)	Chlorophyll content	Foliar, 0.1 µM EBL	Drought stress	Zafari and Ebadi 2016

My own research on brassinosteroids in relation to the genetic variability in photosynthetic characteristics started some ten years ago. Steroid chemists from the research group then existing at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, were interested in the biological testing of some of their new synthetic analogues of brassinosteroids and I was interested in the question whether the inbred and hybrid genotypes respond to these compounds in a similar manner. This initial cooperation was based mostly on the long-term field studies conducted with maize plants and resulted in two published papers that are included in this part of my thesis.

The four-year analysis of plant morphology, flowering and yield performed in two field-grown inbred lines of maize and their F_1 hybrid subjected to the exogenous application of 24-epibrassinolide or the synthetic androstane analogue of castasterone either early or later during plant vegetative development was summarised in the paper **Holá *et al.* (2010)**. It indicated that both examined brassinosteroids could affect maize growth initially after their application but that this effect did not persist: the final plant height and the number of leaves in the brassinosteroid-treated plants was not significantly different from that in the non-treated plants. The brassinosteroid-associated effects on plant morphology and yield depended on the developmental stage in which these compounds were applied and also on the respective genotype. A very interesting observation was related to the brassinosteroid effect on the time plants begun to flower: when applied at the earlier stage of plant development, brassinosteroids delayed both anthesis and silking (and decreased the number of female inflorescences), whereas when applied at the later stage of plant development, the situation was completely opposite. This was a completely new finding because no one has previously reported such influence of brassinosteroids on the timing of the development of female reproductive organs and only one earlier study described an earlier emergence of the male inflorescence in some maize genotypes as the effect associated with brassinosteroids. This role of brassinosteroids in the regulation of the flowering course could perhaps be utilised in practical maize breeding, *e.g.*, for the synchronisation of flowering of various genotypes as necessary. Moreover, both our inbred lines responded to brassinosteroids more strongly in regard to the development of secondary ears compared to their F_1 hybrid. Actually, the response of one inbred line to brassinosteroids was often the opposite to the response of the second inbred line, and the hybrid usually followed its paternal parent in this aspect.

The accompanying study conducted during the same time period with the same genotypes and steroid compounds focused on the effect of brassinosteroids on selected photosynthetic parameters. Its results were published in the same year in the paper **Kočová *et al.* (2010)**. Contrary to our original expectations, we found that brassinosteroids applied to the field-grown maize plants do affect neither the efficiency of primary photosynthetic processes in their leaves nor the content of photosynthetic pigments. No genotype×treatment interaction was, of course, observed in this case. This led us to speculate that the photosynthetic electron transport chain is not the primary site of brassinosteroid action in maize under non-stress conditions (although it can be in some other plant species or in unfavourable environments; see below).

At the same time we also experimented with young, greenhouse-grown maize plants stressed by low temperature and tried to improve their photosynthetic efficiency by the application of brassinosteroids. The results of this study were published in another paper included in this part of my thesis, **Honnerová *et al.* (2010)**. Again, neither the efficiency of the Hill reaction nor the PS I activity were affected by the exogenous application of 24-epibrassinolide or the synthetic androstane analogue of castasterone. However, the content of chlorophylls in leaves of the chilling-stressed plants positively responded to extremely low (0.01 pM) concentration of both tested steroids (with the synthetic analogue having a greater effect compared with 24-epibrassinolide) suggesting that the synthesis of photosynthetic pigments can possibly be regulated by brassinosteroids in this plant species but only under unfavourable environmental conditions such as low temperature.

Brassinosteroids are not the only sterol compounds that are naturally present in plants. Progesterone, a typical sex steroid of mammals, has also been shown to be synthesised in various plant species, together with substances similar to 17β-estradiol, and the same applies for other mammalian sex hormones such as androsterone or testosterone. Similarly to brassinosteroids, they affect plant growth, flower development, pollen germination and pollen tube growth but it seems that they can also influence other plant processes such as the response to biotic or abiotic stressors or photosynthesis (Janeczko and Skoczowski 2005, Speranza 2010, Janeczko 2012, Janeczko *et al.*

2012, 2013a, b, Lindemann 2015). Some plant species contain and synthesise also ecdysteroids, sterol compounds originally found in insects. They are present mostly in young organs, flowers or seeds. Their function in plants is largely unknown, but they are usually proposed to be involved in the plant defence against various phytophages or to act as allelochemicals (Dinan 2001, 2009, Dinan *et al.* 2009, Tarkowská and Strnad 2016).

In 2008, some steroid chemists from the Institute of Organic Chemistry and Biochemistry and the Institute of Chemical Technology, Prague published a paper which demonstrated that the Rubisco protein can directly bind ecdysteroids and that this interaction increases carboxylation yield of the Rubisco activity *in vitro* (Uhlík *et al.* 2008). They continued to identify other possible protein targets for ecdysteroid binding in plants and found that some proteins of PS II OEC are also able to specifically interact with these oxysterols under *in vitro* conditions (unpublished data). They wanted to know whether such interactions can improve the photosynthetic efficiency also *in vivo* and because this seemed a very exciting possibility (it would reveal a completely new role for ecdysteroids in plants), a new period of my research was initiated.

We soon demonstrated that ecdysteroids can indeed have a positive effect on photosynthesis in living plants. The exogenous application of 20-hydroxyecdysone in a "natural" (mM) concentration improved, at least temporarily (in a short-time range), the net photosynthetic rate in New Zealand spinach. The further analysis of photosynthetic characteristics revealed that this improvement could be at least partially caused by a better activation of OEC; the changes in the functionality of this complex in our experiments correlated rather well with the observed changes in the net photosynthetic rate. This further supported the possibility of the direct activation of OEC by ecdysteroids (as suggested by the *in vitro* observations of its high binding affinity to the OEC proteins). However, other components of the photosynthetic electron transport chain or photosynthetic pigments were not affected by this oxysterol. The results of these experiments were published in the paper **Holá *et al.* (2013)** and, at least for me, were rather encouraging and suggested a new direction my research on plant steroids would probably take.

However, the concentration of 20-hydroxyecdysone used in the above-described study was still rather high, close to that naturally occurring in plants but unsuitable for potantial future practical application in agriculture. Thus, we wanted to examine whether the ecdysteroids applied in a much lower concentration (similar to that usually used for brassinosteroids) will also have a significant effect on the efficiency of photosynthetic apparatus. We decided to compare two plant species, common spinach as a plant with the naturally high content of ecdysteroids, and maize, a plant in which these steroids do not naturally occur in detectable levels. We also tested the effect of 24-epibrassinolide and 20-hydroxyecdysone applied separately or jointly. We showed that the lower concentration (0.1 nM) of 20-hydroxyecdysone is indeed effective but that the response of the plant photosynthetic apparatus depends on plant species. In common spinach, the effect was clearly negative, which applied both for the net photosynthetic rate and for the parameters of the primary photosynthetic processes (with PS I and OEC being the most strongly affected). The other species examined (maize) responded to the exogenous application of 20-hydroxyecdysone to a much smaller degree. The negative effect observed in common spinach was rather interesting and led us to speculate that this species needs to maintain a delicate balance in the content of ecdysteroids (having a naturally high content of these compounds) and even a slight deviation from the optimum (induced by their exogenous addition) can result in the changes in the efficiency of photosynthetic processes. For other plant species that do not contain ecdysteroids, higher concentrations of these compounds would be probably needed to be applied in order to have their effect on photosynthesis manifested. We also found that there were some developmental differences in the response of photosynthetic complexes to ecdysteroids (mature leaves showing a more pronounced response compared to young ones). The joint application of both types of oxysterols in nanomolar concentrations to spinach or maize resulted in the diminishion of the effects these compounds had on the primary photosynthetic processes and the overall photosynthetic efficiency when applied individually. This suggested either some direct competition, mutually antagonistic interactions, or perhaps some indirect effects of one type of oxysterol on the other one, which could be on the level of synthesis/degradation or some other mechanism. The paper **Rothová *et al.* (2014)** presents the above-described results and conclusions in a more detail.

Further investigation into the matter of the possible relationship between brassinosteroids and ecdysteroids in plants brought another set of interesting results (**Kamlar *et al.* 2015**). One of

our hypotheses at the beginning of our research postulated that the well-known effects of brassinosteroids on photosynthesis probably cannot be explained by the direct interactions of these compounds with photosynthetic proteins (because brassinosteroids have been known to act in extremely low concentrations). However, brassinosteroids could act as the regulators of the levels of final effector(s) and this final effector could be some ecdysteroid, as suggested by the results of the *in vitro* and *in vivo* studies mentioned above. We thus assessed whether the treatment with brassinosteroids causes any change in the ecdysteroid levels in plant leaves (with common spinach as an experimental model). We confirmed that such is indeed the case and that the exogenous application of 24-epibrassinolide resulted in the changed levels of several major and minor ecdysteroids present in this species. The nature of these changes depended on the concentration of 24-epibrassinolide used for treatments, on the age of treated leaves (young *vs* older) and differed also between various types of ecdysteroids (20-hydroxyecdysone, polypodine B, ajugasterone C, ponasterone A, stachysterone C). The exact relationship between the individual brassinosteroids and ecdysteroids therefore seems to be worth further attention.

Both brassinosteroids and ecdysteroids will certainly remain at the centre of my scientific interest in the near future. Our experiments presented in this part of my thesis, together with the detailed study of literature associated with these topics, opened for me various new questions that, in my opinion, deserve answers. One area I would like to concentrate on is the relationship between brassinosteroids and the primary photosynthetic processes. The potential participation of brassinosteroids in the regulation of this part of photosynthesis is currently being rather ignored. Most studies that provide some data on the efficiency of the photosynthetic electron transport chain in connection with the excess or lack of brassinosteroids do not do so as a main purpose and usually show only the results of the simplest analysis of the maximum quantum efficiency of PS II (the F_v/F_m parameter derived from chlorophyll fluorescence measurements). What is perhaps worse, they have been mostly conducted in only two plant species (cucumber and tomato) and often in plants exposed to some unfavourable environment. Our analysis of the effect of 24-epibrassinolide on the efficiency of primary photosynthetic processes in maize and spinach clearly revealed not only that the individual parts of the photosynthetic electron transport chain can respond to this brassinosteroid differently, but that different plant species can provide very different results. We also suspect that the positive effects of brassinosteroids on the primary photosynthetic processes, presented in the scientific literature as an established fact, have more to do with the reality that the evaluated plants were subjected to some stressor or manifested symptoms of senescence. This would mean that the effects that were observed in, *e.g.*, chlorophyll fluorescence parameters could in fact be only of a secondary character. We think that under non-stress conditions such effects will be seen more rarely and will strongly depend on plant species (this is partially supported by our earlier work with field- or greenhouse-grown maize). Thus, we have decided to start a systematic study of the effect of oxysterols on various plant species grown under similar, non-stress conditions; this analysis is currently well under way. A comparison of the response of the dicotyledonous and monocotyledonous plants to various types of sterols will be particularly interesting, because these two plant groups differ in the final steps of brassinosteroid synthesis and contain different endogenous sterols.

The relationship between brassinosteroids and ecdysteroids (or, possibly, also some other plant steroids), together with the more detailed examination of the potential role of ecdysteroids in the regulation of photosynthesis *per se* (*i.e.*, not only the primary photosynthetic processes) will be probably another research topic my Laboratory will be engaged in during the coming years. This is a research area that is yet almost completely blank, so any data we will obtain, no matter whether negative or positive, should be of an interest to the community of plant sterol scientists.

Besides the interspecific variability, I would like to return to/continue with the assessment of the genetic variability in the response to brassinosteroids *within* one species, particularly in relation to plant resistance to stressors such as drought. We will probably continue with the experiments designed to use exogenously applied brassinosteroids, because the utilisation of brassinosteroid mutants is, in my opinion, not particularly suitable for this purpose (all mutants described so far show either dwarf phenotype or are at least significantly smaller than *wild type*, which would strongly bias the obtained results, because the size/general morphology of plants is of a very great importance in the plant drought response). Recently, I have made a very thorough survey of the scientific literature dealing with the effect of brassinosteroids on drought-stressed plants (I hope to publish this as a review paper in some scientific journal) and I was dismayed by

the discovery of how many of these studies suffer from various shortcomings, often very serious ones. The data presented in these papers have frequently not been subjected to any statistical evaluation or have not been based on true biological replicates of experiments, the statements/conclusions in the text often did not agree with the documentation of the results, many studies did not present the necessary information on plant cultivation conditions, the age/developmental stage of plants at the start of the drought period, at the time of the brassinosteroid treatment or at the time of the measurements of the respective parameters, *etc*. Moreover, I think that the changes observed by the authors of these papers and attributed to the role of brassinosteroids in plant drought response are in fact of a more general, not drought-specific character, because they were mostly already induced in plants not yet experiencing drought. I do not mean to diminish the value of such studies – they were usually aimed at ascertaining whether the application of brassinosteroids can improve plant drought resistance in case plants ever encounter this stressor, and as such they are perfectly valid. However, in order to learn more about the mechanisms by which these hormones could specifically regulate plant drought response, the experiments should be designed differently than they were in most published studies. Additionally, the overwhelming majority of brassinosteroid/drought analyses focused on a relatively small number of parameters. No one has yet tried to make a really complex study which would assess parameters describing various aspects of plant morphology, water status, photosynthesis, cell damage, main protective systems, other phytohormones, gene expression, *etc.*, and at the same time would focus not only on one genotype of one species, but would also take into an account possible intra- and interspecific variability. This is of course a very ambitious wish of mine; however, I believe that in order to obtain a true picture of the role of brassinosteroids in the regulation of plant drought response, resistance or sensitivity, such approach is really necessary. If at all possible, I would like to engage in achieving such a long-distance goal in my future career as a scientist.

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