Univerzita Karlova v Praze
Přírodovědecká fakulta
Katedra fyziologie rostlin

Plasticita ultrastruktury chloroplastů a fotosyntetická aktivita listů

Ph.D. práce

RNDr. Anna Vičánková

Praha 2006

Školitel:

Doc. RNDr. Jaromír Kutík, CSc.
Katedra fyziologie rostlin
Univerzita Karlova v Praze

Konzultanti:

RNDr. Dana Holá, Ph.D.
Katedra genetiky a mikrobiologie
Univerzita Karlova v Praze

RNDr. Jana Opatrná, CSc.
Ústav experimentální botaniky
Akademie věd České republiky

Práce je součástí grantového projektu GA UK 103/1998/B BIO/PřF „Ultrastruktura chloroplastů a fotosyntetická aktivita listů kukuřice na různých místech listové čepele a v průběhu cirkadienního cyklu", projektu J 13/98113100004 „Ekologické a evoluční aspekty rozmanitosti organismů" Ministerstvo školství, mládeže a sportu, a projektu GAČR 206/03/0369.

V Praze 16. 5. 2006 **Anna Vičánková**

Na tomto místě bych chtěla poděkovat mému školiteli Doc. RNDr. Jaromíru Kutíkovi, CSc., konzultantům RNDr. Daně Holé, Ph.D. z Katedry genetiky a mikrobiologie PřF UK a RNDr. Janě Opatrné. CSc. z Ústavu experimentální botaniky AV ČR. Zvláštní poděkování patří RNDr. Ivaně Macháčkové, CSc. z Ústavu experimentální botaniky AV ČR, za nezištnou osobní podporu, které si velice vážím. Dále děkuji všem členům Katedry fyziologie rostlin PřF UK i mým kolegům z Ústavu experimentální botaniky AV ČR, kde má práce vznikala, a také mým nejbližším.

Obsah

OBSAH **3**
ÚVODNÍ KOMENTÁŘ **5**
1 ÚVOD A CÍLE PRÁCE **6**
1.1 ÚVOD 6
1.2 CÍLE PRÁCE 8
2 LITERÁRNÍ PŘEHLED **9**
2.1 PLASTIDY A JEJICH KLASIFIKACE 9
2.2 CHLOROPLASTY VYŠŠÍCH ROSTLIN 10
2.2.1 Ultrastruktura a funkce chloroplastů *10*
2.2.2 Dělení chloroplastů *15*
2.2.3 Fotosyntéza *16*
2.3 PLASTICITA ULTRASTRUKTURY CHLOROPLASTŮ 19
2.3.1 Vývoj chloroplastů během ontogeneze listu *19*
2.3.2 Heterogenita ultrastruktury a fotosyntetických funkcí chloroplastů na listové čepeli *26*
2.3.3 Vliv amitrolu na vývoj chloroplastů *28*
2.3.4 Vliv imobilizace a různé hladiny cytokininů na chloroplasty *29*
3 ZÁVĚRY / CONCLUSIONS **31**
4 SEZNAM CITOVANÉ LITERATURY **34**
5 SEZNAM PUBLIKACÍ AUTORKY **46**
5.1 PUBLIKACE, KTERÉ TVOŘÍ PH.D. PRÁCI 46
5.2 OSTATNÍ PUBLIKACE 46

Úvodní komentář

Tato práce je součástí širšího studia dynamiky fotosyntetického aparátu zelených rostlin z pohledu plasticity ultrastruktury buněčných organel – chloroplastů. Doktorskou práci, kterou v této podobě předkládám k obhajobě zakončující mé Ph.D. studium, tvoří soubor šesti publikací doplněných literárním přehledem. Tento přehled uvádí mé publikace do kontextu vědeckého poznání na poli ultrastruktury chloroplastů a podává informace o současném poznání této fotosyntetizující organely.

Chloroplasty byly jako zelené částice v listech rozeznány již sto let před vznikem buněčné teorie. Jejich velikost a zbarvení usnadňují jejich pozorovatelnost. Studium buňky elektronovým mikroskopem umožňujícím sledování ultrastruktury jednotlivých buněčných organel se začalo rozvíjet hlavně po druhé světové válce. V současnosti jsou chloroplasty nejlépe prostudovaným typem plastidů z hlediska fyziologického, genetického, biochemického a molekulárně biologického.

Předkládané práce vznikaly na základě experimentální činnosti od počátku mého Ph.D. studia v roce 1998 na Katedře fyziologie rostlin Univerzity Karlovy v Praze, pod vedením mého školitele Doc. RNDr. Jaromíra Kutíka, CSc. a zejména ve spolupráci s mým konzultantem RNDr. Danou Holou, Ph.D. z týmu genetické zahrady Katedry genetiky a mikrobiologie Univerzity Karlovy v Praze. V průběhu studia, pod vedením mého druhého konzultanta RNDr. Jany Opatrné, CSc. z Ústavu experimentální botaniky AV ČR, jsem k získání kvantitativních hodnot vedle stereologické metody k hodnocení ultrastruktury chloroplastů začala využívat i softwarový systém analýzy obrazu Lucia. Čtyři roky (až téměř do konce roku 2005) jsem pak pracovala v laboratoři RNDr. Ivany Macháčkové, CSc. v Ústavu experimentální botaniky AV ČR. Zde jsem se kromě ultrastruktury chloroplastů v souvislosti s metabolismem cytokininů zabývala také imunodetekcí fytohormonů *in situ*. Tato zkušenost vhodně navazovala na dříve osvojenou metodu imunolokalizace bílkovin koloidním zlatem v laboratoři mého školitele Doc. Jaromíra Kutíka.

1 Úvod a cíle práce

Úvodem své práce chci stručně připomenout hlavní poznatky o zelených plastidech – chloroplastech, které představují na úrovni buněk fotosyntetický aparát vyšších rostlin, a metodách jejich studia, a dále vytýčit cíle předkládané práce.

1.1 Úvod

Plastidy jsou typické buněčné rostlinné organely. Podle jejich přítomnosti nebo nepřítomnosti, dělíme eukaryotické organismy na autotrofní a heterotrofní. V rostlinných buňkách se nacházejí různé typy plastidů, jedním z nich jsou chloroplasty. Tyto buněčné organely zajišťují fotoautrotrofní metabolismus rostlinných buněk. Patří spolu s mitochondriemi mezi geneticky semiautonomní organely endosymbiotického původu.

Poznatky o chloroplastech jako organelách, kde probíhá jeden ze základních životních procesů na Zemi – fotosyntéza (Lawlor 2001), shrnují například Hudák a kol. (1983, 1997), Staehelin a van der Staay (1996).

Chloroplasty byly jako zelené částice v listech rozeznány již sto let před vznikem buněčné teorie. Jejich velikost a zbarvení usnadňují jejich pozorovatelnost. Studium buňky elektronovým mikroskopem, umožňujícím sledování ultrastruktury jednotlivých buněčných organel, se začalo rozvíjet hlavně po druhé světové válce. V současnosti jsou chloroplasty nejlépe prostudovaným typem plastidů z hlediska fyziologického, genetického, biochemického a molekulárně biologického. Je znám úplný plastidový genom kukuřice, tabáku a dalších rostlin (játrovky *Marchantia polymorpha*, rýže atd.).

Plastidy se obecně rozdělují podle zbarvení (Kutík 1985) a jejich vzájemné přeměny (metamorfózy plastidů) jsou běžným jevem pro tyto funkčně i strukturně plastické organely (Thomson a Whatley 1980, Biswal a kol. 2003).

Chloroplasty jsou v posledních letech předmětem studia pomocí molekulárně biologických metod, zejména v oblastech bádání o jejich vzniku primární endosymbiózou fotosyntetizujících prokaryotických buněk (Cavalier-Smith 2000)

v souvislosti se skutečností, že ačkoliv má chloroplast vlastní genom, většina chloroplastových proteinů je jaderně kódována (Bauer a kol. 2001, Bodyl 2002). Chloroplastový proteinový import velmi názorně shrnují Jarvis a Soll (2002). V posledních pěti letech se obracela pozornost i k mechanismům dělení plastidů (např. Reski 2002, Maple a Moller 2005) a k prostorovým rekonstrukcím těchto fascinujících organel (Kiessling a kol. 2000, Shimoni a kol. 2005).

Značná pozornost je stále věnována jak ultrastruktuře chloroplastů na úrovni stavby fotosystémů I a II, enzymatických komplexů thylakoidních membrán (např. Staehelin a van der Staay 1996, Biswal a kol. 2003), tak i architektuře systému thylakoidů (Mustárdy 1996, Shimoni a kol. 2005). V průběhu vývoje listů se ultrastruktura chloroplastů mění. Tyto ontogenetické změny těsně souvisejí se změnami fotosyntézy listu. Poznání vztahů struktury a funkce fotosyntetického aparátu je významné i z hlediska praktického v zemědělství a lesnictví. Vývoj ultrastruktury plastidů bývá většinou studován na ultrastrukturálních změnách při zezelenání etiolovaných listů (přeměna etioplastů v chloroplasty) a dále pak na změnách v průběhu senescence (stárnutí) listů. Studiu přeměny etioplastů ve chloroplasty se důkladně věnovali např. Virgin a Egnéus (1983), Link (1991) a Biswal a kol. (2003). Stárnutí chloroplastů spojené s postupným poklesem fotosyntézy popisují např. Biswal a Biswal (1988) a Smart (1994) a shrnují Biswal a kol. (2003).

Celá řada dosud neobjasněných otázek však vybízí k dalšímu studiu kvantitativních charakteristik ultrastruktury chloroplastů v průběhu přirozeného vývoje listu (od jeho rozvinutí do zežloutnutí). U rostlin s C4 fotosyntézou, jejichž typickou představitelkou je kukuřice, je tento vývoj o to zajímavější, že tyto rostliny disponují dvěma typy chloroplastů, které vzájemně spolupracují a ontogenetický vývoj těchto chloroplastů se dá sledovat podél listové čepele. Problematiku změn ultrastruktury chloroplastů v průběhu ontogeneze listu shrnují Kutík (1985, 1998), Biswal a kol. (2003), u kukuřice se jí zabývali Kutík a kol. (1999).

Na mnoho procesů týkajících se změn vývoje a funkce chloroplastů mají vliv rostlinné hormony cytokininy. Po objevení rostlinného regulátoru kinetinu (Miller a kol., 1955) se začal zkoumat jeho vliv na diferenciaci chloroplastu z proplastidu (Stetler a Laetsch 1965). Exogenně aplikované cytokininy mají ve tmě zejména vliv na vývoj chloroplastů z proplastidů, amyloplastů a etioplastů (Chory a kol., 1994, Kusnetsov a kol., 1994). Výzkum pokračuje i na opětovném zezelenání senescentních chloroplastů pod vlivem cytokininů (Zavaleta-Mancera a kol., 1999 a, b).

Ve své práci jsem používala řadu metod, které jsou podrobně popsány v jednotlivých publikacích a pracích připravených pro publikaci. Za nejdůležitější z nich považuji metody sloužící pozorování ultrastruktury chloroplastů a jejímu kvantitativnímu hodnocení, které bylo základem mé práce. Šlo o transmisní elektronovou mikroskopii (včetně elektronmikroskopické imunocytochemie), stereologii a automatickou analýzu obrazu. Klasickou příručkou první z uvedených skupin metod je kniha Hall (1978), která dává podrobné návody pro fixaci, zalévání a řezání rostlinného materiálu, kontrastování ultratenkých řezů, elektronmikroskopickou cytochemii a některé speciální elektronmikroskopické metody. Stereologie umožňuje kvantitativní hodnocení trojrozměrné stavby objektů pozorovaných pomocí světelné nebo elektronové mikroskopie na prakticky dvojrozměrných řezech. Klasickou metodickou práci zde napsali Gundersen a Jensen (1987). Automatická analýza obrazu se v elektronové mikroskopii zatím používá málo, pro komplikovanost elektronmikroskopického obrazu. Ještě více to platí pro studium chloroplastů.

1.2 Cíle práce

Předkládaná práce se zabývá studiem změn ultrastruktury chloroplastů z několika různých hledisek, přičemž sleduje zejména tyto cíle:

1. Výzkum rozdílů v ultrastruktuře dimorfních chloroplastů a dále ve fotosyntetické aktivitě dospělých listů na různých místech listové čepele u kukuřice seté.
2. Studium projevů postupné destrukce chloroplastů a snižování fotosyntetické aktivity listů se zvyšující se koncentrací herbicidu amitrolu u kukuřice seté.
3. Zhodnocení vlivu imobilizace na ultrastrukturu izolovaných chloroplastů ve vztahu k jejich viabilitě a cytokininovému metabolismu u rostlin tabáku.
4. Sledování ultrastruktury chloroplastů v korelaci s hladinami cytokininů u normálních rostlin tabáku a u rostlin tabáku s nadprodukcí těchto rostlinných hormonů.

2 Literární přehled

2.1 Plastidy a jejich klasifikace

Geneticky semiautonomní organely rostlinné buňky, plastidy, jsou endosymbiotického původu a patří k organelám energetického metabolismu, jak již bylo zmíněno v úvodu této práce. Je známo několik typů plastidů. Přehled o funkční struktuře plastidů podává Ryberg a kol. (1993). Nejznámější klasifikace je založena na rozdílné barvě plastidů (Kutík 1985). Máme tedy plastidy nebarevné – leukoplasty – se slaběji vyvinutým membránovým systémem a barevné – chromoplasty *sensu lato.* Leukoplasty dělíme podle látek se v nich hromadících na amyloplasty, elaioplasty, a proteinoplasty. Etioplasty jsou téměř bezbarvé plastidy tvořící se ve tmě, které se po ozáření přeměňují na chloroplasty. Chromoplasty dělíme na fotosynteticky neaktivní plastidy – chromoplasty *sensu stricto* (obsahující různé karotenoidy) a fotosynteticky aktivní plastidy – chloroplasty – obsahující zelené barvivo chlorofyl. Avšak toto dělení se může lišit podle jednotlivých autorů, např. Hudák a kol. (1983) řadí mezi leukoplasty také proplastidy. Hudák (1997) také dělí plastidy podle schopnosti fotosyntetizovat na fotosynteticky aktivní (chloroplasty) a fotosynteticky neaktivní (leukoplasty a chromoplasty).

Podle stupně diferenciace buněk se vyvíjí i strukturální heterogenita plastidů. Všechny typy plastidů jsou v zásadě vzájemně přeměnitelné (Thomson a Whatley 1980). Dvě sousední buňky mohou mít plastidy s různou vnitřní architekturou (např. dimorfní chloroplasty v asimilačních listech C_4 rostlin). Fisher a Evert (1982) pozorovali v listech *Amaranthus retroflexus* dokonce sedm typů chloroplastů. Chloroplasty jsou vždy přítomny ve stomatálních buňkách.

Leukoplasty se vyskytují v kořenech, v zásobních pletivech, v meristematických pletivech a mohou sloužit i jako zásobárna lipidů (elaioplasty). Lipidy se vyskytují v plastidech ve formě globulů. Leukoplasty se často podílejí na různých metabolických procesech (např. syntéza sacharidů, aminokyselin, proteinů, lipidů a isoprenoidů).

V zásobních pletivech, v meristémech a ve statocytech v centrální části kořenové čepičky se vyskytují amyloplasty obsahující škrob ve formě škrobových zrn. Škrob je tvořen amylózou a amylopektinem, přičemž amylóza může ve škrobových zrnech i chybět. Při procesu zezelenání rostlinného orgánu (např. hlízy bramboru) dochází ke transformaci amyloplastů v chloroamyloplasty (Virgin a Egnéus 1983, Hudák 1997). Chloroamyloplasty se vyskytují v buňkách mezofylu stále zelených rostlin v jarním období. Hudák (1997) nazývá chloroamyloplasty i chloroplasty buněk pochev cévních svazků C_4 rostlin.

Proteinoplasty obsahují proteinové inkluze, které se mohou nacházet také volně v cytosolu.

Chromoplasty obsahují karoteny a xantofyly a jejich různý poměr pak způsobuje červené, oranžové a žluté zabarvení květů, listů, plodů a některých kořenů (Ryberg a kol. 1993, Hudák 1997). Vyvíjejí se z leukoplastů, nebo chloroplastů a klasifikujeme je na globulární, membránové, krystalové a retikulotubulární. Stárnoucí chloroplasty, ve kterých se rozkládají chlorofyly a které nehromadí aktivně karotenoidy, byly nazvány gerontoplasty (Ryberg a kol. 1993), i když jsou podobné globulárním chromoplastům.

2.2 Chloroplasty vyšších rostlin

2.2.1 Ultrastruktura a funkce chloroplastů

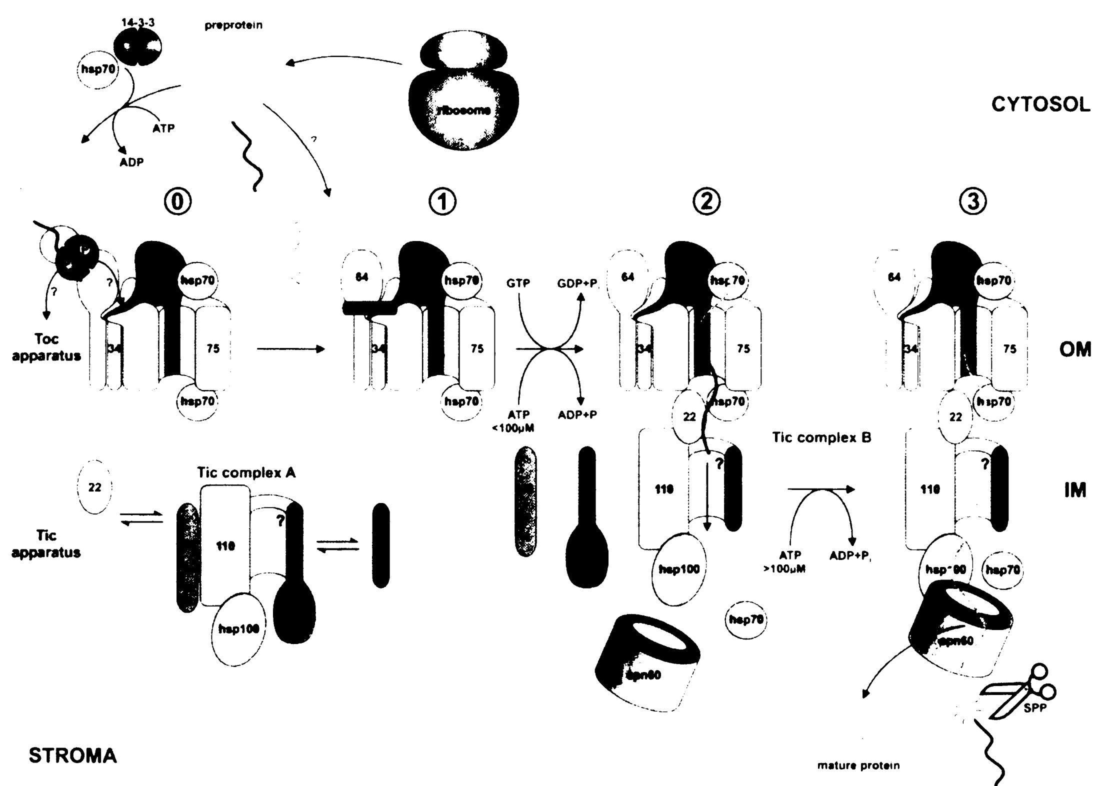
Chloroplasty jsou nejstudovanější plastidy díky své složité submikroskopické struktuře, kde probíhá fotosyntéza, která je základem produkce biomasy na Zemi. Chloroplasty a obecně řečeno plastidy vznikly primární endosymbiózou fotosyntetizujících prokaryotických mikroorganismů typu sinic (Cavalier-Smith 2000). Vznikly plastidy s dvojitou povrchovou membránou, přičemž vnitřní membrána je odvozena z plasmalemy endosymbionta a vnější membrána je pravděpodobně původem z hostitelské fagosomální membrány. Takto je tomu u řas skupiny *Glaucophyta, ruduch*, zelených řas a vyšších rostlin. Předpokládá se, že k procesu endosymbiózy došlo v evoluci opakovaně, tzv. sekundární endosymbiózou, což dokazuje až čtyřmembránová plastidová obálka u mnohých skupin řas, jako jsou *Chlorarachniophyta, Cryptophyta, Heterokonta* a *Haptophyta* (Cavalier-Smith 2000).

Chloroplasty vyšších, cévnatých rostlin se nacházejí v největší míře v mezofylových buňkách listů. Vyskytují se také ve vnějších vrstvách buněk stonků a v nezralých plodech. V dospělém chlorenchymu se chloroplasty nacházejí v tenké nástěnné vrstvě cytoplazmy spolu s menšími mitochondriemi a peroxizómy. V blízkosti chloroplastů se často v hojném počtu nacházejí cisterny endoplazmatického retikula, které hraje roli při transportu polypeptidů z cytoplazmy do chloroplastů (Hudák 1997).

Počet chloroplastů v buňce se liší u jednotlivých rostlinných druhů i typů buněk. S velikostí buňky roste i velikost a počet chloroplastů. Různé tvary chloroplastů můžeme pozorovat zejména u řas, avšak chloroplasty vyšších rostlin mají zpravidla bochníčkovitý tvar (Hudák 1997) a jejich průměr je asi pět až deset µm. Köhler a Hanson (2000) popsali a pojmenovali u chloroplastů výběžky, tzv. stromuly. Nejprve byly pozorovány u chloroplastů ve světelném mikroskopu, později také pomocí elektronmikroskopické techniky mrazového lámání (Bourett a kol. 1999). Stromuly pravděpodobně propojují jednotlivé chloroplasty, či umožňují transport molekul a tak komunikaci mezi chloroplasty (Gray a kol. 2001, Kwok a Hanson 2003, Gunning 2005). Tvar chloroplastů je závislý na ozářenosti. Na světle se zplošťují a jejich objem se zmenšuje, naopak ve tmě se chloroplasty se zvětšujícím se objemem zakulacují. Tvar chloroplastů a jeho změny určuje také plastoskeleton (Kiessling 2000), který hraje roli i při dělení chloroplastů. Chloroplasty se mohou pohybovat pomocí cytoskeletárního komplexu v nástěnné vrstvě cytoplazmy. Dalším pohybem chloroplastů je pohyb orientační, kdy ve tmě jsou chloroplasty rozmístěné náhodně, při slabém nebo dlouhovlnném ozáření se chloroplasty přibližují buněčným stěnám kolmým k dopadajícímu záření (epistrofe nebo diastrofe) a naopak při silném nebo krátkovlnném ozáření se přemísťují k buněčným stěnám rovnoběžným se směrem záření, což je označováno jako parastrofe (Kutík 1985).

Na povrchu chloroplastů vyšších rostlin jsou dvě obalové membrány, které oddělují vnitřní prostor chloroplastů od základní cytoplazmy buňky, cytosolu. Obě tyto membrány se liší chemickým složením, propustností a funkcí, což je v souladu s teorií endosymbiotického vzniku chloroplastů. Tloušťka obalových membrán se pohybuje mezi šesti až deseti nm, přičemž jejich vzájemná vzdálenost činí asi 20 nm a prostor mezi nimi se nazývá periplastidový. Obě povrchové membrány neobsahují chlorofyly a neprobíhají v nich primární děje fotosyntézy. Obsahují však karotenoidy, které chrání chlorofyly před fotooxidací. Mezi hlavní funkce obalových membrán dále patří transport proteinů a metabolitů mezi stromatem chloroplastu a buněčnou cytoplazmou.

Vnější membrána primárně hraje roli fyzické bariéry a kontroly vstupu velkých molekul z cytoplazmy do chloroplastu (Staehelin a van der Staay 1996). Transportní a syntetické aktivity jsou spojeny hlavně s vnitřní obalovou membránou. Důležitou roli hrají obě obalové membrány při transportu proteinů, syntetizovaných na cytoplazmatických ribosomech a kódovaných v buněčném jádře (Bauer a kol. 2001, Bodyl 2002) do chloroplastu. V obalových membránách byly popsány transmembránové transportní komplexy označované jako Tic – pro vnitřní a Toc – pro vnější membránu (Schleiff a Soll 2000, Jarvis a Soll 2002, viz obr. 1).

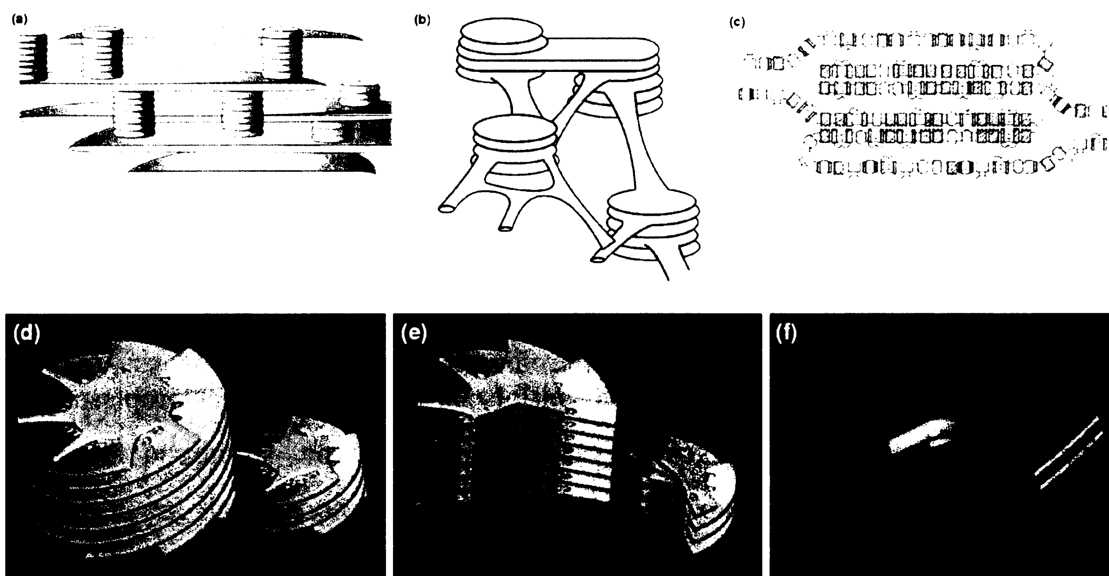


Obr. 1: Model pro proteinový importní mechanismus v chloroplastu (Jarvis a Soll 2002). Preprotein se navazuje na membránu po spojení s jinými proteiny nebo přímo na jádro Toc komplexu (1). Toto stádium importního systému je na energii nezávislá vazba proteinu na Toc komplex. Stádium 2 znázorňuje tvorbu středně pokročilého komplexu importu proteinu, kde je zapotřebí v nízké koncentraci ATP v intermembránovém prostoru a GTP. V tomto stádiu importu protein prochází vnější chloroplastovou membránou (OM) prostřednictvím Toc komplexu a je v kontaktu s komponenty Tic aparátu (typ A a B) vnitřní chloroplastové membrány (IM). Stádium 3 (kompletní translokace) požaduje vysokou koncentraci ATP ve stromatu chloroplastu. Preprotein je translokován přes obě membrány a po následném oddělení transitního peptidu nabývá konečné konformace.

Od vnitřní obalové membrány chloroplastu je, kromě thylakoidů, odvozeno také periferní retikulum. Jde o komplikovaný systém kanálků a měchýřků lokalizovaný v periferní oblasti chloroplastů, který zřejmě usnadňuje transport produktů fotosyntézy z chloroplastu do cytosolu. Periferní retikulum se vyskytuje především v chloroplastech rostlin s C_4 fotosyntézou, kde je pravděpodobně zapojeno do transportu primárních asimilátů z mezofylových buněk do buněk pochev cévních svazků (Hudák 1997, Kratsch a Wise 2000).

Další strukturou odvozenou od vnitřní obalové membrány chloroplastů jsou thylakoidy – ploché měchýřkovité váčky. Jsou klíčovou strukturou oxygenní fotosyntézy. Přikládáním těchto diskovitých útvarů na sebe vznikají grana. Stupeň přitisknutí thylakoidů je ovlivňován koncentrací dvojmocných kationtů Ca^{2+} a Mg^{2+}, ale také konformačními změnami membránových bílkovin. Téměř 50 let je znám mírou ozářenosti řízený tzv. „přechod stavu 1 do stavu 2“ a naopak. Dochází zde ke změně rozdělení excitační energie mezi PS I a PS II (Allen a Forsberg 2001). Thylakoidní membrány jsou velmi dynamickou strukturou. Grana mohou být tvořena dvěma a více thylakoidy, jsou obklopena a propojena šroubovitě uspořádanými stromatálními (intergranálními) thylakoidy, jež propojují jednotlivá grana, ale i jednotlivé části téhož grana (Staehelin a van der Staay 1996). Intergranální thylakoidy tvoří kolem gran pravotočivé šroubovice. Prostorovou stavbu thylakoidů popisuje např. Mustárdy (1996). Mustárdy a Garab (2003) shrnují aktuální vývoj trojrozměrných modelů systému thylakoidů (obr. 2).

Thylakoidní membránu si lze představit dle modelu „tekuté mozaiky“ – lipidová dvojvrstva se zanořenými integrálními či periferními molekulami bílkovin. V granech je především lokalizován fotosystém II (PS II) a v membránách intergranálních thylakoidů je z větší části lokalizován fotosystém I (PS I). Detailní strukturu fotosystému II popsali např. Rhee a kol. (1998). Thylakoidní membrány obsahují čtyři základní typy funkčních komplexů: světlosběrné komplexy (LHC) obou fotosystémů, reakční centra obou fotosystémů, komplexy přenašečů elektronů a spojovací faktor (chloroplastová ATP syntáza) syntetizující ATP na účet energie protonů, vracejících se po koncentračním spádu ze vnitřku thylakoidů do stromatu (Allen a Forsberg 2001). Uvnitř lumina thylakoidů je kyselé prostředí dosahující hodnot pH mezi 5,8 a 6,5. Pouze za podmínek světelného stresu pH lumina klesá pod hodnoty 5 a vede fotosyntetizující aparát ke fotoinhibici (Kramer a kol. 1999).



Obr. 2: Vývoj trojrozměrných modelů systému thylakoidů (převzato z publikace Mustárdy a Garab 2003). Autoři ukazují nejvíce používané zastaralé nebo nesprávné prostorové modely thylakoidů: (a) – model podle Wilhelma Menkeho, (b) – tubulární model, (c) – záhybový membránový model (Andersson a Anderson 1980), kde thylakoidní membrány tvoří „vidličku". Tento zastaralý model je používán podle autorů stále častěji než adekvátnější počítačový 3D model (d), (e) a (f), kde pravotočivá šroubovice stromatálních thylakoidů kryje „sloup" thylakoidů granálních. Stromatální thylakoidy propojují mezi sebou jednotlivá grana. Detailní rekonstrukce na obr. (f) znázorňuje přehyb membrán a sklon závitu šroubovice.

Vnitřek chloroplastů, kromě intrathylakoidního a mezimembránového prostoru, vyplňuje stroma (matrix). Obsahuje ribosomy, nukleoidy, enzymy Calvinova cyklu a různé typy inkluzí (škrobové, lipoidní, bílkovinné a shluky fytoferritinu). Probíhá zde také syntéza bílkovin kódovaných chloroplastovou DNA.

Škrobové inkluze jsou tvořeny směsí amylózy a amylopektinu. V chloroplastech se vyskytují v menší míře než v plastidech zásobních orgánů. Na snímcích z transmisního elektronového mikroskopu jsou zřetelně ohraničená zrna škrobu obklopena bílým lemem, tzv. elektrontransparentním dvůrkem. Škrobové inkluze jsou pravděpodobně tvořeny krystalickým jádrem, které obaluje amorfní materiál z méně větvených glukanových polymerů. Součástí těchto inkluzí jsou i enzymy biosyntézy škrobu a některé lipidy (Martin a Smith 1995). Syntézu a odbourávání škrobu v listech shrnují Zeeman a kol. (2002) a Lloyd a kol. (2005)

Plastoglobuly se vyskytují ve všech typech plastidů. Jejich četnost a velikost souvisí s vývojovým stadiem a typem plastidů. Zejména však ve stárnoucích chloroplastech vznikají při degradaci thylakoidních membrán. Plastoglobuly chloroplastů obsahují plastidové chinony, triacylglyceroly a karotenoidy (Dahlin a Ryberg 1986).

V matrix chloroplastů se také vyskytují mikrotubulární struktury o menším průměru (13 nm) než cytoplazmatické (Ryberg a kol. 1993), které popsali již v minulosti např. Lawrence a Possingham (1984). Filamentární síť uvnitř chloroplastů byla vizualizovaná a nazvána „plastoskeleton" (Kiessling a kol. 2000). Tuto síť tvoří FtsZ proteiny, které mají multifunkční vlastnosti a podílejí se i na plastidovém dělení jak shrnuje např. McFadden (2000).

Vesikulární struktury uvnitř chloroplastů byly již pozorovány pomocí elektronového mikroskopu téměř před 50. lety. Možnost transportu lipidů vesikuly z vnitřní chloroplastové membrány k thylakoidům navrhoval Douce (1984). Vesikulární transport, podobný vesikulárnímu transportu v cytoplasmě, nepřetržitě probíhá i v dospělých chloroplastech a reprezentuje důležitou cestu formování a obnovy thylakoidních membrán (Westphal a kol. 2001).

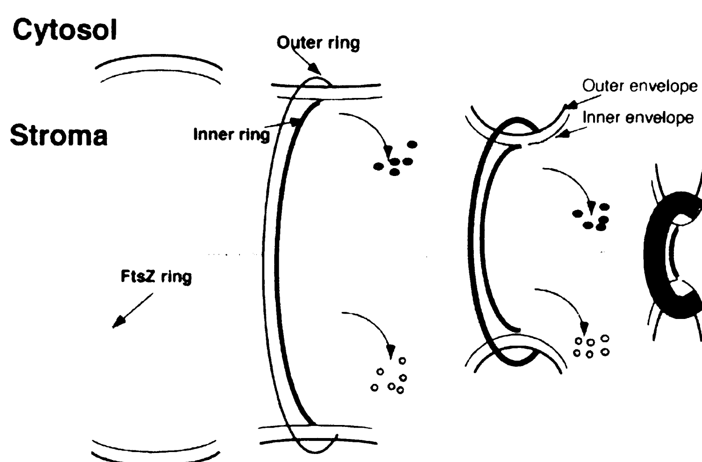
2.2.2 Dělení chloroplastů

Plastidy, a tedy i chloroplasty, nevznikají *de novo*, ale dělením již existujících plastidů, které se do dceřinných buněk dostávají při buněčném dělení (Hudák a kol. 1983). Přenos z rodičovské rostliny na dceřinnou při pohlavním rozmnožování je zpravidla po mateřské linii. Plastidy pocházejí z nediferencovaných meristematických proplastidů (Pyke 1999) a vznikají dělením preexistujících plastidů v cytosolu (Aldridge a kol. 2005). Plastidy si udržují schopnost dělit se až do stádia úplné zralosti, pak frekvence dělení rychle klesá (Hudák a kol. 1983). Počet plastidů v buňce se zvyšuje úměrně s velikostí buňky (Hudák 1997). Dělení plastidů může předcházet dělení buňky a naopak (Butterfass 1983).

Na základě vědeckého poznání posledních deseti let víme, že dělení plastidů je vysoce koordinovaný proces, na němž se podílejí a společně jej kontrolují proteiny prokaryotického i eukaryotického původu.

Chloroplastové dělení je zahájeno polymerizací čtyř jaderně kódovaných FtsZ stromálních proteinů, které jsou původem blízké bakteriální formě dělícího Z-prstenu

(Lutkenhaus a Addinall 1997). FtsZ-prsten byl popsán u chloroplastů vyšších rostlin autory Vitha a kol. (2001) a Mori a kol. (2001). Kromě FtsZ-prstence se na dělení chloroplastů podílejí i PD-prsteny (plastid-dividing rings), které jsou zformovány od časných stadií dělení chloroplastů až po stádia konečná. Chování PD-prstenů při dělení bylo poprvé důkladně popsáno na červené řase *Cyanidioschyzon merolae* (Miyagishima a kol. 1998, 2001a). Kooperaci prstenů při dělení chloroplastů znázorňuje model autorů Kuroiwa a kol. (2002) na obr. 3.



Obr. 3. Model dělícího aparátu chloroplastů (Kuroiwa a kol. 2002). Nejprve se zformuje FtsZ - prsten uvnitř chloroplastu. Ve stejném místě se vytvoří vnější a vnitřní PD prsten. Dále dochází ke kontrakci celého dělícího aparátu a začíná rozklad FtsZ-prstenu a vnitřního PD-prstenu. Sílící vnější PD-prsten dokončuje dělení chloroplastu.

Důležitou roli při dělení plastidů hrají proteiny ARTEMIS (integrální protein vnitřní membrány chloroplastu, Fulgosi a kol. 2002) a GIANT CHLOROPLAST 1 (GC1, Raynaud a kol. 2004). Dalším proteinem důležitým při ukotvení FtsZ-prstenu je ACCUMULATION AND REPLICATION OF CHLOROPLASTS (ARC6, stromální protein, Vitha a kol. 2003, Maple a kol. 2005). Úlohu těchto proteinů a současně plastid dělících proteinů v cytosolu ARC5 a ARC3 shrnují Maple a Moller (2005).

2.2.3 Fotosyntéza

Fotosyntéza je základní biologický proces na této planetě, při kterém dochází s využitím energie slunečního záření k syntéze energeticky bohatých organických látek z jednoduchých látek anorganických.

Fotosyntézu lze definovat jako oxidoredukční reakci, při které dochází k přenosu vodíku z donoru na akceptor, kterým je nejčastěji CO_2. Podle donoru rozlišujeme kyslíkovou a bakteriální fotosyntézu. Dále se budu zabývat pouze kyslíkovou fotosyntézou, kde primárním donorem je voda. Tento proces je spojený s přírůstkem volné energie a uvolněním kyslíku. Nejnovější a ucelené informace o fotosyntéze podává např. Lawlor (2001).

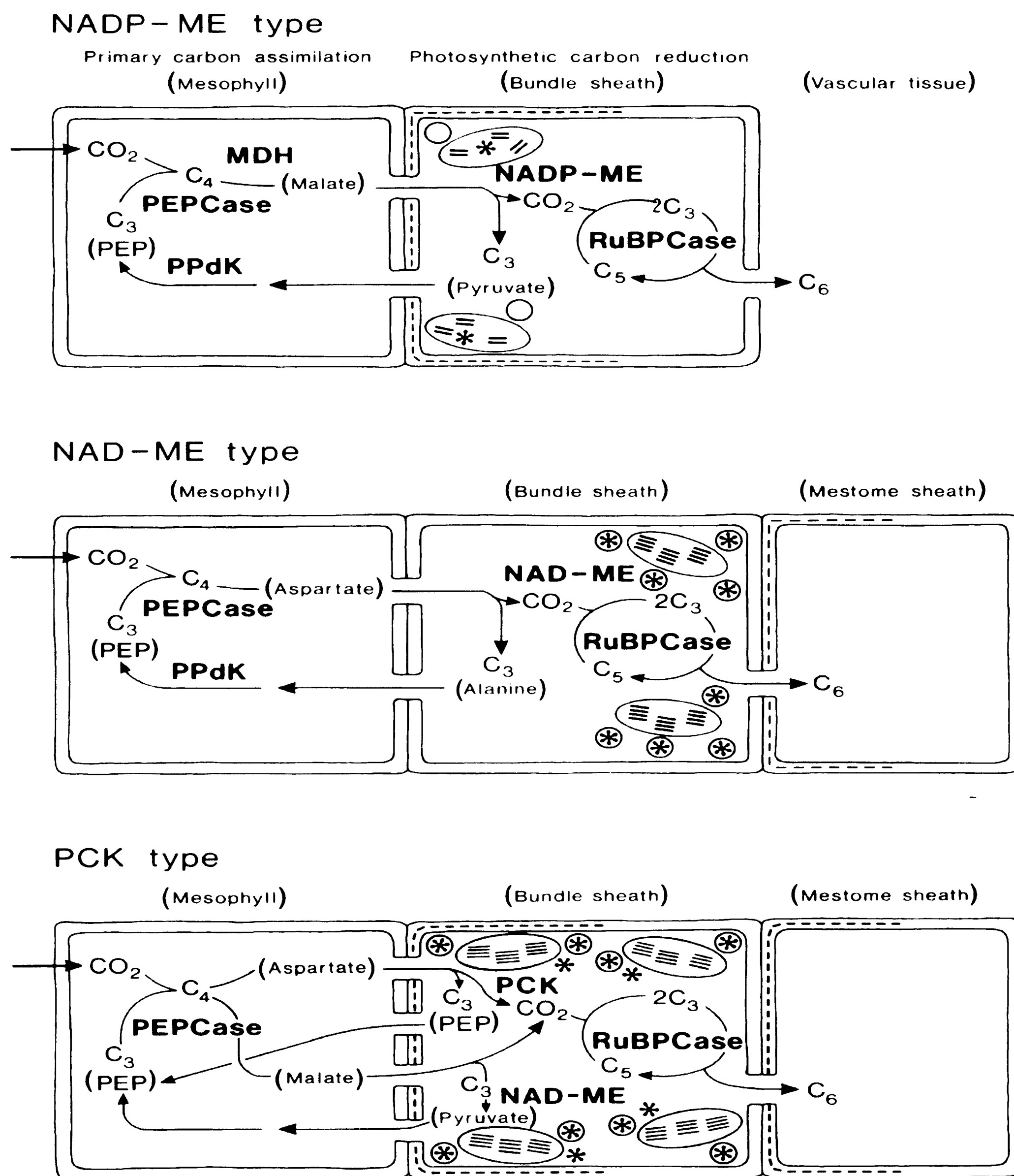
Při primárních (světelných) fotosyntetických procesech vznikají makroergické sloučeniny – adenosintrifosfát (ATP) a redukovaný nikotinamidadenindinukleotidfosfát (NADPH), které jsou pak využívány v temnotních reakcích při fixaci a redukci CO_2. V Calvinově cyklu je CO_2 za katalýzy enzymem Rubisco (ribulosa–1,5-bisfosfát karboxyláza –oxygenáza) vázán na ribulosa-1,5-bisfosfát.

Oxygenázová aktivita enzymu Rubisco se projevuje tím, že fotosyntéza je inhibována kyslíkem. Na tuto aktivitu enzymu Rubisco navazují další procesy, které vyúsťují v paradoxní jev označovaný jako fotorespirace.

V rostlinách s C_3 fotosyntézou je CO_2 fixován na pětiuhlíkatou sloučeninu a prvním stabilním produktem této fixace jsou dvě molekuly tříuhlíkaté sloučeniny kyseliny 3-fosfoglycerové (PGA). Rychlost fotosyntézy je u těchto rostlin fotorespirací snižována (Furbank a Taylor 1995)

Rostliny s C_4 fotosyntézou CO_2 primárně fixují na kyselinu fosfoenolpyrohroznovou (PEP). Fixaci CO_2 katalyzuje enzym PEP-karboxyláza. Tato reakce probíhá v cytosolu mezofylových buněk. Navázáním na tříuhlíkatou PEP vzniká čtyřuhlíkatá kyselina oxaloctová. Fotosyntéza C_4 rostlin má prostorově oddělenou primární fixaci CO_2 od sekundární fixace v Calvinově cyklu, kde je v chloroplastech buněk pochev cévních svazků CO_2 refixován enzymem Rubisco. Účinná fixace CO_2 PEP-karboxylázou vede k potlačení fotorespirace. Podle přítomnosti enzymů katalyzujících dekarboxylační reakce C_4 rostliny třídíme do podskupin (viz obr. 4):

1. NADP-ME typ – Nejběžnější typ C_4 fotosyntézy.
2. NAD-ME typ
3. PEP-CK typ



Obr. 4: Enzymová kompartmentace a strukturální charakteristiky mezofylu a pletiva buněk pochev cévních svazků v C_4 biochemických variantách (Nelson a Dengler 1992 podle Hattersley).

C_4 fotosyntézu popisují např. práce autorů Nelson a Langdale (1992), Nelson a Dengler (1992), Hatch (1992), Furbank a Taylor (1995) a Hudák (1997). Pro většinu rostlin s C_4 fotosyntézou je typické věncovité uspořádání dvou typů fotosyntetických pletiv (na příčném řezu listem). V nedávné době však byla u dvou polopouštních rostlin z čeledi *Chenopodiaceae* popsána C_4 fotosyntéza, při níž jsou dva typy chloroplastů a všechny potřebné enzymy metabolicky odděleny uvnitř jediného typu buněk chlorenchymu (Edwards a kol. 2004).

Typickou představitelkou rostlin s C_4 fotosyntézou (nejběžnějšího, NADP-malátového typu) je kukuřice, která byla také mojí hlavní modelovou rostlinou. Důvodem její obliby jako experimentálního objektu je její ekonomický význam a také znalost jejího chloroplastového genomu a genetiky vůbec.

CAM rostliny (odvozeno od Crassulacean Acid Metabolism) mají metabolismus přizpůsobený k životu v prostředí s nedostatkem vody. Jsou to sukulenty mezi které řadíme tučnolisté – *Crassulaceae,* některé *Liliaceae, Bromeliaceae, Orchidaceae* a další. U CAM fotosyntézy je na rozdíl od C_4 fotosyntézy dvojitá fixace CO_2 oddělena časově. CAM rostliny fixují CO_2 hlavně v noci, kdy jsou otevřené průduchy. Kyselina jablečná se hromadí během noci ve vakuolách a ve dne, kdy jsou průduchy zavřené, se vrací zpět do cytosolu, kde dochází k uvolnění CO_2, který je v chloroplastech opět fixován Calvinovým cyklem.

2.3 Plasticita ultrastruktury chloroplastů

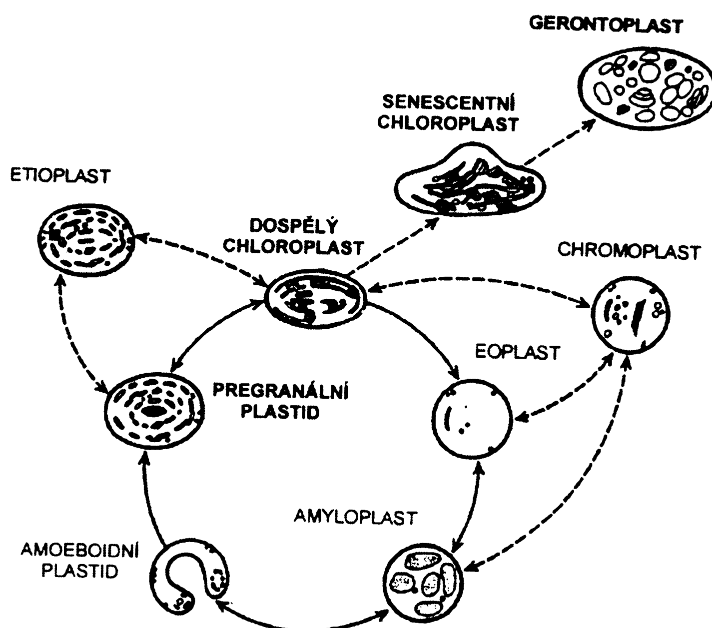
U vyšších rostlin dochází během jejich ontogenetického vývoje při diferenciaci jejich pletiv a buněk také k diferenciaci plastidů. Vznikají různé typy plastidů, fotosyntetizujících i fotosynteticky neaktivních. Jejich biogeneze je stále fascinující z hlediska strukturálního, biochemického, genetického a molekulárního. Následující kapitoly by měly nastínit plasticitu těchto organel během ontogeneze listu, jejich heterogenitu na ploše listové čepele, jejich modifikace v závislosti na druhu rostliny či genotypu a změny ve vývoji vlivem stresu, prostředí nebo experimentálního působení.

2.3.1 Vývoj chloroplastů během ontogeneze listu

Vývoj chloroplastů v průběhu ontogeneze – individuálního vývoje – listu je shrnut v přehledech Kutík (1985), Kutík (1998) a monografii Biswal a kol. (2003). Již před čtyřiceti lety vydal Sironval (1967) monografii zabývající se vývojem chloroplastů v průběhu ontogeneze listu z různých pohledů na dynamiku struktury a funkcí těchto organel. Jako první prostudovala vývoj plastidů v průběhu celé ontogeneze listu již od embryonální fáze v primárních listech fazolu Whatley (1974). Vývoj plastidů v rámci celé rostliny má v zásadě cyklický charakter a je to řetězec strukturálních a funkčních

přeměn, jak je patrné z obrázku 5. V listech jsou to procesy představující změny ve vývoji plastidů ze strukturálně jednoduchých proplastidů, zvaných též eoplasty v chloroplasty a až po jejich poslední, nevratnou přeměnu na gerontoplasty. Každé vývojové stádium plastidů je charakteristické určitou úrovní membránové diferenciace (Hudák 1997). Chloroplasty představují plastidy s nejsložitější vnitřní organizací. Vývoj ultrastruktury chloroplastů v buňkách mezofylu dospívajících, dospělých a stárnoucích listů fazolu sledovali z hlediska jejich fotosyntetické aktivity Kutík a kol. 1984).

Vzájemné přeměny plastidů, jak je popisují např. Virgin a Egnéus (1983), bývají nazývány metamorfózy plastidů. Model těchto přeměn znázorňuje schéma podle Thomson a Whatley (1980) na obr. 5.



Obr. 5: Vzájemné přeměny plastidů. Upravené schéma podle Thomson a Whatley (1980).

Metamorfózy plastidů probíhají několika způsoby, které shrnují Virgin a Egnéus (1983). Všechny vývojové přeměny plastidů probíhají v zásadě obousměrně, pouze stádium gerontoplastu již nelze zvrátit. V listech se na světle proplastidy či eoplasty vyvíjejí, zpravidla přes mezistádia bohatá na škrob a mezistádia laločnatého tvaru, v chloroplasty. Ty se pak s poklesem fotosyntetické aktivity mění v senescentní chloroplasty a nakonec v gerontoplasty, neschopné již dalších přeměn. U senescentních chloroplastů ještě může za určitých okolností dojít k „omlazení" a návratu fotosyntetické aktivity, důležitou úlohu zde zřejmě hrají rostlinné hormony cytokininy

(Hudák 1997). Proces postupné transformace proplastidů na chloroplasty vyžaduje světlo, pak jsou syntetizovány chlorofyly a diferencuje se membránový systém. Proplastidy se nacházejí především v meristematických buňkách. Lze tu rozeznat několik vývojových stupňů (Ryberg a kol. 1993). Ultrastruktura proplastidů je zpravidla velmi jednoduchá, vnitřní povrchová membrána tvoří často invaginace. Ve stonku a listech rostlin vyvíjejících se za nedostatku světla se vyvíjejí z proplastidů etioplasty. Etioplast je intermediální fáze vývoje plastidů. Etioplasty obsahují prolamelární těleso tvořící prothylakoidy, ze kterých se při ozáření tvoří stromální a granální thylakoidy a z etioplastů vznikají chloroplasty (Leech 1986). Přeměna etioplastu ve chloroplast je velmi rychlá (řádově hodiny), regulovaná světlem. Z toho vyplývá, že všechny chemické konstituenty nutné pro vývoj chloroplastu jsou již přítomné v etioplastu. Pro svoji rychlost a snadnou manipulovatelnost byla tato přeměna dlouho hlavním modelem studia vývoje chloroplastů.

Zájem výzkumu se proto především soustředil na zezelenání etioplastů, a druhým tématem zájmu byla senescence chloroplastů. Již méně bylo věnováno pozornosti změnám struktury chloroplastů v buňkách mezofylu zelených, fotosynteticky aktivních listů.

Ultrastruktura chloroplastů vyšších rostlin se v průběhu ontogeneze fotosynteticky aktivního listu mění, jak detailně popisuje review Kutík (1998). Po rozvinutí listové čepele mají chloroplasty vyvinutý systém thylakoidů z několika paralelně vrstvených nepřitisklých thylakoidů a malá, pouze z několika thylakoidů tvořená, grana. Tyto mladé chloroplasty mají ve stromatu mnoho ribosomů, malé plastoglobuly a větší či menší škrobové inkluze. Při dospívání chloroplastu dochází k jeho zvětšování. Před ukončením vývoje listu také nabývají největší objemové hustoty škrobové inkluze. V dospělém listu, který právě ukončil svůj růst, byl popsán největší počet velkých gran i integranálních thylakoidů. Přibývá i velkých plastoglobulů. Na konci listové ontogeneze dochází k chloroplastové senescenci, kdy se objem chloroplastů začíná zmenšovat a tvar chloroplastů se z čočkovitého mění na téměř kulatý. Dochází k dilataci a destrukci intergranálního i granálního thylakoidního systému, ztrátě paralelního uspořádání thylakoidů, stroma vakuolizuje a zvětšuje se počet i velikost plastoglobulů. Senescentní chloroplast, který postrádá DNA a není schopen jiné transformace je pak označen jako gerontoplast (Sitte 1977). Senescenci listů a chloroplastů v nich studovali detailně např. Matile (1991) a Hudák (1997). Tento proces z hlediska molekulární biologie souhrnně popsala Buchanan-Wollaston (1997).

Spolu s vývojem chloroplastů během ontogenetického vývoje listu se mění a vyvíjejí fotosyntetické aktivity listu (Čatský a Šesták 1997, Hudák 1997). Poznatky ohledně strukturálních změn v průběhu normálního vývoje listu během diurnálního cyklu světlo/tma shrnul Kutík (1985, 1998). Whatley a kol. (1982) studovali prostorové uspořádání thylakoidního systému chloroplastů na primárních listech fazolu šarlatového (*Phaseolus vulgaris*). Na tuto práci navázala Silaeva (1982), která s využitím rostlinného materiálu jako cukrová řepa, kukuřice nebo pšenice navrhovala „blízcegranální thylakoidy" jako specifickou oblast thylakoidního systému. Uspořádáním thylakoidního systému se dále zabývali Albertsson (1995) a Mustárdy (1996). Dělení dospělých chloroplastů nebo chloroamyloplastů v buňkách listů žijících více než jedno vegetační období bylo dříve odmítáno (Gamaleï a Kulikov 1978), ale později prokázáno (Sagisaka (1993 a, b, 1994 a, b). „Plastidová iniciála" vzniká během zimy a časného jara jako malý membránový váček téměř bez vnitřní struktury inekválním dělením dospělého chloroplastu. Podobné poznání učinili Miroslavov a Alekseeva (1990) v jehlicích tisu. Endler a kol. (1990) popsali v jehlicích *Pinus sylvestris* v jedné buňce chloroplasty dobře diferencované i proplastidy. Během přezimování se dočasně objevuje periferní retikulum v chloroplastech C_3 rostlin (Modrušan a Wrischer 1987, Morré a kol. 1991).

Vývoj chloroplastů v listech špenátu studovali Rascio a kol. (1985). Vývoj chloroplastů, tak jako i růst rostlin špenátu byl zpomalen na podzim oproti vývoji v létě. Prolamelární tělíska zůstávaly v chloroplastech i na světle. Vývoj chloroplastů během listové ontogeneze kapradiny Phyllitis scolopendrium ukázal na šest vývojových stádií chloroplastů během listové ontogeneze od báze ke středu a apexu listu. Vývoj chloroplastů z hlediska fylogeneze byl prováděn zejména u *Cycadaceae* (Bonatti a Sabato 1984, Bonatti a Fornasiero 1990, Bonzi a kol. 1992). Pro ultrastrukturu chloroplastů těchto rostlin jsou typická prolamelární tělesa v mladých chloroplastech i za světla a ohromná grana (magnograna) v chloroplastech dospělých.

Pro mnoho detailních studií vývoje chloroplastů během ontogeneze listu byly použity rostliny některých význačných zemědělských plodin, např. stereologická studie vývoje chloroplastu a dalších organel v buňkách palisádového parenchymu rostoucího slunečnicového listu (Fagerberg 1984). Na tuto práci navázala studie chloroplastové senescence a formování gerontoplastů u bobu (*Vicia faba*) v mezofylových buňkách, kde žloutnoucí listy ztratily 95% chlorofylu a gerontoplast má jen několik nepřitisklých thylakoidů, které obsahují světlosběrné komplexy (LHC) a mají pak velmi vysokou

fotochemickou aktivitu, plastoglobuly obsahují karotenoidy (Schmidt 1988). Somersalo a Aro (1987) sledovali vývoj chloroplastů na rostlinách hrachu, kde stereologická objemová hustota gran během celého vývoje klesala, škrobové inkluze byly největší v dospělých chloroplastech a plastoglobulů přibývalo se stářím chloroplastů. V nejstarších listech se chloroplasty stávaly zakrnělými. Senescenci chloroplastů palisádových buněk listů broskvoně, kdy poklesl obsah chlorofylu v listech, thylakoidy chloroplastů jsou dilatovány a objevují se plastoglobuly, popsali Nii a kol. (1988).

Vývoj chloroplastů mezofylových buněk během ontogeneze „primárního" (prvního po děložních listech) a následujícího „sekundárního" listu fazolu šarlatového (*Phaseolus vulgaris*) studovali Kutík a kol. 1984, 1988, Kutík 1988).

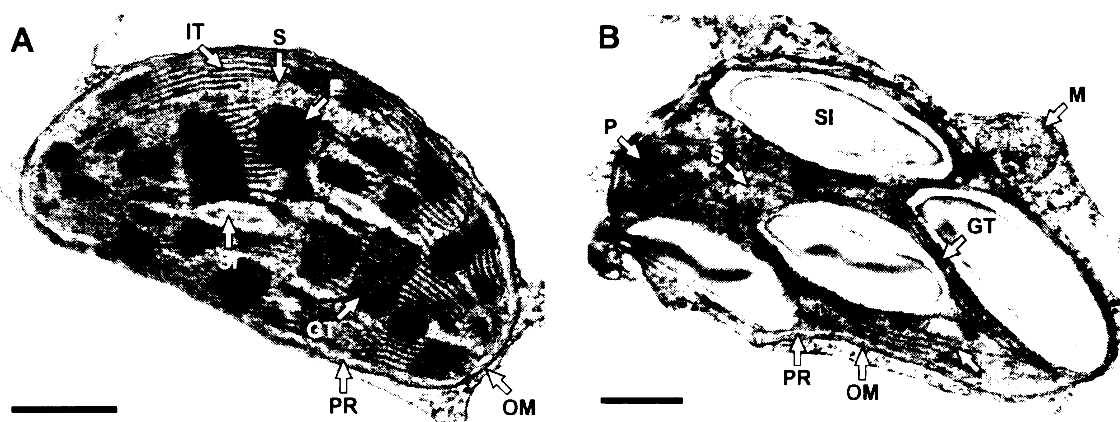
U těchto rostlin objemová hustota intergranálních a granálních thylakoidů byla největší ve velmi mladých „primárních"listech, klesala během zvětšování chloroplastu a opět rostla až do fáze, kdy začala senescence listů. Škrobové inkluze dosahovaly maxima během dospělosti chloroplastu, těsně před dosažením maximální objemové hustoty thylakoidů, a druhého maxima škrobové inkluze dosahovaly až při senescenci chloroplastů. U „sekundárního" fazolového listu vývoj chloroplastů byl velmi podobný jako u listu „primárního". V dekapitovaných rostlinách fazolu se ontogeneze „primárních" listů výrazně prodloužila díky akumulaci cytokininů tvořících se v kořeni rostliny. Nebyly nalezeny významné rozdíly ve struktuře chloroplastů v buňkách palisádového a houbového parenchymu listového mezofylu stejného stáří (Kutík 1989).

Často používaným objektem pro studium vývoje chloroplastů jsou jednoděložné plodiny s dlouhými protáhlými listy, jako jsou pšenice, ječmen, rýže nebo kukuřice. U těchto rostlin lze na základě listové heterogenity studovat různé stáří chloroplastů, ale o tom se více zmíním v další kapitole.

Studiem vývoje chloroplastů za nízké ozářenosti na různých segmentech listové čepele ječmene *(Hordeum vulgare)* se zabývali Wellburn a kol. (1982). V souladu s Whatley (1977) pozorovali eoplasty, amyloplasty, amoeboidní plastidy, pregranální a dospělé chloroplasty, někdy s prolamelárními tělesy. Během vývoje mezofylových chloroplastů pšenice v nich byly pozorovány mikrotubulům podobné struktury (Kutík 1992). Podobné struktury byli popsány i u jiných vyšších rostlin a zelených řas (Lawrence a Possingham 1984, Artus a kol. 1990), jejich složení a funkce jsou stále nejasné.

Jednou z důležitých modelových rostlin pro studium vývoje chloroplastů je kukuřice setá (*Zea mays* L.). Kukuřice je představitelkou jednoděložných rostlin s C_4

fotosyntézou NADP–malátového typu, kdy díky anatomické stavbě listu dochází jak k prostorovému oddělení primární a sekundární fixace CO_2, tak i oddělena je produkce a spotřeba NADPH. Tyto procesy probíhají ve dvou typech buněk obsahujících chloroplasty: v mezofylových buňkách a buňkách pochev cévních svazků, které na příčném řezu listem jako věnec obklopují cévní svazky. Proto se toto uspořádání pletiv uvnitř listu nazývá Kranz-anatomie (věnčitá anatomie, Furbank a Foyer 1988, Nelson a Langdale 1989, Nelson a Dengler 1992, Edwards a kol. 2001, Leegood 2002, Brown a kol. 2005). V souvislosti s jejich fotosyntetickými funkcemi jsou buňky pochev cévních svazků větší se silnější buněčnou stěnou než buňky mezofylové a chloroplasty jsou v nich uspořádány centrifugálně. V obou těchto typech buněk se nacházejí i ultrastrukturálně odlišné chloroplasty. Tento chloroplastový dimorfismus je dobře vidět na obr.6.



Obr. 6: Chloroplast mezofylové buňky (A) a chloroplast buňky pochvy cévního svazku (B) dospělého listu kukuřice seté (*Zea mays* L.). IT- intergranální thylakoidy, GT- granální thylakoidy, SI- škrobové inkluze, S- stroma, PR- periferní retikulum, P- plastoglobuly, OM- obalové membrány, M mitochondrie, délka měřítka = 1µm.

Chloroplastový dimorfismus mezofylových buněk a buněk pochev cévních svazků kukuřice souvisí s různou úlohou při fixaci CO_2. Chloroplasty mezofylových buněk mají velká grana, kde je soustředěno veliké množství PS2 k produkci NADPH a ATP, které jsou potřeba v mezofylových buňkách k přeměně oxalacetátu na malát a pyruvátu na PEP a na další procesy (Chow a kol. 2005, Majeran a kol. 2005). Pro chloroplasty buněk pochev cévních svazků jsou zase typické nepřitisknuté, intergranální thylakoidy, velmi malá grana (maximálně ze čtyř thylakoidů) a tvorba škrobových inkluzí (Spilarto a Preiss 1987, Nelson a Langdale 1989, Lunn a Hatch 1995, Majeran a

kol. 2005). Diferenciaci listů kukuřice z meristému a jejich další ontogenetický vývoj popsali Sylvester a kol. (1996). Pro řadu pokusů je tato modelová rostlina používána díky dobré znalosti již zmíněných anatomických vlastností, tak i genetických parametrů (Maier a kol. 1995).

Na ontogenezi dimorfních chloroplastů v listech této rostliny se zaměřila řada autorů. Postupnou diferenciaci chloroplastů mezofylových buněk a buněk pochev cévních svazků z nelišících se proplastidů sledovala Brangeon (1973a) na druhém listu 3 až 11 dní po přesazení naklíčených obilek. V chloroplastech mezofylových buněk granálních a intergranálních thylakoidů během vývoje přibývalo a naopak u chloroplastů buněk pochev cévních svazků začalo ubývat granálních thylakoidů pátý den po přesazení. Zřejmě zvýšením ozářenosti, při vyrůstání druhého listu z pochvy prvního, dochází k rozrušování granálních thylakoidů. Ozářenost má patrně vliv na rozpad gran v chloroplastech buněk pochev cévního svazku. Umělé stínění zpomaluje odbourávání gran v chloroplastech buněk pochev cévních svazků a naopak zvýšení ozářenosti druhého listu kukuřice odbourávání gran urychluje (Brangeon 1973b).

Vztahy mezi anatomickou stavbou listu a rychlostí fotosyntézy u tří inbredních linií kukuřice a jejich hybridů sledoval ve své diplomové práci Babůrek (1973). Nevěnoval se však kvantitativnímu hodnocení ultrastruktury chloroplastů, ani nestudoval průběh vývoje chloroplastů během ontogeneze listu.

Změny aktivity PS2 a PS1 během vývoje dimorfních chloroplastů u kukuřice lze sledovat i cytochemickým průkazem jejich aktivity. Aktivita PS2 v dospělých listech je výrazná v granech chloroplastů buněk mezofylu, oproti tomu velmi slabá v intergranálních a rudimentálních granálních thylakoidech chloroplastů buněk pochev cévního svazku (Wrischer 1989).

Vývoj gran u chloroplastů buněk pochev cévních svazků u kukuřice a *Portulaca grandiflora*, NADP-ME jednoděložných a dvouděložných C_4 rostlin studovali Nishioka a kol. (1993). U obou druhů dimorfismus chloroplastů není zpočátku ontogeneze listu patrný. K rozvoji gran dochází pouze u chloroplastů buněk mezofylu, což se shoduje s Brangeon 1973a). Autoři vyvodili, že kdyby chloroplasty buněk pochev cévních svazků měly dobře vyvinutá grana, byla by v těchto chloroplastech inhibována oxidativní dekarboxylace malátu kompeticí o $NADP^+$ redukovaného na NADPH.

Kutík a Kočová (1996) a Kutík a kol. (1999) se zabývali vývojem srovnatelné části (střední části listové čepele) různě starých listů kukuřice, nikoliv vývojovým

gradientem podél listů kukuřice (viz též Kutík a kol. 2001, Vičánková a kol., nabídnuto Photosynthetica). Vývoj chloroplastů mezofylových buněk a aktivitu Hillovy reakce u dvou nových fotosynteticky kontrastních genotypů kukuřice a jejich hybridů sledovali Kutík a Kočová (1996). Srovnávali mladé, dospělé a stárnoucí chloroplasty ve střední části čepele třetího listu. Objemová hustota thylakoidů byla nejvyšší v dospělých chloroplastech. Rozdíly byly i mezi genotypy. Fotosynteticky více účinný genotyp (v aktivitě Hillovy reakce) měl také více thylakoidů, zejména granálních. Se stárnutím se chloroplasty zakulacovaly a ve starých chloroplastech výrazně přibylo plastoglobulů.

Na práci Kutík a kol. (1999) navazovala má vlastní práce. Pomocí stereologických metod jsem se zabývala vývojem chloroplastů buněk pochev cévních svazků u kukuřice seté (Vičánková a Kutík 2005). Doplnila jsem výsledky pozorování ultrastrukturálních změn ve vývoji chloroplastů v mezofylových buňkách (Kutík a kol. 1999) výsledky sledování těchto změn u chloroplastů buněk pochev cévních svazků také u výše zmiňovaných fotosynteticky kontrastních genotypů kukuřice a jejich hybridů. Šlo patrně o první kvantitativní hodnocení vývojových změn chloroplastů v buňkách pochev cévních svazků. Objemová hustota thylakoidů byla v dospělých a stárnoucích listech vyšší než v mladých, zajímavé je nejvyšší zastoupení granálních thylakoidů ve stárnoucích listech, kde současně výrazně přibylo plastoglobulů a výrazně ubylo škrobu. To vše nasvědčuje horšímu fungování C_4 fotosyntézy ve stárnoucích listech. Oba rodičovské genotypy se příliš nelišily. U hybrida, kde mateřským genotypem byl fotosynteticky výkonnější rodič, se hromadilo v dospělých listech několikanásobně více škrobu, než u reciprokého hybrida.

2.3.2 Heterogenita ultrastruktury a fotosyntetických funkcí chloroplastů na listové čepeli

Změny ultrastruktury chloroplastů a jejich funkčních charakteristik během ontogenetického vývoje listu jsou často sledovány na listové čepeli trav (např. *Triticum, Hordeum, Zea*). Vývoj těchto protáhlých úzkých listů umožňuje sledování vývojového gradientu chloroplastů podél listové čepele, přičemž nejmladší chloroplasty se nacházejí v bazální části listu a nejstarší v apexu listu. Široké listy dvojděložných rostlin, často s komplikovaným tvarem listové čepele, jsou pro tento účel méně vhodné. Inspirací pro hledání možných gradientů ultrastruktury chloroplastů na listu nám byly dlouho známé a na naší katedře studované gradienty anatomických charakteristik na listu (např.

hustota průduchů, velikost epidermálních buněk), o kterých referuje Tichá (1985). Různá stádia vývoje chloroplastů podél listové čepele trav jsou charakterizována také změnami v ultrastruktuře těchto organel odpovídajícími vývojovým změnám chloroplastů během ontogeneze listu popsaným v review Kutík 1998.

V návaznosti na výsledky z prací Kutík a kol. (1999), Vičánková a Kutík (2005), kde jsme sledovali ultrastrukturu chloroplastů v buňkách mezofylu i pochev cévních svazků fotosynteticky aktivního listu rostlin kukuřice různých genotypů v průběhu listové ontogeneze, jsme se zaměřili na rozdíly ve strukturních i fotosyntetických charakteristikách dvou fotosynteticky kontrastních genotypů (rodičovská linie a F1 kříženec kukuřice) v závislosti na místě odběru asimilačního pletiva z čepele dospělého listu kukuřice (Kutík a kol. 2001, Vičánková a kol. – *Photosynthetica nabídnuto*).

Vztahy mezi ultrastrukturou chloroplastů a funkčními charakteristikami fotosyntetického aparátu ve vztahu k heterogenitě listové čepele se detailně dosud nikdo nezabýval. Byl popsán vývojový gradient ultrastruktury plastidů od báze k vrcholu ještě rostoucích listů Brangeon (1973a). Chloroplasty v buňkách listového mezofylu kukuřice se také mohou lišit v závislosti na vzdálenosti těchto buněk od cévních svazků. Tyto možné rozdíly, stejně jako možné rozdíly mezi mezofylovými buňkami na svrchní a spodní straně monofaciálního listu kukuřice, jsme se rozhodli zanedbat, protože je považujeme za méně významné. Většina prací studujících vývojové gradienty na čepeli listů trav je zaměřena na mladé chloroplasty na bázi listu, které mají prolamelární tělesa (Brangeon 1973a), nebo obsahují škrobová zrna a pak je nazýváme chloroamyloplasty (Wellburn a kol. 1982, Chonan a kol. 1991).

Heterogenitu ultrastruktury chloroplastů buněk mezofylu a heterogenitu fotosyntetické aktivity podél listové čepele jsme sledovali u dvou kontrastních genotypů kukuřice porovnáním bazální, střední a apikální části listové čepele dospělých listů a listů začínajících stárnout (Kutík a kol. 2001). Zjištěné gradienty byly podobné jako v předešlých pracích, kdy byly studovány vzorky ze střední části listové čepele mladého, dospělého a stárnoucího listu (Kutík a kol. 1999, Vičánková a Kutík 2005). Výhodou použití dvou genotypů kukuřice je, že jsou tyto rostliny ontogeneticky posunuty ve vývoji(kříženec se vyvíjí rychleji), takže lze i takto sledovat časovou dynamiku prostorových gradientů. Tyto výsledky doplňuje publikace Vičánková a kol. –Photosynthetica, nabídnuto. Kvantitativně pomocí analýzy obrazu jsem hodnotila chloroplasty mezofylových buněk a buněk pochev cévních svazků. Změny

ultrastruktury dimorfních chloroplastů podél listové čepele v obou těchto pracích doplňují změny fotosyntetických charakteristik (Kutík a kol. 2001) a jsou v souladu se změnami biochemických charakteristik během vývoje listu (Čatský a Šesták 1997, Šesták a Šiffel (1997). Zajímavý je rozdíl zjištěný mezi MC a BSC chloroplasty: zatímco změny MC chloroplastů u hybrida sledují změny u mateřského rodiče, chloroplasty BSC se u rodiče a hybrida chovají různě (např. různé hromadění škrobových inkluzí).

2.3.3 Vliv amitrolu na vývoj chloroplastů

K získání mnohostranného obrazu o plasticitě a dynamice fotosyntetického aparátu vyšších rostlin se při studiu vztahů mezi strukturou a funkcí fotosyntetického aparátu často využívají různé experimentální zásahy, které působí na ultrastrukturu chloroplastů, funkční charakteristiky thylakoidních membrán a složení fotosyntetických barviv.

V návaznosti na naše předchozí práce, popisující vývoj dimorfních chloroplastů kukuřice a gradienty fotosyntetických charakteristik na jejím listu (Kutík a kol.1999, Kutík a kol. 2001), jsme ovlivnili vývoj dimorfních chloroplastů kukuřice působením herbicidu amitrolu (3-amino-1,2,4 triazol), který inhibuje biosyntézu karotenoidů thylakoidních membrán (beta-karoten, xantofyly). Funkce karotenoidů v chloroplastech, zejména jako ochranných komponentů pigment-proteinových komplexů, je významná (např. Demmig-Adams a kol. 1996, Philip a kol. 2002). Jejich roli ve vývoji a funkci fotosyntetického aparátu můžeme pozorovat na rostlinách deficientních na karotenoidy, které získáme působením herbicidů (Sandmann a Böger 1982) nebo použitím mutantů (Fambrini a kol. 1993, Wetzel a kol. 1994, La Rocca a kol. 2000a). Amitrol působí na ochranné mechanismy rostlin různými cestami. Inhibice katalázy rostlin *Solanum tuberosum* L. působením amitrolu vede ke vzrůstu koncentrace H_2O_2 v perixosomech (Muraja-Ljubičić a kol. 1999). Aplikace amitrolu však nejvíce působí na obsah karotenoidů (La Rocca a kol. 2001, Hirschberg 2001). Přerušením syntézy karotenoidů amitrol negativně ovlivňuje i jiné fotosyntetické pigmenty, jako např. absencí β-karotenu dochází k degradaci chlorofylů (Wrischer a kol. 1992).

Změny ve struktuře pigment- proteinových komplexů se odrážejí i ve změnách ultrastruktury chloroplastů, které závisejí na použité koncentraci herbicidu, světle a teplotě (Anderson a Robertson 1960, Rascio a kol. 1996, La Rocca a kol. 1998, 2001).

Nejčastější jsou studie vlivu vysokých koncentrací na ultrastrukturu chloroplastů (Zito a kol. 1995, Agnolucci a kol. 1996, Rascio a kol. 1996, La Rocca a kol. 2000a,b, Dalla Vecchia a kol. 2001), které popisují téměř úplnou destrukci chloroplastů.

Sledovali jsme působení tří různých koncentrací amitrolu (20, 60 a 120 mikromolů na litr) na vývoj dimorfních chloroplastů dvou fotosynteticky kontrastních genotypů kukuřice (Pechová a kol. 2003). Zjistili jsme, že zatímco téměř jediným vlivem nízké koncentrace amitrolu je pokles objemové hustoty škrobových inkluzí v chloroplastech BSC, střední a vysoká koncentrace amitrolu působí nápadnou a téměř úplnou destrukci vnitřní stavby obojích chloroplastů, pokles obsahu a téměř úplné vymizení karotenoidů a chlorofylů a pokles a vymizení fotochemické aktivity listů. Mezofylové chloroplasty jsou více citlivé na poškození ultrastruktury pod vlivem amitrolu než chloroplasty buněk pochev cévních svazků, i ve značně narušených chloroplastech však bylo možné imunocytochemicky prokázat světlosběrné komplexy. Oba studované genotypy kukuřice se chovaly podobně, ale některé charakteristiky ukazují na větší citlivost k amitrolu u fotosynteticky výkonnějšího genotypu. Je zajímavé, že u rostlin ovlivněných amitrolem i kontrolních jsme občas pozorovali u chloroplastů MC i BSC výběžky - stromuly (Gray a kol. 2001). Herbicidy amitrol a norflurazon, negativně ovlivňující biosyntézu karotenoidů, zůstávají významným nástrojem pro studium vývoje struktury a funkcí plastidů (Moro a kol. 2004). Správná syntéza polárních karotenoidů (xantofylů) je zřejmě nezbytná pro udržování stabilních membrán plastidů.

2.3.4 Vliv imobilizace a různé hladiny cytokininů na chloroplasty

Rostlinné hormony cytokininy hrají důležitou roli ve vývoji a diferenciaci plastidů. Po objevení rostlinného regulátoru kinetinu (Miller a kol. 1955), byl prokázán jeho vliv na vývoj chloroplastů z proplastidů v tabákových kulturách (Stetler a Laetsch 1965). Cytokininy regulují vývoj chloroplastů, zejména pak při de-etiolaci (Zavaleta-Mancera a kol. 1999 a, b, Chory a kol. 1994). Vliv cytokininů na počet a velikost chloroplastů a na rozvoj vnitřní struktury chloroplastů popisují Longo a kol. (1979). V rostlinách tabáku bylo ve chloroplastech detekováno celé spektrum cytokininů (Benková a kol. 1999).

V současnosti je v popředí vědeckého zájmu cytokininový metabolismus a jeho vliv na rostlinu. K objasnění regulace metabolismu rostlinných hormonů se často

používají izolované organely, které nejsou ovlivňovány zbytkem buňky. Studie cytokininového metabolismu v izolovaných chloroplastech jsou limitovány zejména jejich nízkou stabilitou při pokojové teplotě. Zakomponování chloroplastů do vhodného materiálu má ochranný efekt, protože umožňuje vytvořit vhodné mikroprostředí pro organelu a ještě ji chránit před mechanickým poškozením. Stabilizující hmota, matrix, zabraňuje také difusi např. O_2 (Guiseley 1989). Vliv imobilizace závisí jak na typu a koncentraci použitého polymeru tak na použitém organismu (Junter a kol. 2002). Synková a Šesták (1991) srovnávali fotochemické aktivity u volných a imobilizovaných chloroplastů do agaru, Ca- alginátu a glutaraldehydem zesíťovaného bovin-albumin séra. Hara a kol. (1999) srovnávali metody a materiály při imobilizaci chloroplastů rostlin špenátu.

K prodloužení viability izolovaných chloroplastů z listů tabáku *Nicotiana tabacum* L. cv. Petit Havana SR1 jsme použili jejich imobilizaci do nízkoviskozitního alginátu (Polanská a kol. 2004). Imobilizace téměř neměla vliv na ultrastrukturu chloroplastů a oddálila jejich totální desintegraci. Ultrastruktura volných chloroplastů byla totálně desintegrovaná po 7 h. Více než 50% imobilizovaných chloroplastů zůstalo intaktních po 24 h.

Součástí studia hladin fytohormonů v listech a chloroplastech u rostlin tabáku se změněným metabolismem cytokininů bylo i sledování změn ultrastruktury chloroplastů (Polanská a kol. – rukopis v poslední fázi přípravy). Rostliny transgenního tabáku s nadprodukcí cytokininů mají pozměněnou vnitřní strukturu chloroplastů. Tyto změny se většinou týkají akumulace škrobových zrn, což většinou vede i ke změně tvaru chloroplastu. Chloroplasty u rostlin s konstitutivně overexprimovaným *Sho* genem a trvalou nadprodukcí cytokininů měly nejvíce změn v ultrastruktuře, které se projevovaly zvýšeným počtem thylakoidů v granech, zvýšením množství plastoglobulů a občasnou přítomností krystaloidů ve stromatu.Podle Synkové a kol. (2005) může jít o agregáty světlosběrných komplexů chloroplastů, což nasvědčuje narušení fotosyntetického aparátu trvale zvýšenou hladinou cytokininů. Exogenně aplikované cytokininy, které ve tmě chrání fotosyntetický aparát před senescencí, ho mohou na světle poškozovat (Vlčková a kol. 2006). Snížená hladina cytokininů v našich pokusech na ultrastrukturu chloroplastů výrazný vliv neměla.

3 Závěry / Conclusions

ZÁVĚRY

Ve své práci jsem sledovala plasticitu ultrastruktury chloroplastů ve vztahu k fotosyntéze listů u kukuřice a ve vztahu k metabolismu cytokininů u tabáku. Dospěla jsem k následujícím hlavním závěrům.

1. Během vývoje fotosynteticky aktivních listů kukuřice (mladé, dospělé a stárnoucí listy) se nenáhodně kvantitativně mění ultrastruktura chloroplastů v buňkách pochev cévních svazků (BSC) ve střední části listové čepele, podobně jako to bylo dříve zjištěno pro chloroplasty v buňkách mezofylu těchto listů (MC). Zjistila jsem dále heterogenitu v ultrastruktuře MC i BSC chloroplastů, obsahu fotosyntetických barviv a fotochemické aktivitě částí listu při srovnání bazální, střední a vrcholové třetiny listové čepele dospělých listů kukuřice. Vrchol čepele má charakteristiky nejstarší, báze čepele nejmladší části. Tím se konzervuje vývojový gradient listu. Podoba gradientu závisí na genotypu kukuřice a mění se při nástupu senescence listu. Na uvedeném materiálu jsem také úspěšně porovnala kvantitativní hodnocení ultrastruktury chloroplastů stereologicky a pomocí automatické analýzy obrazu.

2. Herbicid amitrol (3-amino-1,2,4-triazol), který brzdí syntézu ochranných a pomocných barviv fotosyntetického aparátu karotenoidů, způsobuje u kukuřice v nižší koncentraci (20 mikromolů na litr) jen nevelké kvantitativní změny v ultrastruktuře MC a BSC chloroplastů a příliš neovlivňuje ani obsah fotosyntetických barviv a fotochemickou aktivitu listů. Ve vyšších koncentracích (60 a zejména 120 mikromolů na litr) způsobuje výrazný pokles obsahu karotenoidů, fotodestrukci chlorofylů a vážné narušení ultrastruktury MC a BSC chloroplastů, které ztrácejí funkčnost. Chloroplasty MC jsou citlivější než BSC. Dva vybrané genotypy kukuřice se vzájemně příliš neliší.

3. Imobilizace izolovaných chloroplastů z listů tabáku do nízkoviskozitního alginátu umožňuje studovat u nich metabolismus rostlinných hormonů cytokininů bez ovlivnění zbytkem buňky. Imobilizace nemá výrazný vliv na ultrastrukturu těchto chloroplastů a podstatně zpomaluje jejich destrukci po izolaci.

4. Cytokininy se podílejí na regulaci vývoje chloroplastů v listech tabáku. U rostlin tabáku s konstitutivní nadprodukcí cytokininů je narušena ultrastruktura chloroplastů, zejména zvýšeným množstvím plastoglobulů a občasnou přítomností krystaloidních struktur ve stromatu. Při nadprodukci cytokininů s pomocí indukovatelného promotoru enzymu isopentenyltransferasy nejsou patrné krystaloidy, ale pouze zvýšená tvorba gran ve chloroplastech. Snížený obsah cytokininů ultrastrukturu chloroplastů příliš neovlivňuje.

CONCLUSIONS

In my thesis I studied ultrastructural plasticity of chloroplasts in relation to leaf photosynthesis in maize and in relation to cytokinin metabolism in tobacco plants. Main conclusions of my studies are as follows.

1. During development of photosynthetically active leaves of maize plants (young, mature, and senescing leaves) chloroplast ultrastructure in vascular bundle sheath cells (BSC) in middle part of leaf blade is non randomly quantitatively changed similarly as it was found formerly for the chloroplasts in mesophyll cells (MC) of these leaves. I also found heterogeneity in MC and BSC chloroplast ultrastructure, photosynthetic pigments content and photochemical activities comparing basal, middle, and apical third of the blade of mature maize leaves. Leaf blade apex has characteristics of the oldest, leaf blade base of the youngest leaf part. In such a manner, leaf developmental gradient is conserved. The form of the gradients depends on maize genotype studied and it is somewhat changed after start of leaf senescence. On this material I also successfully compared the quantitative evaluation of chloroplast ultrastructure using stereology or automatic image analysis.

2. Herbicide amitrole (3-amino-1,2,4-triazol) which is inhibiting the biosynthesis of protective and auxiliary pigments of photosynthetic apparatus, carotenoids, is causing in lower concentration (20 micromoles per litre) some quantitative changes in the ultrastructure of MC and BSC maize chloroplasts only and it does not much influence also the content of photosynthetic pigments and photochemical leaf activities. In higher concentrations (60 and especially 120 micromoles per litre) amitrol is causing a striking decrease of the content of carotenoids, photodestruction of chlorophylls and a serious damage of chloroplast ultrastructure and loss of function of MC and BSC chloroplasts, the MC ones being more sensitive. Two maize genotypes studied behave rather similarly.

3. Immobilisation of isolated chloroplasts from tobacco leaves into low viscosity alginate makes possible to study metabolism of plant hormones cytokinins in them without influence of other cell compartments. This immobilisation has no striking impact on the ultrastructure of these chloroplasts and it is slowing down substantially their destruction after isolation.

4. Cytokinins take part in the control of chloroplast development in tobacco leaves. In tobacco plants with constitutive cytokinin overproduction the ultrastructure of leaf chloroplasts is altered, especially by large amount of plastoglobuli and occurence of crystalloid structures in chloroplast stroma. During cytokinin overproduction brought about by an inducible promotor of isopentenyl transferase, only increased thylakoid membrane stacking is observed. Lowered cytokinin content has no striking impact on the chloroplast ultrastructure.

4 Seznam citované literatury

- Albertsson PA (1995): The structure and function of the chloroplast photosynthetic membrane - A model for the domain organization. Photosynth Res 46: 141-149
- Aldridge C, Maple J, Moller SG (2005): The molecular biology of plastid division in higher plants. J Exp Bot 56: 1061-1077
- Allen JF, Forsberg J (2001): Molecular recognition in thylakoid structure and function. Trends Plant Sci 6: 317-326
- Anderson C, Robertson DS (1960): Role of carotenoids in protecting chlorophyll from photodestruction. Plant Physiol 35: 531-534
- Andersson B, Anderson JM (1980): Lateral heterogeneity in the distribution of chlorophyll – protein complexes of the thylakoid membranes of spinach chloroplasts. Biochim Biophys Acta 593: 427–440
- Artus NN, Ryberg M, Sundqvist C (1990): Plastid microtubule-like structures in wheat are insensitive to microtubule inhibitors. Physiol Plant 79: 641-648
- Babůrek I (1973): Kvantitativní genetické studium některých struktur listu významných pro fotosyntézu. Diplomová práce PřF UK, Praha
- Baker NR, Barber J (eds.). Chloroplast Biogenesis, Elsevier Science Publishers, Amsterdam. Pp. 71-132
- Bauer J, Hiltbrunner A, Kessler F (2001): Molecular biology of chloroplast biogenesis: gene expression, protein import and intraorganellar sorting. Cell Mol Life Sci 58: 420-433
- Benková E, Witters E, van Dongen W, Kolář J, Motyka V, Brzobohatý B, Van Onckelen HA, Macháčková I (1999): Cytokinins in tobacco and wheat chloroplasts. Occurrence and changes due to light/dark treatment. Plant Physiol 121: 245-251
- Biswal UC, Biswal B (1988): Ultrastructural modifications and biochemicalchanges during senescence of chloroplasts. Int Rev Cytol 113: 271-321
- Biswal UC, Biswal B, Raval MK (2003): Chloroplast Biogenesis. From Proplastid to Gerontoplast. Kluwer Academic Publishers, Dordrecht, Boston, London
- Bodyl A (2002): Is protein import into plastids with four-membrane envelopes dependent on two Toc systems operating in tandem? Plant Biol 4: 423-431
- Bonatti PM, Fornasiero RB (1990): Developmental pattern and structural organization of leaf chloroplasts in Lepidozamiaperoffskyana. Aust J Bot 38: 53-62

- Bonatti PM, Sabato S (1984): Plastid ultrastructure in leaves of Macrozamiamoorei (Zamiaceae). Caryologia 37: 133-139
- Bonzi LM, Bonatti PM, Marini C, Fornasiero RB, Paoletti C (1992): Ultrastructural studies on differentiating chloroplasts in the forma fuscoviridis of Ceratozamiamexicana Brongn. New Phytol 120: 427-434
- Bourett TM, Czymmek KJ, Howard RJ (1999): Ultrastructure of chloroplast protuberances in rice leaves preserved by high-pressure freezing. Planta 208: 472-479
- Brangeon J (1973a): Compared ontogeny of the two types of chloroplasts of Zea mays. J Microscopie 16: 233-242
- Brangeon J (1973b): Effect of irradiance on granal configurations of Zea mays bundle sheath chloroplasts. Photosyntetica 7: 365-372
- Brown NJ, Parsley K, Hibberd JM (2005): The future C4 research - maize, Flaveria or Cleome? Trends Plant Sci 10: 215-221
- Buchanan-Wollaston V (1997): The molecular biology of leaf senescence. J Exp Bot 48: 181-199
- Butterfass T (1983): A nucleotypic control of chloroplast reproduction. Protoplasma 118: 71-74
- Cavalier-Smith T (2000): Membrane heredity and early chloroplast evolution. Trends Plant Sci 5: 174-182
- Čatský J, Šesták Z (1997): Photosynthesis during leaf development. In: Pessarakli M (ed.): Handbook of Photosynthesis. Pp. 633-660. Marcel Dekker, Inc., New York, Basel, Hong Kong
- Dahlin C, Ryberg H (1986): Accumulation of phytoene in plastoglobuli of San-9789 (norflurazon)-treated dark-grown wheat. Physiol Plant 68: 39-45
- Dalla Vecchia F, Barbato R, La Rocca N, Moro I, Rascio N (2001): Responses to bleaching herbicides by leaf chloroplasts of maize plants grown at different temperatures. J Exp Bot 52: 811-820
- Demmig-Adams B, Gilmore AM, Adams WW (1996): Carotenoids.3. In vivo functions of carotenoids in higher plants. FASEB J 10: 403-412
- Edwards GE, Franceschi VR, Ku MSB, Voznesenskaya EV, Pyankov VI, Andreo CS (2001): Compartmentation of photosynthesis in cells and tissues of C4 plants. J Exp Bot 52: 577-590
- Edwards GE, Franceschi VR, Voznesenskaya EV (2004): Single-cell C4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Biol 55: 173-196

- Endler W, Barsig M, Weese G, Hafner L (1990): Cytomorphological investigations of pine needles (Pinus-silvestris L.) in a conurbation.1. The ultrastructure of chloroplasts in mesophyll according to season, age, and pollution burden. Angew Bot 64: 289-302

- Fagerberg WR (1984): Cytological changes in palisade cells of developing sunflower leaves. Protoplasma 119: 21-30

- Fambrini M, Pugliesi C, Vernieri P, Giuliano G, Baroncelli S (1993): Characterization of a sunflower (Helianthus-annuus L) mutant, deficient in carotenoid synthesis and abscisicacid content, induced by in-vitro tissue-culture. Theor Appl Genet 87: 65-69

- Fisher DG, Evert RF (1982): Studies on the leaf of Amaranthusretroflexus (Amaranthaceae) - chloroplast polymorphism. Bot Gaz 143: 146-155

- Fulgosi H, Gerdes L, Westphal S, Glockmann C, Soll J (2002): Cell and chloroplast division requires ARTEMIS. Proc Natl Acad Sci USA 99: 11501-11506

- Furbank RT, Foyer CH (1988): C plants as valuable model experimental systems for the study of photosynthesis. New Phytol 109: 265-277

- Furbank RT, Taylor WC (1995): Regulation of photosynthesis in C3 and C4 plants - a molecular approach. Plant Cell 7: 797-807

- Gamaleï YV, Kulikov GV (1978): Razvitie Khlorenkhimy Lista [Development of Leaf Chlorenchyma]. Nauka, Leningrad

- Gray JC, Sullivan JA, Hibberd JM, Hansen MR (2001): Stromules: Mobile protrusions and interconnections between plastids. Plant Biol 3: 223-233

- Guiseley KB (1989): Chemical and physical-properties of algal polysaccharides used for cell immobilization. Enzyme Microb Technol 11: 706-717

- Gundersen HJG, Jensen EB (1987): The efficiency of systematic sampling in stereology and its prediction. J Microsc-Oxford 147: 229-263

- Gunning BES (2005): Plastid stromules: video microscopy of their outgrowth, retraction, tensioning, anchoring, branching, bridging, and tip-shedding. Protoplasma 225: 33-42

- Hall JL (ed.) (1978): Electron Microscopy and Cytochemistry of Plant Cells. Elsevier/ North-Holland Biomedical Press, Amsterdam, Oxford, New York

- Hara M, Iazvovskaia S, Ohkawa H, Asada Y, Miyake J (1999): Immobilization of P450 monooxygenase and chloroplasts for use in light-driven bioreactors. J Biosci Bioeng 87: 793-797

- Hatch MD (1992): C4 photosynthesis: An unlikely process full of surprises. Plant Cell Physiol 33: 333-342

- Hirschberg J (2001): Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol 4: 210-218

- Hudák J (1997): Photosynthetic apparatus. In: Pessarakli M (ed.): Handbook of Photosynthesis. Pp. 27-48. Marcel Dekker, Inc., New York, Basel, Hong Kong

- Hudák J, Herich R, Bobák M (1983): Plastidy. Veda, Bratislava

- Chonan N, Matsuda T, Tsuchiya T (1991): Ultrastructural-changes in chloroplasts and phloem of rice leaves during senescence under different nitrogen supply. Jpn J Crop Sci 60: 247-254

- Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994): A role for cytokinins in de-etiolation in Arabidopsis - det mutants have an altered response to cytokinins. Plant Physiol 104: 339-347

- Chow WS, Kim EH, Horton P, Anderson JM (2005): Granal stacking of thylakoid membranes in higher plant chloroplasts: the physicochemical forces at work and the functional consequences that ensue. Photochem Photobiol Sci 4: 1081-1090

- Jarvis P, Soll J (2002): Toc, Tic, and chloroplast protein import. Biochim Biophys Acta-Mol Cell Res 1590: 177-189

- Junter GA, Coquet L, Vilain S, Jouenne T (2002): Immobilized-cell physiology: current data and the potentialities of proteomics. Enzyme Microb Technol 31: 201-212

- Kiessling J, Kruse S, Rensing SA, Harter K, Decker EL, Reski R (2000): Visualization of a cytoskeleton-like FtsZ network in chloroplasts. J Cell Biol 151: 945-950

- Köhler RH, Hanson MR (2000): Plastid tubules of higher plants are tissue-specific and developmentally regulated. J Cell Sci 113: 81-89

- Kramer DM, Sacksteder CA, Cruz JA (1999): How acidic is the lumen? Photosynth Res 60: 151-163

- Kratsch HA, Wise RR (2000): The ultrastructure of chilling stress. Plant Cell Environ 23: 337-350

- Kuroiwa H, Mori T, Takahara M, Miyagishima S, Kuroiwa T (2002): Chloroplast division machinery as revealed by immunofluorescence and electron microscopy. Planta 215: 185-190

- Kusnetsov VV, Oelmuller R, Sarwat MI, Porfirova SA, Cherepneva GN, Herrmann RG, Kulaeva ON (1994): Cytokinins, abscisicacid and light affect accumulation of chloroplast proteins in Lupinusluteus cotyledons without notable effect on steady-state messenger-RNA levels - specific protein response to light/phytohormone interaction. Planta 194: 318-327

- Kutík J (1985): Chloroplast development. In: Šesták Z (ed.): Photosynthesis during Leaf Development. Pp. 51-75. Academia, Praha, Dr W Jung Publishers, Dordrecht, Boston, Lancaster

- Kutík J (1988): A quantitative study on ontogenetic development of chloroplast ultrastructure. Acta Univ Carol Biol 31: 51-54

- Kutík J (1989): Ultrastructure of chloroplasts in palisade and spongy parenchyma cells during development of primary bean-leaves. Photosynthetica 23: 678-681

- Kutík J (1992): Microtubule-like structures in developing wheat-leaf chloroplasts. Biol Plant 34: 355-357

- Kutík J (1998): The development of chloroplast structure during leaf ontogeny. Photosynthetica 35: 481-505

- Kutík J, Holá D, Vičánková A, Šmídová M, Kočová M, Körnerová M, Kubínová L (2001): The heterogeneity of structural and functional photosynthetic characteristics of mesophyll chloroplasts in various parts of mature or senescing leaf blade of two maize (Zea mays L.) genotypes. Photosynthetica 39: 497-506

- Kutík J, Kočová M (1996): The development of chloroplast ultrastructure and photochemical activity during leaf ontogeny in maize (Zea mays L.). Biológia, Bratislava 51: 59

- Kutík J, Kočová M, Holá D, Körnerová M (1999): The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (Zea mays L.) genotypes. Photosynthetica 36: 497-507

- Kutík J, Šesták Z, Volfová A (1984): Ontogenetic changes in the internal limitations to bean-leaf photosynthesis.8. Primary leaf blade characteristics and chloroplast number, size and ultrastructure. Photosynthetica 18: 1-8

- Kutík J, Zima J, Šesták Z, Volfová A (1988): Ontogenetic changes in the internal limitations to bean-leaf photosynthesis.10. chloroplast ultrastructure in primary and first trifoliate leaves. Photosynthetica 22: 511-515

- Kwok EY, Hanson MR (2003): Microfilaments and microtubules control the morphology and movement of non-green plastids and stromules in Nicotiana tabacum. Plant J 35: 16-26

- La Rocca N, Barbato R, Dalla Vecchia F, Rascio N (2000a): Cab gene expression in bleached leaves of carotenoid-deficient maize. Photosynth Res 64: 119-126

- La Rocca N, Bonora A, Dalla Vecchia F, Barbato R, Rascio N (1998): Effects of amitrole and norflurazon on carotenogenesis in barley plants grown at different temperatures. In: Garab G (ed.).

- La Rocca N, Dalla Vecchia F, Barbato R, Bonora A, Bergantino E, Rascio N (2000b): Plastid photodamage and Cab gene expression in barley leaves. Physiol Plant 109: 51-57

- La Rocca N, Rascio N, Oster N, Rüdiger W, (2001): Amitrole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of Lhc and RbcS genes. Planta 213: 101-108

- Lawlor DW (2001): Photosynthesis (Third Edition). BIOS Scientific Publishers, Ltd., Oxford

- Lawrence ME, Possingham JV (1984): Observations of microtubule-like structures within spinach plastids. Biol Cell 52: 77-82

- Leegood RC (2002): C4 photosynthesis: principles of CO_2 concentration and prospects for its introduction into C3 plants. J Exp Bot 53: 581-590

- Leech RM (1986): Stability and plasticity during chloroplast development. In: Jennings DH, Trewavas AJ (ed.). Plasticity in Plants. Pp. 121-153. The Company of Biologists, Cambridge

- Link G (1991): Photoregulated development of chloroplasts. In: Bogorad L, Vasil IK (eds.). The Photosynthetic Apparatus: Molecular Biology and Operation. Pp. 365-394. Academic Press, San Diego

- Lloyd JR, Kossmann J, Ritte G (2005): Leaf starch degradation comes out of the shadows. Trends Plant Sci 10: 130-137

- Longo GP, Pedretti M, Rossi G, Longo CP (1979): Effect of benzyladenine on the development of plastids and microbodies in excised watermelon cotyledons. Planta 145: 209-217

- Lunn JE, Hatch MD (1995): Primary partitioning and storage of photosynthates in sucrose and starch in leaves of C4 plants. Planta 197: 385-391

- Lutkenhaus J, Addinall SG (1997): Bacterial cell division and the Z ring. Annu Rev Biochem 66: 93-116

- Maier RM, Neckermann K, Igloi GL, Kossel H (1995): Complete sequence of the maize chloroplast genome - gene content, hotspots of divergence and fine-tuning of genetic information by transcript editing. J Mol Biol 251: 614-628

- Majeran W, Cai Y, Sun Q, van Wijk KJ (2005): Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. Plant Cell 17: 3111-3140

- Maple J, Aldridge C, Moller SG (2005): Plastid division is mediated by combinatorial assembly of plastid division proteins. Plant J 43: 811-823

- Maple J, Moller SG (2005): An emerging picture of plastid division in higher plants. Planta 223: 1-4

- Martin C, Smith AM (1995): Starch biosynthesis. Plant Cell 7: 971-985

- Matile Ph (1991): Chloroplast senescence. In: Baker NR, Thomas H (ed.). Crop Photosynthesis: Spatial and Temporal Determinants. Pp. 413-440. Elsevier, Amsterdam

- McFadden GI (2000): Skeletons in the closet: How do chloroplasts stay in shape? J Cell Biol 151: F19-F21

- Miller CO, Skoog F, von Saltza MH, Strong F (1955): Isolation, structure and synthesis of kinetin, a substance promoting cell division. J Am Chem Soc 78: 1375-1380

- Miller CO, Skoog F, von Saltza MH, Strong F (1955): Isolation, structure and synthesis of kinetin, a substance promoting cell division. J Am Chem Soc 78: 1375-1380

- Miroslavov EA, Alexeeva OA (1990): On the life duration of plastids in chlorenchima cells of Taxus leaves. Dokl Akad Nauk SSSR. 313: 1274-1276

- Miyagishima SY, Itoh R, Toda K, Takahashi H, Kuroiwa H, Kuroiwa T (1998): Orderly formation of the double ring structures for plastid and mitochondrial division in the unicellular red alga Cyanidioschyzon merolae. Planta 206 (4): 551-560

- Miyagishima SY, Takahara R, Kuroiwa T (2001): Novel filaments 5 nm in diameter constitute the cytosolic ring of the plastid division apparatus. Plant Cell 13: 707-721

- Modrušan Z, Wrischer M (1987): Seasonal changes in chloroplasts of blackberry leaves. Acta Bot Croat 46: 23-31

- Mori T, Kuroiwa H, Takahara M, Miyagishima S, Kuroiwa T (2001): Visualization of an FtsZ ring in chloroplasts of Lilium longiflorum leaves. Plant Cell Physiol 42: 555-559

- Moro I, Dalla Vecchia F, La Rocca N, Navari-Izzo F, Quartacci MF, Di Baccio D, Rudiger W, Rascio N (2004): Impaired carotenogenesis can affect organization and functionality of etioplast membranes. Physiol Plant 122: 123-132

- Morré DJ, Selldén G, Sundqvist C, Sandelius AS (1991): Stromal lowtemperature compartment derived from the inner membrane of the chloroplast envelope. Plant Physiol 97: 1558-1564

- Muraja-Ljubičić J, Wrischer M, Ljubešić N (1999): Influence of the herbicides amitrole and norflurazon on greening of illuminated potato microtubers. Z Naturforsch 54: 333-336

- Mustárdy L (1996): Development of thylakoid membrane stacking. In: Ort DR, Yocum CF (eds.). Oxygenic Photosynthesis: The Light Reactions. Pp. 59-68. Kluwer Academic Publishers, Dordrecht, Boston, London

- Mustárdy L, Garab G (2003): Granum revisited. A three-dimensional model - where things fall into place. Trends Plant Sci 8: 117-122

- Nelson T, Dengler NG (1992): Photosynthetic tissue differentiation in C 4 plants. Int J Plant Sci 153: S93-S105

- Nelson T, Langdale JA (1989): Patterns of leaf development in C4 plants. Plant Cell 1: 3-13

- Nelson T, Langdale JA (1992): Developmental genetics of C4 photosynthesis. Annu Rev Plant Physiol Plant Molec Biol 43: 25-47

- Nii N, Kawano S, Nakamura S, Kuroiwa T (1988): Changes in the finestructure of chloroplasts and chloroplast DNA of peach leaves during senescence. J Jpn Soc Hortic Sci 57: 390-398

- Nishioka D, Brisibe EA, Miyake H, Taniguchi T (1993): Ultrastructural observations in the suppression of granal development in bundlesheath chloroplasts of NADP-ME type C4 monocot and dicot species. Jpn J Crop Sci 62: 621-627

- Pechová R, Kutík J, Holá D, Kočová M, Haisel D, Vičánková A (2003): The ultrastructure of chloroplasts, content of photosynthetic pigments, and photochemical activity of maize (Zea mays L) as influenced by different concentrations of the herbicide amitrole. Photosynthetica 41: 127-136

- Phillip D, Hobe S, Paulsen H, Molnar P, Hashimoto H, Young AJ (2002): The binding of xanthophylls to the bulk light-harvesting complex of photosystem II of higher plants - A specific requirement for carotenoids with a 3-hydroxy-beta-end group. J Biol Chem 277: 25160-25169

- Photosynthesis: Mechanisms and Effects. Vol. V. Pp. 3865-3868. Kluwer Academic Publishers, Dordrecht, Boston, London

- Polanská L, Vičánková A, Dobrev PI, Macháčková I, Vaňková R (2004): Viability, ultrastructure and cytokinin metabolism of free and immobilized tobacco chloroplasts. Biotechnol Lett 26: 1549-1555

- Polanská L, Vičánková A, Nováková M, Malbeck J, Dobrev PI, Brzobohatý B, Vaňková R, Macháčková I (rukopis v poslední fázi přípravy): Altered cytokinin metabolism affects cytokinin, auxin and abscisic acid contents in leaves and chloroplasts and chloroplast ultrastructure in transgenic tobacco

- Pyke KA (1999): Plastid division and development. Plant Cell 11: 549-556

- Rascio N, DallaVecchia F, Agnolucci L, Barbato R, Tassani V, Casadoro G (1996): Amitrole effects on barley etioplasts. J Plant Physiol 149: 295-300

- Rascio N, Mariani Colombo P, Dalla Vecchia F, Chitani P (1985): Intrathylakoidal crystal appearance during the vital cycle of spinachchloroplasts. Protoplasma 126: 153-157

- Raynaud C, Cassier-Chauvat C, Perennes C, Bergounioux C (2004): An Arabidopsis homolog of the bacterial cell division inhibitor SulA is involved in plastid division. Plant Cell 16: 1801-1811

- Reski R (2002): Rings and networks: the amazing complexity of FtsZ in chloroplasts. Trends Plant Sci 7: 103-105

- Rhee KH, Morriss EP, Barber J, Kuhlbrandt W (1998): Three-dimensional structure of the plant photosystem II reaction centre at 8 angstrom resolution. Nature 396: 283-286

- Ryberg H, Ryberg M, Sundqvist C (1993): Plastid ultrastructure and development. In: Sundqvist C, Ryberg M (eds.). Pigment - Protein Complexes in Plastids: Synthesis and Assembly. Pp. 25-62. Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto

- Ryberg M (eds.): Pigment - Protein Complexes in Plastids: Synthesis and Assembly. Pp. 25-62. Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto

- Sagisaka S (1993a): Formation of plastid initials, the presumed precursors of plastids, in corticalcells of living bark, leaf buds, and flower buds of appletrees in midwinter. Biosci Biotechnol Biochem 57: 1641-1645

- Sagisaka S (1993b): The general occurrence of plastid initials and their development into plastids in corticalcells of woodyplants in winter. Trees-Struct Funct 8: 93-98

- Sagisaka S (1994a): Occurrence of a putative precursor to plastids, plastid initials in apple callus Biosci Biotechnol Biochem 58: 1153-1154

- Sagisaka S (1994b): Evidence for the occurrence of plastid initials in a free state in corticalcells of poplar. Biosci Biotechnol Biochem 58: 1527-1529

- Sandmann G, Boger P (1982) Mode of action of herbicidal bleaching. In: Moreland, DE, St. John, JB, Hess, FD (eds.): Biochemical Responses Induced by Herbicides. Pp. 111-130, American Chemical Society, Washington

- Shimoni E, Rav-Hon O, Ohad I, Brumfeld V, Reich Z (2005): Three-dimensional organization of higher-plant chloroplast thylakoid membranes revealed by electron tomography. Plant Cell 17: 2580-2586

- Schleiff E, Soll J (2000): Travelling of proteins through membranes: translocation into chloroplasts. Planta 211: 449-456

- Schmidt HO (1988): The structure and function of grana-free thylakoid membranes in gerontoplasts of senescent leaves of Vicia faba L. Z Naturforsch 43c: 149-154

- Silaeva AM, Tkachuk ES (1982): Ultrastructural organization of wheat leaf chloroplasts under the conditions of varying water-supplies. Fiziol Biokhim Kult Rast 14: 148-152

- Sironval C (ed.) (1967): Le Chloroplaste. Croissance et Vieillissement. Masson et Cie. Paris

- Sitte P (1977): Chloroplasten - bunte Objekte der modernen Zellbiologie. Biologie in unserer Zeit 7: 65-74

- Smart CM (1994): Gene-expression during leaf senescence. New Phytol 126: 419-448

- Somersalo S, Aro EM (1987): Fluorescence induction in pea leaves of different ages. Photosynthetica 21: 29-35

- Spilatro SR, Preiss J (1987): Regulation of starch synthesis in the bundle sheath and mesophyll of Zea mays L - intercellular compartmentalization of enzymes of starch metabolism and the properties of the ADPglucose pyrophosphorylase. Plant Physiol 83: 621-627

- Staehelin LA, van der Staay GWM (1996): Structure, composition, functional organization and dynamic properties of thylakoid membranes. In: Ort DR, Yocum CF (eds.): Oxygenic Photosynthesis: The Light Reactions. Pp. 11-30. Kluwer Academic Publishers,Dordrecht, Boston, London

- Stetler DA, Laetsch WM (1965): Kinetin-induced chloroplast maturation in cultures of tobacco tissue. Science 149: 1387-1388

- Sylvester AW, Smith L, Freeling M (1996): Acquisition of identity in the developing leaf. Annu Rev Cell Dev Biol 12: 257-304

- Synková H, Schnablová R, Hušák M, Šiffel P, Bumba L, Hunalová I, Čeřovská N, Vácha F (2005): Isolation and characterization of paracrystalline structures from transgenic Pssu-ipt tobacco. Photosynthetica 43: 509-517

- Synková H, Šesták Z (1991): Immobilization of chloroplasts from young and old leaves of lettuce, pea, spinach and tobacco. Photosynthetica 25: 1-10

- Šesták Z, Šiffel P (1997): Leaf-age related differences in chlorophyll fluorescence. Photosynthetica 33: 347-369

- Thomson WW, Whatley JM (1980): Development of non-green plastids. Annu Rev Plant Physiol 31: 375-394

- Tichá I (1985): Ontogeny of leaf morphology and anatomy. In: Šesták Z (ed.). Photosynthesis during Leaf Development. Pp. 16-50. Academia, Praha. Dr W. Junk Publishers, Dordrecht, Boston, Lancaster

- Vičánková A, Holá D, Kutík J (Photosynthetica nabídnuto) Maize F1 hybrid differs from its maternal parent in the development of bundle sheath, but not mesophyll, cells chloroplasts: the quantitative analysis of chloroplast ultrastructure and dimensions in different parts of leaf blade at the beginning of its senescence

- Vičánková A, Kutík J (2005): Chloroplast ultrastructural development in vascular bundle sheath cells of two different maize (Zea mays L.) genotypes. Plant Soil Environ 51: 491-495

- Virgin HI, Egnéus HS (1983): Control of plastid development in higher plants. In: Shropshire W, Mohr H (eds.): Photomorphogenesis. Encyclopedia of Plant Physiology, New Series 16. Pp. 289-311. Springer-Verlag, Berlin

- Vitha S, Froehlich JE, Koksharova O, Pyke KA, van Erp H, Osteryoung KW (2003): ARC6 is a J-domain plastid division protein and an evolutionary descendant of the cyanobacterial cell division protein Ftn2. Plant Cell 15: 1918-1933

- Vitha S, McAndrew RS, Osteryoung KW (2001): FtsZ ring formation at the chloroplast division site in plants. J Cell Biol 153: 111-119

- Vlčková A, Špundová M, Kotábová E, Novotný R, Doležal K, Nauš J (2006): Protective cytokinin action switches to damaging during senescence of detached wheat leaves in continuous light Physiol Plant 126: 257-267

- Wellburn AR, Robinson DC, Wellburn FAM (1982): Chloroplast development in low light-grown barley seedlings. Planta 154: 259-265

- Westphal S, Soll J, Vothknecht UC (2001): A vesicle transport system inside chloroplasts. FEBS Lett 506: 257-261

- Wetzel CM, Jiang CZ, Meehan LJ, Voytas DF, Rodermel SR (1994): Nuclear organelle interactions - the immutans variegation mutant of Arabidopsis is plastid autonomous and impaired in carotenoid biosynthesis. Plant J 6: 161-175

- Whatley JM (1974): Chloroplast development in primary leaves of Phaseolus vulgaris. New Phytol 73: 1097-1110

- Whatley JM (1977): Variations in the basic pathway of chloroplast development. New Phytol 78: 407-420

- Whatley JM (1982): Finestructure of the endothecium and developing xylem in Phaseolus vulgaris. New Phytol 91: 561-570

- Wrischer M (1989): Ultrastructurallocalization of photosynthetic activity in thylakoids during chloroplast development in maize. Planta 177: 18-23

- Wrischer M, Ljubešić N, Devidé Z (1992): Ultrastructural studies of degradational processes in amitrole-damaged photosynthetic membranes. J Struct Biol 108: 1-5

- Zavaleta-Mancera HA, Franklin KA, Ougham HJ, Thomas H, Scott IM (1999a): Regreening of senescent Nicotiana leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. J Exp Bot 50: 1677-1682

- Zavaleta-Mancera HA, Thomas BJ, Thomas H, Scott IM (1999b): Regreening of senescent Nicotiana leaves. II. Redifferentiation of plastids. J Exp Bot 50: 1683-1689

- Zeeman SC, Tiessen A, Pilling E, Kato KL, Donald AM, Smith AM (2002): Starch synthesis in Arabidopsis. Granule synthesis, composition, and structure. Plant Physiol 129: 516-529

5 Seznam publikací autorky

5.1 Publikace, které tvoří Ph.D. práci

- Kutík J, Holá D, **Vičánková A**, Šmídová M, Kočová M, Körnerová M, Kubínová L (2001): The heterogeneity of structural and functional photosynthetic characteristics of mesophyll chloroplasts in various parts of mature or senescing leaf blade of two maize (*Zea mays* L.) genotypes. Photosynthetica 39: 497-506

- Pechová R, Kutík J, Holá D, Kočová M, Haisel D, **Vičánková A** (2003): The ultrastructure of chloroplasts, content of photosynthetic pigments, and photochemical activity of maize (*Zea mays* L.) as influenced by different concentrations of the herbicide amitrole. Photosynthetica 41: 127-136

- Polanská L, **Vičánková A**, Dobrev PI, Macháčková I, Vaňková R (2004): Viability, ultrastructure and cytokinin metabolism of free and immobilized tobacco chloroplasts. Biotechnol Lett 26: 1549-1555

- **Vičánková A**, Kutík J (2005): Chloroplast ultrastructural development in vascular bundle sheath cells of two different maize (*Zea mays* L.) genotypes. Plant Soil Environ 51: 491-495

- **Vičánková A**, Holá D, Kutík J (Photosynthetica nabídnuto) Maize F1 hybrid differs from its maternal parent in the development of bundle sheath, but not mesophyll, cells´ chloroplasts: the quantitative analysis of chloroplast ultrastructure and dimensions in different parts of leaf blade at the beginning of its senescence.

- Polanská L, **Vičánková A**, Nováková M, Malbeck J, Dobrev PI, Brzobohatý B, Vaňková R, Macháčková I (rukopis v poslední fázi přípravy): Altered cytokinin metabolism affects cytokinin, auxin and abscisic acid contents in leaves and chloroplasts and chloroplast ultrastructure in transgenic tobacco.

5.2 Ostatní publikace

- Radochová B, **Vičánková A**, Kutík J, Tichá I (2000): Leaf structure of tobacco *in vitro* grown plantlets as affected by saccharose and irradiance. Biologia Plantarum 43: 633-636

- Schnablová R, Synková H, **Vičánková A**, Burketová L, Eder J, Cvikrová M. (Plant Cell Rep. –nabídnuto): Transgenic ipt tobacco overaccumulates phenolic compounds during in vitro growth.

- Synková H, Schnablová R, **Vičánková A**, Burketová L, Eder J, Cvikrová M (rukopis v přípravě): Grafting includes the accumulation of phenolic acids in transgenic ipt tobacco overproducing cytokinins.

The heterogeneity of structural and functional photosynthetic characteristics of mesophyll chloroplasts in various parts of mature or senescing leaf blade of two maize (*Zea mays* L.) genotypes

J. KUTÍK*, D. HOLÁ**, A. VIČÁNKOVÁ*, M. ŠMÍDOVÁ*, M. KOČOVÁ**, M. KÖRNEROVÁ**, and L. KUBÍNOVÁ+

Department of Plant Physiology and Department of Genetics and Microbiology**, Faculty of Sciences, Charles University, Viničná 5, CZ-128 44 Praha 2, Czech Republic*
Physiological Institute, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Praha 4, Czech Republic+

Abstract

Differences in ultrastructural parameters of mesophyll cell (MC) chloroplasts, contents of photosynthetic pigments, and photochemical activities of isolated MC chloroplasts were studied in the basal, middle, and apical part of mature or senescing leaf blade of two maize genotypes. A distinct heterogeneity of leaf blade was observed both for structural and functional characteristics of chloroplasts. In both mature and senescing leaves the shape of MC chloroplasts changed from flat one in basal part of leaf to nearly spherical one in leaf apex. The volume density of granal thylakoids decreased from leaf base to apex in both types of leaves examined, while the amount of intergranal thylakoids increased in mature leaves but decreased in senescing leaves. The most striking heterogeneity was found for the quantity of plastoglobuli, which strongly increased with the increasing distance from leaf base. The differences in chloroplast ultrastructure were accompanied by differences in other photosynthetic characteristics. The Hill reaction activity and activity of photosystem 1 of isolated MC chloroplasts decreased from leaf base to apex in mature leaves. Apical part of senescing leaf blade was characterised by low contents of chlorophyll (Chl) *a* and Chl *b*, whereas in mature leaves, the content of Chls as well as the content of total carotenoids (Car) slightly increased from basal to apical leaf part. This was reflected also in the ratio Chl (*a*+*b*)/total Car; the ratio of Chl *a*/*b* did not significantly differ between individual parts of leaf blade. Both genotypes examined differed in the character of developmental gradient observed along whole length of leaf blade.

Additional key words: chloroplast development; chloroplast dimensions; electron microscopy; Hill reaction activity; peripheral reticulum; photosynthesis; photosystem 1 activity; plastoglobuli; thylakoids.

Introduction

Changes in chloroplast structural or functional characteristics during the development of leaf blade have been often studied in grasses (*e.g.*, *Triticum*, *Hordeum*, *Zea*). A gradient of chloroplasts with respect to their developmental stage has been usually found along whole length of developing leaf blade, with the youngest ones in basal part and mature, photosynthetically fully active chloroplasts in leaf apex. The various stages of chloroplast development can be characterised by changes in chloroplast ultrastructure, especially as regards their inner membrane system (see Kutík 1998 for review). Basal part of young leaves usually contains plastids with prolamel-

Received 20 March 2001, *accepted* 1 October 2001.
Fax: 420-02/2195-3306, e-mail: kutik@natur.cuni.cz
Abbreviations: BSC – bundle sheath cells; Car – carotenoids; CCU – chlorophyll content unit; Chl – chlorophyll; DCMU – 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenol-indophenol; DMA – dry matter expressed per leaf area unit; DMU – dry matter unit; ES – experimental set; HRA – Hill reaction activity; LAU – leaf area unit; L/W – chloroplast cross section length to width ratio; MC – mesophyll cells; P – plastoglobuli; PAR – photosynthetically active radiation; PR – peripheral reticulum; PS – photosystem; S – stroma; SI – starch inclusions; TG – granal thylakoids; TI – intergranal thylakoids; TT – total thylakoids.
Acknowledgements: This research was supported by the grant No. 103/1998 of the Grant Agency of the Charles University, and by the grant No. J13/98113100004 of The Ministry of Youth, Sports, and Education of the Czech Republic. The authors are also grateful to Dr. D. Hulínská and Ing. M. Doubek of the State Institute of Health, Prague, for facilitation of transmission electron microscopy studies, and to Dr. J. Poruba, Maize Breeding Station CEZEA, Čejč, for supply of maize seeds.

lar bodies, which are subsequently transformed into immature, pre-granall chloroplasts with a simple thylakoid system. In some cases, these young plastids contain starch inclusions and can be therefore denoted as amyloplasts (Wellburn *et al.* 1982, Chonan *et al.* 1991). Degradation of starch and synthesis of various components of photosynthetic apparatus is typical phenomenon associated with the development of immature plastids into mature, photosynthetically fully active chloroplasts found usually in parts of leaf blade more distant from leaf base (Wellburn *et al.* 1982). Transcription activity of chloroplast genes as well as the content of cpDNA increases from basal to middle part of developing leaves of some plant species, whereas in other species a further increase from middle to apical part has been also observed (Lawrence and Possingham 1986). In C4 plants, which are characterised by typical dimorphism of mesophyll (MC) and bundle sheath (BSC) chloroplasts, the diversification of chloroplast ultrastructure begins at leaf apex and gradually continues with the chloroplast development toward leaf base (Rascio *et al.* 1984, Nishioka *et al.* 1993, and others).

The majority of these studies, however, concentrated on the young leaves, whose growth is still unfinished. This is why we tried to find out whether the ultrastructural changes associated with the developmental gradient observed in young leaves are conserved also in mature, non-growing, or even senescing leaves. To our knowledge, such heterogeneity of chloroplast ultrastructure with respect to various parts of mature or senescing leaf blade has not yet been studied. Gradients in anatomical characteristics (*e.g.*, stomata density or epidermal cell size) on mature leaf blade area are known for a long time (for review see, *e.g.*, Tichá 1985). Several studies dealing with the differences in net photosynthetic rate, contents of photosynthetic pigments, or activities of pigment-protein complexes of thylakoid membranes with respect to heterogeneity of mature (or, in some cases, senescing) leaf blade were also published. The amount of photosynthetic pigments and pigment-protein complexes of thylakoid membranes usually increases with the increasing distance from leaf base. This applies also for the activity of these complexes and the net photosynthetic rate (Wellburn *et al.* 1982, Lebedev *et al.* 1986, Bredenkamp and Baker 1988, Davies *et al.* 1989, 1990, Hew *et al.* 1998, and others). For comprehensive review on various changes in photosynthetic characteristics associated with leaf development see, *e.g.*, Čatský and Šesták (1997) or Šesták and Šiffel (1997).

The aim of this work was therefore to determine possible differences in ultrastructure of chloroplasts in three parts of mature and senescing leaf blade of maize, and to analyse the relationship between these structural parameters and various photosynthetic characteristics of mesophyll chloroplasts. Another object of our study was to ascertain whether the genotypic variability found for many photosynthetic characteristics reflects not only in chloroplast development during leaf ontogeny (Kutík *et al.* 1999) but also in the differences of chloroplast structural and functional parameters along the length of leaf blade.

Materials and methods

Plants: The ultrastructural characteristics of MC chloroplasts together with their photochemical activity and the contents of photosynthetic pigments in leaves were studied in two maize (*Zea mays* L.) genotypes: inbred line 2023 and its F1 hybrid 2023×CE810. Two sets of experiments (*i.e.*, ES 1 and ES 2) were performed with similar experimental pattern. Seeds, obtained from Maize Breeding Station CEZEA in Čejč (Czech Republic), were sown to low planting dishes with soil and placed in the growth chamber (*Klimabox RK1-007, Kovodružstvo Slaný*, Czech Republic) at day/night regime 16/8 h, irradiance 470/0 (ES 1) or 230/0 (ES 2) µmol m^{-2} s^{-1} (PAR), temperature 25/16 °C, and relative air humidity 70/80 %. 50-60 plants represented each genotype.

The mature, non-growing third leaf (numbering from coleoptile as the leaf 0) has been studied in both experimental sets. In ES 1, plants were 27-28 d old; however, since their leaves have already started to show various symptoms of senescence, the age of plants used for ES 2 was lowered to 23-24 d, and the irradiance was also reduced. The leaves were taken three hours after beginning of light period, the midrib was excised, and the leaf blade was cut into three parts of approximately equal length, referred to as 'basal', 'middle', and 'apical' part. The samples for the analysis of MC chloroplast structure and function, the photosynthetic pigment contents, and the dry matter were prepared from each third of leaf blade.

Chloroplast ultrastructure: Four plants of each genotype were used for electron microscopic and stereological evaluation following the standard procedure described in Kutík *et al.* (1999). This procedure consisted of double fixation of leaf blade samples with glutaraldehyde followed by osmic acid treatment, their dehydration through ethanol series, and embedding into Spurr's low viscosity resin. Chloroplast ultrastructure was evaluated on transverse ultrathin sections of embedded objects contrasted with uranyl acetate solution, followed by lead citrate solution treatment. The transmission electron microscope *Philips EM 300* (the Netherlands) was used at primary magnifications of about 7 000×. On electron microphotographs, at final magnifications of about 30 000×, chlo-

roplast cross section's length and width were determined together with the volume densities of individual chloroplast compartments. Relative partial volumes of granal and intergranal thylakoids, peripheral reticulum, starch inclusions, plastoglobuli, and stroma (including the space between the outer and inner envelope membranes) were evaluated using morphometric grids with regularly distributed points. Five chloroplasts were analysed for each leaf sample.

Photochemical activity of isolated chloroplasts: The isolation of MC chloroplasts from three parts of maize leaf blade was performed as described in Körnerová and Holá (1999). 18 to 20 plants were usually needed to get sufficient amount of leaf tissue. Photochemical activity of isolated chloroplasts was measured polarographically as Hill reaction activity (HRA) and photosystem 1 (PS1) activity, *i.e.*, the amount of oxygen formed (or, in case of PS1 activity, consumed) by the suspensions of isolated chloroplasts irradiated by "white light" (800 μmol m^{-2} s^{-1}, PAR) after the addition of artificial electron acceptors or donors. In case of HRA, 7 mM $K_3[Fe(CN)_6]$ served as artificial electron acceptor whereas 0.15 mM reduced DCPIP as electron donor, 0.1 mM methylviologen as electron acceptor, and 0.1 mM DCMU as PS2 activity inhibitor were used for measurement of PS1 activity. Each sample was measured four to eight times and the values were expressed per leaf area unit (LAU), dry matter unit (DMU), or chlorophyll content unit (CCU), and time unit.

Contents of photosynthetic pigments: Six leaf discs, each corresponding to 0.5 cm^2, were cut into small pieces which were put into 10 cm^3 of N,N-dimethylformamide and stored in a dark and cool place. After 2 d, Chl *a*, Chl *b*, and total carotenoids (Car) content in the extracts was determined spectrophotometrically (*Spekol 211*, *Carl Zeiss*, Jena, Germany) (Porra *et al.* 1989, Wellburn 1994). Each genotype/part of leaf blade was represented by six samples and the values were expressed per LAU or DMU. The Chl ($a+b$) content and the ratios of Chl a/b and Chl/Car were also evaluated.

Dry matter expressed per leaf area unit (DMA): Eight samples, each containing six 0.5 cm^2 leaf discs (usually 1 to 2 discs per one plant), were taken from each part of leaf blade analysed. They were fully dried and their mass was determined on analytical balance (*Sartorius*, Germany) with precision of 0.03 mg.

Statistical treatment: Statistical significance of differences in photosynthetic characteristics between various parts of leaf blade as well as between genotypes or experimental sets was tested by one- or two-way analysis of variance followed by Scheffe's non-parametric test, using the 5 % level of statistical significance as the critical one.

Results

Differences between experimental sets: The habit and growth pattern of plants was similar in both experimental sets. At the beginning of measurement of photosynthetic characteristics the plants usually had four leaves, but the fourth leaf was not yet fully developed. The third leaf, used for the analysis of structural and functional characteristics of MC chloroplasts, was therefore the youngest one whose growth has been already completed. Its external appearance, however, strongly differed between both experimental sets. In ES 1, the apical part showed yellow or yellow-green colour and (in 2023×CE810) its extreme tip began to dry. Yellowing of leaf blade as the visible symptom of senescence was also apparent in the middle part; it was more accentuated in the hybrid compared to the parental genotype. In ES 2, on the other hand, the entire leaf was green, photosynthetically fully active, in both genotypes studied.

The external differences between experimental sets in the third leaf appearance were accompanied by the differences in photosynthetic characteristics. With the exception of total Car content, the volume density of starch inclusions in MC chloroplasts and the DMA, the values of all other structural and functional characteristics examined significantly differed between ES 1 and ES 2 (Table 1). The MC chloroplasts analysed in ES 2 showed greater volume density of both granal and intergranal thylakoids, less plastoglobuli, and smaller peripheral reticulum compared to ES 1. They were also more flat, as shown by greater value of their length-to-width ratio (Figs. 1 and 2). These structural differences were accompanied by the differences in photochemical activity (both HRA and PS1) and Chl content: the values of these characteristics were higher in ES 2 (Figs. 3 and 4).

The behaviour of ES 1 and ES 2 was not identical, as proven by the statistically significant interaction between genotypes and experimental sets (Table 1). Whereas in ES 2 the hybrid showed lesser volume density of intergranal thylakoids compared to its parent (Fig. 2*B*), the differences between both genotypes in ES 1 were not statistically significant (Fig. 1*B*). Certain variation was found also for granal thylakoids and plastoglobuli (especially in basal and middle parts of leaf blade), as well as for the content of photosynthetic pigments, ratio Chl/Car, and DMA. The most notable differences between both sets of experiments in parent and hybrid behaviour were recorded for the volume density of peripheral reticulum(Figs. 1*D*, 2*D*) and PS1 activity (Figs. 3*B*, 4*B*).

With the exception of HRA and PS1 activity expressed per CCU, Chl/Car and Chl *a/b* ratios, DMA and some ultrastructural characteristics of MC chloroplasts (volume density of starch inclusions, stroma or granal thylakoids), the interaction between experimental sets and parts of leaf blade was also statistically significant (Table 1). This indicated that the differences in structural and functional characteristics of MC chloroplasts between the examined three parts of leaf blade, found in ES 1, were unlike those observed in ES 2.

Table 1. The differences between experimental sets (ES), genotypes (G), and various parts of leaf blade (LP) in selected structural and functional photosynthetic characteristics of maize leaves. Both experimental sets were analysed together. The statistical significances for individual components of variation are shown.

Characteristic	ES	G	LP	ES×G	ES×LP	G×LP	ES×G×LP
	Photochemical activities of mesophyll chloroplasts						
HRA (LAU)	0	0.01	0.03	0.65	0.02	0.14	0.63
HRA (DMU)	0	0	0	0.23	0	0.06	0.60
HRA (CCU)	0	0.07	0.34	0.17	0.22	0.07	0.57
PS1 (LAU)	0	0.27	0.02	0.02	0.01	0.71	0.54
PS1 (DMU)	0	0.29	0	0.77	0	0.83	0.54
PS1 (CCU)	0	0	0.29	0	0.52	0.60	0.70
	Contents of photosynthetic pigments						
Chl (*a+b*) (LAU)	0	0	0.01	0.03	0	0.48	0.55
Chl (*a+b*) (DMU)	0	0	0	0.20	0	0	0.11
Chl *a* (LAU)	0	0	0.02	0.04	0	0.56	0.61
Chl *a* (DMU)	0	0	0	0.62	0	0.23	0.60
Chl *b* (LAU)	0	0	0	0.01	0	0.20	0.31
Chl *b* (DMU)	0	0	0	0.12	0	0	0.06
Car (LAU)	0.11	0.96	0	0.35	0	0.33	0.84
Car (DMU)	0.17	0	0	0.04	0.02	0.65	0.96
Chl *a/b*	0.01	0.45	0.56	0.58	0.73	0.82	0.86
Chl/Car	0	0	0	0	0.09	0	0.04
	Ultrastructure of mesophyll chloroplasts						
TG	0	0.41	0	0.02	0.07	0.14	0.16
TI	0.01	0.01	0.21	0	0	0.55	0.49
TT	0	0.04	0	0.88	0.03	0.37	0.38
P	0	0	0	0.03	0	0	0.01
SI	0.27	0	0.31	0.23	0.11	0.52	0.88
PR	0	0	0.85	0	0	0.07	0.13
S	0	0.90	0.03	0.75	0.52	0.26	0.21
L/W	0	0	0	0.50	0.04	0.06	0.50
	Dry matter expressed per leaf area unit						
DMA	0.63	0	0	0.01	0.47	0.19	0.51

Differences between genotypes: Plants of the 2023×CE810 genotype were slightly higher and had longer leaves compared to 2023 in both experimental sets. Structural and functional characteristics of MC chloroplasts, as well as the contents of photosynthetic pigments and DMA usually differed between those genotypes. In some cases (especially in ES 1), this difference depended also on the examined part of leaf blade, as proven by the statistically significant interaction between genotypes and leaf parts (Tables 1 and 2).

In ES 1, the parent and its hybrid did not show statistically significant differences either in the amount of granal, intergranal, or total thylakoids, or in the volume density of chloroplast stroma. They differed, however, in some minor chloroplast compartments. The hybrid was characterised by greater volume density of plastoglobuli and starch inclusions per MC chloroplast, and lower amount of peripheral reticulum (with the exception of the middle part of leaf blade) compared to the parental genotype (Figs. 1D,E). The shape of MC chloroplasts in 2023×CE810 was also more flat, especially in basal or middle part of leaf blade (Fig. 1F). In ES 2, on the other hand, the shape of MC chloroplasts in both parent and hybrid was similar, rather flat (Fig. 2F). The differences between genotypes in the volume density of plastoglobuli and starch inclusions were similar to those found in ES 1 (Fig. 2E). As for peripheral reticulum, the hybrid displayed noticeably greater amount of this chloroplast component compared to 2023 in this set of experiments (Fig. 2D). Inversely, the parental line showed significantly more intergranal thylakoids compared to its hybrid (Fig. 2B).

Comparison of parent and hybrid genotype with respect to the photochemical activity of isolated MC chlo-

roplasts showed that 2023 displayed higher HRA values compared to 2023×CE810 in all three parts of leaf blade examined (Figs. 3*A*, 4*A*). This difference was especially pronounced in ES 1. The same phenomenon was recorded for PS1 activity in this experimental set, while in ES 2 the relationship between both genotypes was reverse (Figs. 3*B*, 4*B*).

Higher content of Chls *a* and *b* and higher Chl/Car ratio in 2023 compared to 2023×CE810 was found in both sets of experiments (Figs. 3*D,E*, 4*D,E*). However, only in ES 1 the differences were statistically significant for both types of Chl content expression (LAU or DMU); they were also more pronounced than in ES 2. No significant differences between genotypes were found for total Car content (with the exception of Car per DMU in ES 2) or for the Chl *a/b* ratio (Figs. 3*F*, 4*F*). As for DMA, greater values in the hybrid genotype compared to the parental line were observed only in ES 2 (Fig. 4*C*).

Table 2. The differences between genotypes (G) and various parts of leaf blade (LP) in selected structural and functional photosynthetic characteristics of maize leaves. Each experimental set was analysed separately. The statistical significances for individual components of variation are shown.

Characteristic	First experimental set			Second experimental set		
	G	LP	G×LP	G	LP	G×LP
	Photochemical activities of mesophyll chloroplasts					
HRA (LAU)	0	0.66	0.31	0.22	0.04	0.32
HRA (DMU)	0.01	0.47	0.26	0.01	0.01	0.20
HRA (CCU)	0.02	0.44	0.37	0.78	0.27	0.18
PS1 (LAU)	0	0.14	0.29	0.11	0.04	0.64
PS1 (DMU)	0	0.03	0.08	0.70	0.01	0.69
PS1 (CCU)	0.77	0.03	0.90	0.01	0.49	0.65
	Contents of photosynthetic pigments					
Chl (*a*+*b*) (LAU)	0	0	0.04	0.07	0.01	1.00
Chl (*a*+*b*) (DMU)	0	0	0.04	0	0	0
Chl *a* (LAU)	0	0.01	0.06	0.09	0.02	1.00
Chl *a* (DMU)	0	0	0.04	0	0	0.83
Chl *b* (LAU)	0	0	0.01	0.03	0	0.98
Chl *b* (DMU)	0	0	0.04	0	0	0
Car (LAU)	0.31	0.06	0.57	0.63	0	0.53
Car (DMU)	0.12	0	0.71	0.01	0	0.80
Chl *a*/*b*	0.41	0.50	0.74	0.89	0.91	0.99
Chl/Car	0	0.01	0.01	0	0.12	0.26
	Ultrastructure of mesophyll chloroplasts					
TG	0.07	0.01	0.64	0.19	0	0.01
TI	0.70	0	0.26	0	0	0.88
FT	0.35	0	0.93	0.06	0.05	0.06
P	0	0	0	0.01	0	0.44
SI	0.04	0.03	0.53	0	0.71	0.79
PR	0.04	0	0	0	0	0.98
S	1.00	0.50	0.44	0.88	0.03	0.06
L/W	0	0	0.03	0.10	0	0.34
	Dry matter per leaf area unit					
DMA	0.50	0	0.92	0	0	0.05

Differences between parts of leaf blade: In both experimental sets, the differences between individual parts of leaf blade were statistically significant for most characteristics examined (Tables 1 to 3). Certain general observations were made, concerning mainly various structural characteristics, DMA, or the contents of photosynthetic pigments.

The shape of MC chloroplasts changed from rather flat one in basal part of the leaf to more rounded one in leaf apex (Figs. 1*F*, 2*F*). The difference in this parameter between middle and apical part of leaf blade was statistically significant in ES 1 only (Table 3). In this experimental set, many MC chloroplasts in leaf apex showed various symptoms of advanced senescence, *e.g.*, thylakoid breakdown or envelope membrane rupture. This was characteristic especially for the hybrid 2023×CE810.

The volume density of plastoglobuli increased from middle to apical part of leaf (Figs. 1*E*, 2*E*). The amount of starch did not change with increasing distance from

Table 3. Differences between basal (B), middle (M), and apical (A) parts of leaf blade in selected structural and functional photosynthetic characteristics of maize genotypes 2023 (I) and 2023×CE810 (H). Each experimental set was analysed separately. The statistical significances as determined by Scheffe' test are shown.

Characteristic	First experimental set						Second experimental set					
	B–M		B–A		M–A		B–M		B–A		M–A	
	I	H	I	H	I	H	I	H	I	H	I	H
	Photochemical activities of mesophyll chloroplasts											
HRA (LAU)	0.95	0.16	0.76	0.06	0.61	0.50	0.01	0.70	0	0.73	0.07	1.00
HRA (DMU)	0.86	0.96	0.40	0.48	0.64	0.60	0.01	0.47	0	0.38	0.07	0.97
HRA (CCU)	0.93	0.12	0.73	0.15	0.55	0.95	0.09	0.99	0.03	0.97	0.29	0.93
PS1 (LAU)	0.37	0.33	0.95	0.13	0.49	0.58	0.63	0.21	0.44	0.06	0.91	0.40
PS1 (DMU)	0.45	0.21	0.09	0.56	0.26	0.58	0.29	0.12	0.17	0.04	0.82	0.28
PS1 (CCU)	0.22	0.31	0.99	0.89	0.25	0.20	1.00	0.82	0.99	0.13	0.99	0.22
	Contents of photosynthetic pigments											
Chl (*a*–*b*) (LAU)	0.70	0.47	0.91	0.01	0.49	0	0	0.38	0	0.27	0	0.92
Chl (*a* · *b*) (DMU)	0.01	0.07	0.02	0.93	0.50	0.09	0.04	0.10	0	0.09	0.01	0.02
Chl *a* (LAU)	0.72	0.51	0.89	0.01	0.49	0	0	0.41	0	0.30	0	0.94
Chl *a* (DMU)	0.01	0.07	0.02	0.94	0.44	0.09	0	0.23	0	0.12	0.13	0.74
Chl *b* (LAU)	0.61	0.38	0.98	0.02	0.53	0.01	0	0.25	0	0.15	0.06	0.86
Chl *b* (DMU)	0.01	0.07	0.01	0.85	0.87	0.10	0.02	0.09	0	0.09	0.01	0.01
Car (LAU)	0.44	0.16	0.77	0.15	0.78	0.99	0	0.17	0	0.07	0	0.52
Car (DMU)	0	0.02	0.01	0.01	0.79	0.91	0	0.11	0	0.04	0.07	0.35
Chl *a*/*b*	0.61	0.87	0.97	0.98	0.73	0.95	0.98	1.00	0.92	0.99	0.83	0.99
Chl/Car	0.81	0.69	0.95	0	0.95	0.01	0.75	0.72	0.42	0.24	0.21	0.50
	Ultrastructure of mesophyll chloroplasts											
TG	0.99	0.72	0.06	0.60	0.08	0.20	0	0.77	0	0	0.90	0
TI	0.14	0.04	0.02	0	0.66	0.34	0.32	0.82	0	0	0.01	0.03
TT	0.52	0.65	0	0.03	0.04	0.22	0.98	0.09	0.07	0.62	0.11	0.46
P	0.33	0.12	0	0	0	0	0.10	0.57	0	0	0	0
SI	0.76	0.92	0.02	0.56	0.10	0.81	0.14	0.88	1.00	0.97	0.14	0.76
PR	0.81	0	0.03	0.27	0.12	0.01	0.04	0.16	0.03	0.10	1.00	0.97
S	0.79	0.93	0.21	0.99	0.55	0.97	0.04	0.93	0.53	0.08	0.36	0.16
L/W	0	0	0	0	0.05	0	0	0	0	0	0.57	0.54
	Dry matter per leaf area unit											
DMA	0.01	0.11	0.01	0.10	0.96	0.99	0.04	0.02	0.03	0.01	0.84	0.49

leaf base; the same applied to the volume density of chloroplast stroma except for the statistically significant difference between basal and middle part observed in 2023 in ES 2 (Table 3, Figs. 1*C*, 2*C*). The volume density of granal thylakoids slightly diminished from basal to apical part of leaf blade. The lowest amount of granal thylakoids was observed in leaf apex, while the difference between basal and middle part was usually less pronounced (Figs. 1*A*, 2*A*). However, there were some differences between experimental sets or genotypes (Table 3). As regards intergranal thylakoids, the values clearly differed between experimental sets. In ES 1, the basal part of leaf was characterised by the highest volume density, while in ES 2 it displayed the lowest volume density of these thylakoids (Figs. 1*B*, 2*B*). The MC chloroplasts in this part of leaf blade also showed the lowest (in ES 1) or the highest (in ES 2) content of peripheral reticulum (Figs. 1*D*, 2*D*), respectively.

No statistically significant differences in photochemical activities of MC chloroplasts were found in ES 1 (Table 3). However, in ES 2, the basal part of leaf blade in the parental line displayed the lowest HRA compared to the middle or apical part (Fig. 2*A*). The differences in PS1 activity were usually statistically insignificant in either experimental set (Table 3).

The contents of Chls *a* and *b* and total Cars in fully developed, non-senescing leaves (*i.e.*, those analysed in ES 2) increased from basal to apical part of leaf blade (Fig. 4*D-F*). However, the differences between leaf parts were statistically significant in the parental line only (Table 3). No differences between various parts of leaf blade in Chl or Car content per LAU were observed in senescing leaves (ES 1) of 2023 genotype, but the apex of 2023×CE810 leaves showed significantly lower contents of Chls compared to their basal or middle part (Table 3, Fig. 3*D,E*). When the content of photosynthetic pigments was expressed per DMU, the results were similar to those found in ES 2 (Table 3). The Chl/Car ratio or Chl *a*/*b* ratio did not differ between individual parts of leaf blade in either of genotypes examined (Table 3). The highest DMA was found for leaf base both in 2023 and 2023×CE810 (Figs. 3*C*, 4*C*).

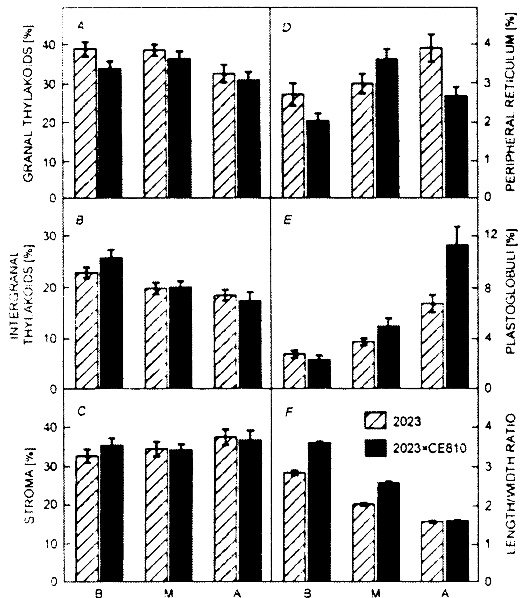


Fig. 1. Structural characteristics of mesophyll chloroplasts in basal (B), middle (M), and apical (A) part of senescing leaf blade (experimental set 1) of two maize genotypes (*hatched bars* – 2023, *solid bars* – 2023×CE810). Means ± standard error of mean (SEM).

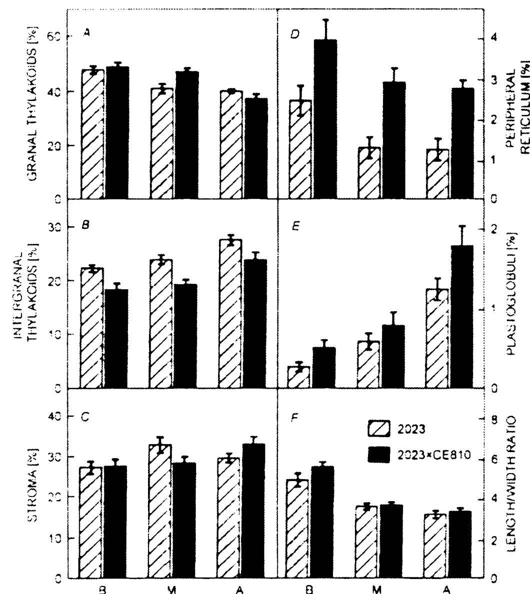


Fig. 2. Structural characteristics of mesophyll chloroplasts in basal (B), middle (M), and apical (A) part of mature leaf blade (experimental set 2) of two maize genotypes (*hatched bars* – 2023, *solid bars* – 2023×CE810). Means ± standard error of mean (SEM).

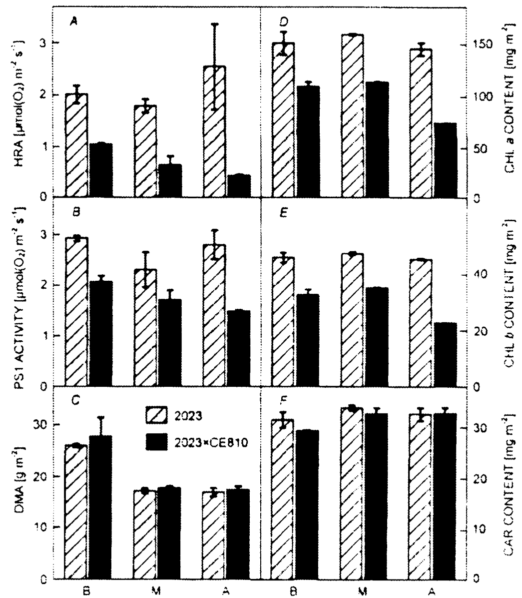


Fig. 3. Photochemical activities of mesophyll chloroplasts, contents of photosynthetic pigments, and dry matter expressed per leaf area unit (DMA) in basal (B), middle (M), and apical (A) part of senescing leaf blade (experimental set 1) of two maize genotypes (*hatched bars* – 2023, *solid bars* – 2023×CE810). Means ± standard error of mean (SEM).

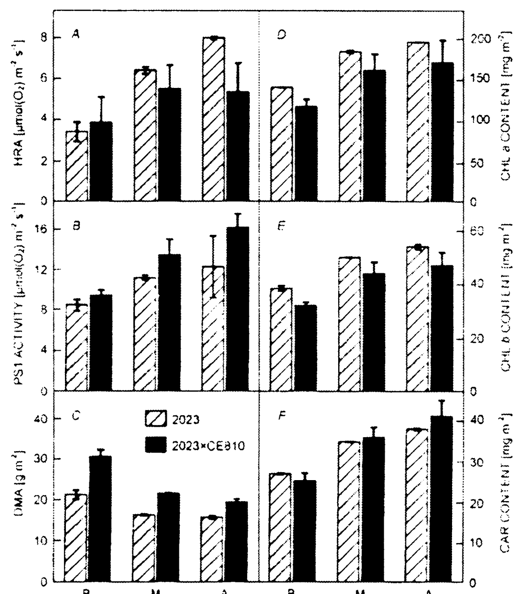


Fig. 4. Photochemical activities of mesophyll chloroplasts, contents of photosynthetic pigments, and dry matter expressed per leaf area unit (DMA) in basal (B), middle (M), and apical (A) part of mature leaf blade (experimental set 2) of two maize genotypes (*hatched bars* – 2023, *solid bars* – 2023×CE810). Means ± standard error of mean (SEM).

Discussion

Our study of MC chloroplasts in maize leaves showed that the chloroplast structural and functional characteristics in various parts of leaf blade were heterogeneous both in mature and senescing leaves. The chloroplasts from leaf apex were characterised by various parameters usually associated with advanced development, while those localised in basal part of leaf blade were clearly younger. This observation applied to both mature and senescing leaves. In both types of leaves, an increasing volume density of plastoglobuli was observed from basal to apical part of leaf blade: the changes were more abrupt in senescing leaves. This increase in plastoglobuli number or volume (or both) is a sign of advancing chloroplast senescence, as the products of breakdown of thylakoid lipids are probably accumulated in this compartment (Kutík *et al.* 1993, Ram *et al.* 1994, Kutík 1985, 1998, Hudák 1997).

Another symptom associated with development of mature chloroplasts and their gradual conversion to gerontoplasts, or senescing chloroplasts, is the change of chloroplast shape and dimensions (Somersalo and Aro 1987, Hashimoto *et al.* 1989, Chonan *et al.* 1991, Ono *et al.* 1995, Kutík 1998, Kutík *et al.* 1999). Senescing leaves of maize examined in our study contained smaller chloroplasts in mesophyll cells compared to non-senescing, mature leaves. A gradual transformation of flat chloroplasts found in leaf base to more rounded ones in middle or apical part of leaf blade was also observed. This phenomenon is characteristic for advanced developmental stage of these organelles (Kutík *et al.* 1999). In senescing leaves, MC chloroplasts of leaf apex were often deformed, with ruptured envelope and broken thylakoid membranes. These chloroplasts could be regarded as the final stage in the chloroplast development (Hudák 1997).

Certain interesting differences in the organisation of thylakoid membranes were also found in MC chloroplasts from various parts of leaf blade. Contrary to the increase of size and amount of granal thylakoids often described for young, growing leaves (Kutík 1998), we observed a slightly decreasing gradient from leaf base to apex for chloroplasts both in mature and senescing leaves. This is in good agreement with general trend depicted in many other developmental studies: once the mature, photosynthetically fully active chloroplasts with large grana are formed, the size of this chloroplast compartment slowly decreases, thylakoid membranes dilate and finally break up (Kutík *et al.* 1988). It can be therefore concluded that the chloroplasts of leaf apex analysed in our study are characterised by more advanced developmental stage compared to those from basal part of leaf blade. As for intergranal thylakoids, our observation that even in mature, fully developed and non-growing leaves their amount increases with the increasing distance from leaf base is fairly interesting. In senescing leaves, the volume density of both granal and intergranal thylakoids decreased with the increasing distance from leaf base which shows that MC chloroplasts have already undertaken further step toward their senescence.

No significant changes associated with the heterogeneity of leaf blade were found in the volume of chloroplast stroma. The same applied to the amount of starch inclusions, which was extremely small; the differences between various parts of leaf blade in the volume density of this chloroplast compartment were usually statistically insignificant. It might be interesting to compare the number and size of starch inclusions along the whole leaf blade in MC and BSC chloroplasts: the ultrastructure of BSC chloroplasts from our experiments is presently evaluated and preliminary results of this analysis have already been published (Vičánková *et al.* 2000). We found interesting differences for the peripheral reticulum: its amount in mature leaves decreased with the increasing distance from leaf base, but in senescing leaves a reverse trend was observed. The role of peripheral reticulum in chloroplasts is still far from being fully solved; it is presumed that this compartment (often found in C4 plants) is involved in various metabolic and transport processes not directly associated with photosynthesis (*e.g.*, Hudák 1997). We can therefore only speculate that the intensity of metabolite efflux from chloroplasts in leaf apex of senescing leaves increased before their final degradation.

The heterogeneity in MC chloroplast ultrastructural parameters observed in various parts of leaf blade was in some cases also accompanied by differences in other photosynthetic characteristics. The content of Chls in mature leaves increased from leaf base to apex, while in senescing leaves this trend was reverse, which is in accord with the findings of other authors (Davies *et al.* 1989, 1990). The content of total Cars increased from basal to middle part of mature, non-senescing leaf blade; no further changes were detected either from middle to apical part of mature leaf blade or in senescing leaves, similarly to Wellburn *et al.* (1982). Lower Chl *a/b* ratio in senescing leaves compared to mature ones could be attributed to more abrupt decrease in the amount of Chl *a*, but no differences in this parameter along the length of leaf blade were detected. The slower decrease in the content of Chl *b* is rather interesting because this pigment is associated with light-harvesting complexes, important in thylakoid stacking (Jackowski and Kluck 1993). On the other hand, Chl *a* is bound in large quantities to PS1 complexes, the number of which strongly decreases during leaf senescence (Lebedev *et al.* 1986), while PS2 or light-harvesting complexes account for much lesser amount of Chl *a*. These results are thus in good agreement with our findings that the reduction in PS1 activity observed in senescing leaves compared to mature ones was more abrupt than the reduction in HRA (which is a measure of PS2 activity). As for various parts of leaf blade, the decrease of photochemical activities of MC

chloroplasts observed from basal to apical part of senescing leaf blade was not statistically significant, due probably to rather large residual variation in those characteristics. On the other hand, an increase both in HRA and (though insignificant) in PS1 activity was observed with an increasing distance from leaf base in mature leaves, similarly to the findings of Webber *et al.* (1986) or Bredenkamp and Baker (1988) in *Triticum*.

The differences in structural and functional photosynthetic characteristics observed between both examined genotypes, as well as the presence of statistically significant interaction between genotypes and leaf parts or genotypes and experimental series can be attributed to the faster development of the hybrid compared to its parental inbred line. The lower content of Chls, lower photochemical activity of isolated MC chloroplasts, greater volume density of plastoglobuli and starch inclusions, more developed peripheral reticulum in mature leaves—all these parameters can be associated with more advanced developmental stage of the 2023×CE810 genotype. The apparent discrepancy in observation that MC chloroplasts of both genotypes contain similar amount of thylakoid membranes while the activities of PS1 and PS2 in senescing leaves are lower in the hybrid than in the parent, can be explained by different methods of evaluation of these parameters. While for the assessment of volume density of individual chloroplast compartments, only undamaged chloroplasts were used, the chloroplasts for measurements of photochemical activity were isolated from much larger part of leaf blade which could contain both functional and partly impaired organelles. This could have great influence especially on HRA or PS1 activity expressed per LAU or DMU.

Our study shows that the developmental gradient of chloroplasts along the whole leaf blade, observed previously for various plant species, is conserved also in mature, non-growing leaves of maize as well as in the senescing ones. Our observation of the ultrastructural changes of mesophyll chloroplasts found from basal to apical part of mature and senescing maize leaves prove for the first time the existence of such gradient in this species. The differences in chloroplast ultrastructure in various parts of leaf blade are accompanied by the changes of photochemical activity and contents of photosynthetic pigments as well, and depend strongly both on the developmental stage of leaf and on the genotype examined.

References

Bredenkamp, G.J., Baker, N.R.: The changing contribution of LHC I to Photosystem I activity during chloroplast biogenesis in wheat. - Biochim. biophys. Acta **934**: 14-21, 1988.

Čatský, J., Šesták, Z.: Photosynthesis during leaf development. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 633-660. Marcel Dekker, New York - Basel - Hong Kong 1997.

Chonan, N., Matsuda, T., Tsuchiya, T.: [Ultrastructural changes in chloroplast and phloem of rice leaves during senescence under different nitrogen supply.] - Jap. J. Crop Sci. **60**: 247-254, 1991. [In Jap.]

Davies, T.G.E., Ougham, H.J., Thomas, H., Rogers, L.J.: Leaf development in *Lolium temulentum*: Plastid membrane polypeptides in relation to assembly of the photosynthetic apparatus and leaf growth. - Physiol. Plant. **75**: 47-54, 1989.

Davies, T.G.E., Rogers, L.J., Thomas, B.J., Thomas, H.: Leaf development in *Lolium temulentum*: Formation of the photosynthetic apparatus in the presence of the porphyrin synthesis inhibitor gabaculine. - J. exp. Bot. **41**: 905-917, 1990.

Hashimoto, H., Kura-Hotta, M., Katoh, S.: Changes in protein content and in the structure and number of chloroplasts during leaf senescence in rice seedlings. - Plant Cell Physiol. **30**: 707-715, 1989.

Hew, C.S., Soh, W.P., Ng, C.K.Y.: Variation in photosynthetic characteristics along the leaf blade of *Oncidium goldiana*, a C_3 tropical epiphytic orchid hybrid. - Int. J. Plant Sci. **159**: 116-120, 1998.

Hudák, J.: Photosynthetic apparatus. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 27-48. Marcel Dekker, New York - Basel - Hong Kong 1997.

Jackowski, G., Kluck, E.: Heterogeneous behaviour of subunits of the light-harvesting chlorophyll-*a/b* protein complex of photosystem 2 during cation-induced aggregation. - Photosynthetica **29**: 401-407, 1993.

Körnerová, M., Holá, D.: The effect of low growth temperature on Hill reaction and photosystem 1 activities and pigment contents in maize inbred lines and their F_1 hybrids. - Photosynthetica **37**: 477-488, 1999.

Kutík, J.: Chloroplast development. - In: Šesták, Z. (ed.): Photosynthesis during Leaf Development. Pp. 51-75. Academia, Praha; Dr W. Junk Publishers, Dordrecht - Boston - Lancaster 1985.

Kutík, J.: A quantitative study on ontogenetic development of chloroplast ultrastructure. - Acta Univ. carol., Biol. **31**: 51-54, 1988.

Kutík, J.: The development of chloroplast structure during leaf ontogeny. - Photosynthetica **35**: 481-505, 1998.

Kutík, J., Činčerová, A., Dvořák, M.: Chloroplast ultrastructural development during the ontogeny of the second leaf of wheat under nitrogen deficiency. - Photosynthetica **28**: 447-453, 1993.

Kutík, J., Kočová, M., Holá, D., Körnerová, M.: The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes. - Photosynthetica **36**: 497-507, 1999.

Kutík, J., Nátr, L., Demmers-Derks, H.H., Lawlor, D.W.: Chloroplast ultrastructure of sugar beet (*Beta vulgaris* L.) cultivated in normal and elevated CO_2 concentrations with two contrasted nitrogen supplies. - J. exp. Bot. **46**: 1797-1802, 1995.

Kutík, J., Zima, J., Šesták, Z., Volfová, A.: Ontogenetic changes in the internal limitations to bean-leaf photosynthesis. 10. Chloroplast ultrastructure in primary and first trifoliate leaves. - Photosynthetica **22**: 511-515, 1988.

Lawrence, M.E., Possingham, J.V.: Microspectrofluorometric measurement of chloroplast DNA in dividing and expanding leaf cells of *Spinacia oleracea*. – Plant Physiol. **81**: 708-710, 1986.

Lebedev, N.N., Šiffel, P., Šesták, Z.: Composition of chlorophyll-protein complexes and fluorescence spectrum changes during ontogeny of primary leaves of *Phaseolus vulgaris* L. – Photosynthetica **20**: 146-152, 1986.

Nishioka, D., Brisibe, E.A., Miyake, H., Taniguchi, T.: Ultrastructural observations in the suppression of granal development in bundle sheath chloroplasts of NADP-ME type C_4 monocot and dicot species. – Jap. J. Crop Sci. **62**: 621-627, 1993.

Ono, K., Hashimoto, H., Katoh, S.: Changes in the number and size of chloroplasts during senescence of primary leaves of wheat grown under different conditions. – Plant Cell Physiol. **36**: 9-17, 1995.

Porra, R.J., Thompson, W.A., Kriedemann, P.E.: Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. – Biochim. biophys. Acta **975**: 384-394, 1989.

Ram, S., Sharma, A., Sen Gupta, U.K.: Photosynthesis and carbon import in developing leaves of groundnut (*Arachis hypogaea* L.). – Photosynthetica **30**: 53-58, 1994.

Rascio, N., Mariani, P., Casadoro, G.: Etioplast-chloroplast transformation in maize leaves: Effects of tissue age and light intensity. – Protoplasma **119**: 110-120, 1984.

Šesták, Z., Šiffel, P.: Leaf-age related differences in chlorophyll fluorescence. – Photosynthetica **33**: 347-369, 1997.

Somersalo, S., Aro, E.-M.: Fluorescence induction in pea leaves of different ages. – Photosynthetica **21**: 29-35, 1987.

Tichá, I.: Ontogeny of leaf morphology and anatomy. – In: Šesták, Z. (ed.): Photosynthesis during Leaf Development. Pp. 16-50. Academia, Praha; Dr W. Junk Publ., Dordrecht – Boston – Lancaster 1985.

Vičánková, A., Holá, D., Kutík, J.: Heterogeneity of chloroplast ultrastructure and functional characteristics in various parts of maize (*Zea mays* L.) leaf blade. – Plant Physiol. Biochem. **38**(Suppl.): 111, 2000.

Webber, A.N., Baker, N.R., Paige, C.D., Hipkins, M.F.: Photosynthetic electron transport and establishment of an associated trans-thylakoid proton electrochemical gradient during development of the wheat leaf. – Plant Cell Environ. **9**: 203-208, 1986.

Wellburn, A.R.: The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. – J. Plant Physiol. **144**: 307-313, 1994.

Wellburn, A.R., Robinson, D.C., Wellburn, F.A.M.: Chloroplast development in low light-grown barley seedlings. – Planta **154**: 259-265, 1982.

PHOTOSYNTHETICA 41 (1): 127-136, 2003

The ultrastructure of chloroplasts, content of photosynthetic pigments, and photochemical activity of maize (*Zea mays* L.) as influenced by different concentrations of the herbicide amitrole

R. PECHOVÁ[*], J. KUTÍK[**], D. HOLÁ[***], M. KOČOVÁ[***], D. HAISEL[*], and A. VIČÁNKOVÁ[+]

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ 160 00 Praha 6, Czech Republic[*]
Department of Plant Physiology[**], *and Department of Genetics and Microbiology*[***], *Faculty of Science, Charles University in Prague, Viničná 5, CZ 128 44 Praha 2, Czech Republic*
Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, CZ 165 02 Praha 6, Czech Republic[+]

Abstract

The effect of three different concentrations of amitrole (AM), a bleaching herbicide affecting carotenogenesis, on chloroplast ultrastructure, photosynthetic pigment contents, and photochemical activity was studied in two maize genotypes differing in photosynthetic characteristics. The content of photosynthetic pigments in leaves of plants treated with low (20 μM) AM concentration was similar to control plants and no damaging effect of the herbicide on the ultrastructure of either mesophyll (MC) or bundle-sheath (BSC) cell chloroplasts was observed. Higher (60 and 120 μM) concentrations of AM caused a significant decrease in the content of carotenoids (especially xanthophylls), which was followed by photooxidative destruction of chlorophylls and some alterations of chloroplast ultrastructure. MC chloroplasts appeared more sensitive to the damaging effect of AM compared to BSC chloroplasts. A significant decrease in the amount of both granal and intergranal thylakoids in MC chloroplasts was observed with the increasing concentration of AM. As regards BSC chloroplasts, rapid decrease in the volume density of starch inclusions was found in plants treated with higher concentrations of AM. When 120 μM AM was used, both MC and BSC chloroplasts contained just a few thylakoid membranes that were strongly altered. The changes in the ultrastructure of MC chloroplasts were accompanied by the changes in their photochemical activity. The formation of chloroplast protrusions after treatment of plants with AM as well as in control plants was also observed.

Additional key words: 3-amino-1,2,4-triazole; carotenoids; chlorophylls; Hill reaction; photosystems 1 and 2.

Introduction

The photosynthetic apparatus localised in chloroplasts is one of the most sensitive systems to various stress factors, both environmental and anthropogenic. Carotenoids (Cars) belong to the most important protective components of photosynthetic pigment-protein complexes. Their function in chloroplast thylakoid membranes is complex (see, *e.g.*, Demmig-Adams *et al.* 1996). Their ability to absorb photons (especially in the blue region of spectrum) enables them to act as additional light-harvesting pigments besides chlorophylls (Chls). They are also necessary for the assembly and stabilisation of various complexes of thylakoid membranes (namely photosystem 2

Received 19 December 2002, *accepted* 26 February 2003.
Fax: (+420) 224 310 113, e-mail: pechova@ueb.cas.cz
Abbreviations: AM, amitrole (3-amino-1,2,4-triazole); BSA, bovine serum albumin; BSC, vascular bundle sheath cell; Car, carotenoid; Chl, chlorophyll; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; HPLC, high performance liquid chromatography; HRA, Hill reaction activity; LHC, light-harvesting complex; MC, mesophyll cell; PAR, photosynthetically active radiation; PBS, buffer used in immunocytochemistry; PS, photosystem.
Acknowledgements: We thank Tim Kunkel and Prof. Eberhard Schäfer (Institute of Biology II, University in Freiburg, Germany) for the LHC antibodies, to Ing. Miloslav Doubek (State Institute of Health, Prague), Dr. Jochen Golecki, and Volker Speth (Institute of Biology II, University in Freiburg, Germany) for help with electron microscopy and immunocytochemistry, to Mgr. Martina Šmídová for help with statistical analysis, and to Prof. Nicoletta Rascio (Department of Biology, University in Padova, Italy) for reading the manuscript. This research was supported by grants No. 103/1998 of the Grant Agency of the Charles University and No. J13/98 113100003 of the Ministry of Youth, Sports, and Education of the Czech Republic.

and LHC), where they fulfil an important structural role (Hoober and Eggink 1999, Phillip *et al.* 2002). However, their most important function in the chloroplast is probably the protection of photosynthetic apparatus from photodestruction caused by strong irradiance or other stress factors (Siefermann-Harms 1987). Their importance for successful development and function of the photosynthetic apparatus is clearly obvious when working with Car-deficient plants. Such plants can be obtained either by treatment with various so-called bleaching herbicides (*e.g.* amitrole, norflurazon, fluridone, flurtamone, and others; see, *e.g.* Sandmann and Böger 1982) or as Car-free mutants, such as *vp9* and *vp2* of maize (*Zea mays* L. – La Rocca *et al.* 2000a), *im* of *Arabidopsis thaliana* L. – Wetzel *et al.* 1994), or *albino* mutant of sunflower (*Helianthus annuus* L. – Fambrini *et al.* 1993).

Amitrole (AM, 3-amino-1,2,4-triazole) can be successfully used to obtain plants with damaged chloroplasts and non-functional photosynthetic apparatus. The effect of this herbicide on plant protective systems seems to be manifold. For example, the inhibition of catalase followed by increased concentration of H_2O_2 in peroxisomes was observed in potato (*Solanum tuberosum* L.) plants treated with AM (Muraja-Ljubičić *et al.* 1999). However, the most conspicuous changes due to the application of this herbicide were found in the content of Cars. AM inhibits cyclisation of lycopene, which leads to the accumulation of its precursors (phytoene and phytofluene; La Rocca *et al.* 2001). The formation of β-ionone or ε-ionone rings is a necessary step in the conversion of lycopene to either β-carotene (and subsequently to zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin) or α-carotene (which is a precursor of lutein; Hirschberg 2001). Therefore the inhibition of this reaction can have dangerous consequences for the structure and function of the whole photosynthetic apparatus. This interruption of the Car synthetic pathway affects negatively the other photosynthetic pigments as well; in the absence of β-carotene the degradation of Chls occurs (Wrischer *et al.* 1992).

The changes in synthesis and/or degradation of Cars and Chls described above are usually followed by changes in the structure of thylakoid pigment-protein complexes, which in turn are associated with alterations in the ultrastructure of chloroplasts. These alterations depend on the concentration of herbicide used, irradiance (Anderson and Robertson 1960), and temperature (Rascio *et al.* 1996, La Rocca *et al.* 1998, 2001). However, most studies concerned with the changes in chloroplast ultrastructure due to AM deal only with the effects of high AM concentrations (125 µM AM: Zito *et al.* 1995, Agnolucci *et al.* 1996, Rascio *et al.* 1996; 200 µM AM: La Rocca *et al.* 2000a,b, Dalla Vecchia *et al.* 2001). Such concentrations lead to the nearly complete destruction of chloroplasts. The use of lower concentrations of AM should facilitate the production of plants with different amounts of Cars, and the correlation between the contents of photosynthetic pigments and the ultrastructure of chloroplasts could therefore be analysed by more subtle methods.

Maize, as a typical C_4 plant belonging to NADP-ME group, exhibits so-called Kranz-type anatomy of leaves with characteristic presence of two distinct cell types (mesophyll and bundle sheaths) containing chloroplasts which differ in many parameters. The possible differences in response of these two types of chloroplasts to various degrees of Car deficiency and its relationship to photosynthetic activity have not—as far as we know—yet been described. The main purpose of this work was therefore to prepare (by the use of AM) maize plants differing in the amount of photosynthetic pigments (especially Cars), to analyse the changes in ultrastructure of MC and BSC chloroplasts in leaves of these plants, and to correlate the level of chloroplast destruction to photosynthetic processes which take place there.

Materials and methods

Plants: Seeds of two genotypes (CE704 and CE810) of maize (*Zea mays* L.) were obtained from the Maize Breeding Station CEZEA in Čejč (Czech Republic). They were germinated for 24 h in water and then placed in nutrient solution (Hoagland 3) with different concentrations of amitrole (20, 60, and 120 µM) or without AM (control plants). Plants were grown in a growth chamber (*Klimabox RK1-007, Kovodružstvo Slaný*, Czech Republic) with the duration of light period 16 h, temperature 21/16 °C, relative humidity 70/80 %, and irradiance 400 $\mu mol\ m^{-2}\ s^{-1}$ PAR. 18 d after germination of seeds, leaf tissue samples were taken from the middle third of leaf blade of the mature third leaves. These samples were used for the transmission electron microscopy and stereological analysis of chloroplast ultrastructure, polarographic measurement of photochemical activity of isolated chloroplasts, and the determination of the contents of photosynthetic pigments.

Analysis of chloroplast ultrastructure: Leaf blade samples were double fixed (glutaraldehyde/osmic acid), dehydrated in ethanol/propylene oxide series, and embedded into Spurr's low viscosity resin. Chloroplast ultrastructure was evaluated on transverse ultra-thin sections of embedded objects contrasted with uranyl acetate followed by lead citrate treatment (Kutík *et al.* 1999) using a transmission electron microscope *Philips EM 300*. The volume densities (relative partial volumes, Gundersen and Jensen 1987) of five chloroplasts for each sample were counted stereologically on microphotographs using morphometric grids with regularly distributed points as described by Kutík *et al.* (1999). Four plants were

evaluated for each variant. In MC chloroplasts, the volume densities of granal and inter-granal thylakoids, peripheral reticulum, starch inclusions, and plastoglobuli were measured and the remaining volume of stroma was counted into 100 %, whereas in BSC chloroplasts only the volume densities of starch inclusions and plastoglobuli were determined. The differences between variants were tested by the Mann-Whitney test, using the 5 % level of statistical significance.

Immunocytochemical analysis: The leaf blade samples were fixed for 2 h in 4 % paraformaldehyde and 0.25 % glutaraldehyde fixative in 0.1 M cacodylate buffer, dehydrated in ethanol, and embedded into *London Resin White* medium. The ultra-thin sections picked up on nickel grids were incubated for 10 min on 1 % bovine serum albumin (BSA) in PBS and treated with the mouse primary antibody against the apoprotein of LHC2 (which labelled both LHC1 and LHC2). After washing with 1 % BSA in PBS, the sections were incubated with colloid gold conjugated with goat-anti-mouse antibodies. The sections were then washed and stained with uranyl acetate for 10 min and examined with the electron microscope (*CM-10 Philips*). Control experiments were performed similarly, but the incubation of the ultra-thin sections with the primary antibody was excluded.

Photochemical activity of chloroplasts: The analysis of photochemical activity of isolated MC chloroplasts was performed as described in Kőmerová and Holá (1999). Hill reaction activity (HRA) was measured polarographically as the amount of oxygen formed by the chloroplast suspensions in the light (750 μmol m^{-2} s^{-1} PAR). 0.007 M $K_3[Fe(CN)_6]$ was added as an artificial electron-acceptor, and a constant temperature of 25 °C was maintained in the measurement chamber (Bartoš *et al.* 1975). Activity of PS1 was measured similarly as the consumption of oxygen by the suspensions of isolated MC chloroplasts. 0.15 mM reduced DCPIP was used as an artificial donor of electrons and 0.1 mM methyl viologen as an electron acceptor. The inhibitor of PS2 activity in this case was 0.1 mM DCMU. Neither HRA nor PS1 activity was analysed in plants treated with 120 μM AM due to the apparent absence of photosynthetic pigments in MC chloroplasts in those plants.

Contents of photosynthetic pigments were determined in acetone extracts of leaf discs by HPLC (*Spectra-Physics*, San Jose, USA) using a reverse phase column (*Sepharon* SGX C18, 5 μm particle size, 150×3 mm, *Tessek*, Prague, Czech Republic). The solvent system was acetonitrile : methanol : water (80 : 12 : 6) followed by 100 % methanol, and the gradient was run from 8 to 12 min. The flow rate was 16.7 mm^3 s^{-1}, the detection wavelength was 445 nm.

Results and discussion

The negative effect of AM was observed almost from the beginning of plant development. As expected, the damage was most pronounced in plants treated with the highest (120 μM) concentration of AM, whereas application of lower concentrations of herbicide (20 or 60 μM) resulted in less perceptible changes. 120 μM AM-treated plants were generally much smaller and could not be cultivated longer than about 18 d, which was obviously due to the fact that the organic nutrients stored in grains were completely consumed at this time. Under normal conditions, the beginning of photosynthetic activity and synthesis of new, energetically rich metabolites would soon compensate loss of these nutrients (used in the first phases of plant development). However, as the application of AM strongly diminishes the amount of photosynthetic pigments in leaves, the consequent damage to photosynthetic apparatus can be severe and no functional chloroplasts might be present in plants treated with high concentration of herbicide. This, of course, would ultimately result in a premature death of plants, as observed in our case.

Differences in the amount of photosynthetic pigments in leaves due to the application of different concentrations of AM were already seen in the outward appearance of plants. Both control and 20 μM AM-treated plants had fully green leaves, whereas the leaves of plants treated with 60 μM AM were pale yellow-green (*i.e.* with diminished amount of Chls and Cars). The leaves of plants treated with the highest concentration of AM were completely white (*i.e.* without photosynthetic pigments). These observations were further supported by HPLC analysis of photosynthetic pigments in tissue of both genotypes. The content of Cars in leaves of AM-treated plants decreased in a concentration-dependent manner (Table 1). A slight decrease in the amount of total Cars—to about 93 % of that found in control plants—was observed in the plants treated with 20 μM AM. A much lower amount of total Cars was found after treatment of plants with 60 μM AM (about 33 % of control). The plants treated with the highest concentration of AM (120 μM AM) contained very limited amounts of protective Cars (0.7 % of control in CE810 to 1.7 % of control in CE704). The loss of xanthophyll cycle pigments (*i.e.* violaxanthin, zeaxanthin, and antheraxanthin) together with the loss of neoxanthin was mainly responsible for this decrease in content of total Cars; this agrees well with some earlier observations (Agnolucci *et al.* 1996, Dalla Vecchia *et al.* 2001, Hirschberg 2001, La Rocca *et al.* 1998). Lutein (synthesised from α-carotene; Young *et al.* 1997) and β-carotene seemed to be less susceptible to AM. However, analysis of the changes in the ratio of lutein and neoxanthin (as representative pigments of LHC) to β-carotene (as a representative Car of reaction

centres) revealed a greater relative decrease in the amount of β-carotene compared to these two xanthophylls. This, together with the significant loss of the pigments of xanthophyll cycle, suggests that the Cars synthesised through β-carotene pathway are more susceptible to AM. AM probably inhibits the cyclisation of lycopene (Dalla Vecchia *et al.* 2001) and the formation of β-ionone ring could be more sensitive than the formation of ε-ionone ring (characteristic for α-carotene and lutein).

The protective role of Cars in plants is widely accepted (Demmig-Adams *et al.* 1996, Young *et al.* 1997). Their absence in chloroplasts leads to severe damage of pigment-protein complexes of thylakoid membranes, which is accompanied by loss of Chls (Anderson and Robertson 1960, Young *et al.* 1997). Moreover, if the Car/xanthophylls binding sites in the complexes can not be occupied, the whole complex becomes instable and Chls will be degraded. The analysis of the content of Chls in leaves of plants treated with various concentrations of AM revealed that even the lowest concentration used (20 μM) had negative influence on these pigments (their amount decreased to about 90 % of control; Table 1). Plants treated with 60 μM AM contained about 49 % of the amount of Chls found in the control plants, and plants treated with 120 μM AM were nearly devoid of Chls (0.7 to 1.1 % of control). The decrease in total Chl concentration was probably due mainly to the decrease in Chl *a* content. This pigment appeared to be more sensitive to AM compared to Chl *b*. Similar results were reported by Kushwaha and Bhowmik (1999) for cucumber (*Cucumis sativus* L.) treated with isoxaflutole, another herbicide inhibiting Car biosynthesis. The greater susceptibility of Chl *a* to AM-induced photooxidative damage was reflected also in the values of Chl *a/b* ratio (Table 1), which decreased with increasing concentration of herbicide. These results, together with the changes observed in the content of individual Cars, suggest that reaction centres of photosystems might be more sensitive to photooxidative damage caused by AM treatment compared to LHC.

Table 1. Contents of photosynthetic pigments [mg m^{-2}] together with Hill reaction activity (HRA) and activity of photosystem 1 (PS1) [μmol(O_2) m^{-2} s^{-1}] in mesophyll chloroplasts isolated from maize plants of two genotypes (CE704, CE810) untreated (C) or treated with different concentrations of amitrole (20, 60, and 120 μM). Plants treated with the highest concentration of AM contained very small amounts of pigments in plant tissue and therefore some values are not given. Car, total carotenoids; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; Chl, chlorophyll; n.d., not determined. Means ± standard errors of mean (SEM).

	Characteristic	C	20 μM	60 μM	120 μM
CE704	Car	46.6±0.0	43.7±0.1	15.0±0.7	0.8±0.0
	neoxanthin	8.3±0.0	7.0±0.1	1.5±0.1	0.0
	lutein	17.9±0.1	17.7±0.1	8.2±0.5	0.5±0.0
	V+A+Z	6.0±0.1	4.8±0.1	1.1±0.1	0.0
	β-carotene	14.5±0.1	14.2±0.2	4.3±0.1	0.3±0.0
	Chl *a+b*	311.0±0.7	281.5±0.7	154.8±6.5	3.4±0.1
	Chl *a/b*	3.33±0.02	3.10±0.00	3.01±0.02	n.d.
	HRA	7.41±0.00	6.07±0.70	3.80±0.00	n.d.
	PS1	12.60±0.21	14.29±0.22	12.81±0.27	n.d.
CE810	Car	43.1±0.0	26.9±10.9	14.1±0.3	0.0
	neoxanthin	9.1±0.1	7.3±0.2	1.7±0.1	0.0
	lutein	14.9±0.1	14.9±0.1	7.4±0.2	0.2±0.1
	V+A+Z	5.4±0.0	4.5±0.1	1.0±0.0	0.0
	β-carotene	13.7±0.1	13.5±0.1	4.1±0.1	0.1±0.0
	Chl *a+b*	268.5±0.2	249.1±1.8	132.3±2.0	0.0
	Chl *a/b*	3.36±0.02	3.01±0.02	2.85±0.03	n.d.
	HRA	11.23±0.00	9.70±0.69	4.75±0.00	n.d.
	PS1	19.88±0.48	15.66±0.12	11.38±0.18	n.d.

Analysis of the photochemical activities of PS1 and PS2 in isolated MC chloroplasts revealed a great sensitivity of PS2 (Table 1). Hill reaction activity (which is a measure of the activity of PS2) significantly decreased with an increasing concentration of herbicide in both genotypes studied. The highest difference was observed between control and 60 μM AM-treated plants (photochemical activity of isolated chloroplasts in 120 μM AM-treated plants could not be measured due to the nearly complete absence of photosynthetic pigments in MC chloroplasts). On the other hand, the activity of PS1 was affected less, which suggests that this pigment-protein complex is more resistant to the photooxidative damage. Similar results were found by Bolychevtseva *et al.* (1995).

Treatment of plants with various concentrations of AM affected not only the content of photosynthetic pigments or photochemical activity of thylakoid pigment-protein complexes, but also the chloroplast ultrastructure (Figs. 1 and 2). MC and BSC chloroplasts in leaves of

control plants were characterised by well-organised thylakoid membranes (see also Kutík *et al.* 2001). MC chloroplasts contained both granal and intergranal thylakoids, whereas thylakoid membranes of BSC chloroplasts were largely non-appressed; these chloroplasts also contained great amounts of starch inclusions. Mesophyll chloroplasts of plants treated with 20 μM AM were ultrastructurally similar to chloroplasts of control plants. However, stereological methods (Table 2) revealed some differences in chloroplast ultrastructure. Statistically significant differences between MC chloroplasts of control and 20 μM AM-treated plants of genotype CE704 were found only for the volume density of starch inclusions and of genotype CE810 for the volume density of starch inclusions and plastoglobuli. On the other hand, the ultrastructure of BSC chloroplasts was more influenced by this low concentration of AM. The volume density of starch inclusions in BSC chloroplasts of 20 μM AM-treated plants of CE704 genotype decreased into about 53.9 % of the values found in control plants and into about 48.1 % in the case of genotype CE810. The decrease in the amount of starch inclusions in chloroplasts is probably connected with either various stress factors or advancing senescence of plants (Mostowska 1997, Kutík 1998). In our case, the decrease observed in BSC chloroplasts of 20 μM AM-treated plants was accompanied by slight tendency of MC chloroplasts of CE810 genotype to accumulate starch under these conditions.

Table 2. Ultrastructural characteristics of mesophyll (MC) and bundle sheath cell (BSC) chloroplasts in two genotypes of maize (CE704 and CE810) untreated (C) or treated with 20 μM amitrole: volume densities [%] of appressed (A) and non-apressed (NA) thylakoids, starch inclusions (ST), plastoglobuli (PL), peripheral reticulum (PR), stroma (S), and proportion [%] of appressed thylakoids to all thylakoids (G). Means ± standard error of mean (SEM). Statistical significance of the differences between AM-treated and untreated plants (D1) or between genotypes (D2) as proven by Mann-Whitney test is also given (**$p \leq 1$ %, *$p \leq 5$ %, $p > 5$ %).

		CE704			CE810			D2	
		C	20 μM	D1	C	20 μM	D1	C	20 μM
MC	A	28.10±1.76	28.29±1.74	-	26.05±1.58	23.37±1.43	-	-	-
	NA	23.34±1.19	21.00±1.24	-	21.17±1.54	18.77±1.34	-	-	-
	G	54.37±1.97	57.08±2.43	-	55.19±2.80	55.24±2.61	-	-	-
	ST	0.10±0.06	0.00	*	1.11±0.32	1.24±0.43	*	**	**
	PL	1.37±0.17	2.10±0.19	-	2.01±0.18	1.68±0.18	*	*	-
	PR	3.84±0.34	3.68±0.20	-	4.78±0.39	3.85±0.34	-	-	-
	S	43.25±2.28	44.92±1.91	-	44.89±1.84	51.09±1.89	-	-	-
BSC	ST	26.00±1.81	14.07±1.77	**	19.14±1.27	9.20±1.36	**	**	*
	PL	0.23±0.05	0.42±0.07	**	0.44±0.08	0.43±0.09	-	*	-

The decrease of Cars to about 33 % of control in plants treated with 60 μM AM was accompanied by dramatic damage of MC chloroplasts especially in genotype CE810 (Fig. 2). MC chloroplasts in leaves of this genotype showed great changes, similar to those observed in plants treated with the highest AM concentration. They were amoeboid in shape and contained only a few thylakoids. MC chloroplasts of genotype CE704 (Fig. 1) subjected to the same experimental conditions were less damaged: they had normal shape and very reduced amount of thylakoids, occasionally being swollen a bit. On the other hand, BSC chloroplasts in leaves of plants treated with 60 μM AM of both genotypes were relatively unaffected. They, however, contained almost no starch inclusions but their thylakoid membranes were still preserved. As it is unlikely to expect that bundle sheath cells contain different AM amount compared to mesophyll, it is probable that BSC chloroplasts are less sensitive to oxidative damage than MC chloroplasts. These two types of chloroplasts differ in the amount of antioxidant compounds (Doulis *et al.* 1997) and this difference seems to be even more pronounced under stress conditions (Pastori *et al.* 2000). Moreover, BSC chloroplasts are relatively physically isolated from oxygen evolved by MC chloroplasts during primary photosynthetic reactions and thus also from reactive oxygen species produced there.

Mesophyll cells in leaves of 120 μM AM-treated plants contained greatly damaged chloroplasts with only a few anomalous thylakoid membranes, sometimes grouped to form very electron-dense masses. Thylakoids were usually either localised only in a part of chloroplast or surrounded the inner chloroplastic membrane. Connections of thylakoids to the inner chloroplastic membrane were also occasionally observed (not shown). Similar phenomenon was found in early stages of chloroplast development (Hudák 1997). BSC chloroplasts in leaves of 120 μM AM-treated plants had very limited amount of thylakoids and never contained starch inclusions. Immunocytochemical analysis of ultra-thin sections of leaf fragments from 120 μM AM-treated plants revealed a great decrease in the amount of LHC particles both in MC and BSC chloroplasts as compared to control plants. However, the decrease in BSC chloroplasts (not shown) was not so prominent as in MC chloroplasts (Fig. 3),

Fig. 1. Chloroplasts of mesophyll cells (MC; *A–D*) or bundle sheath cells (BSC; *E–H*) in the middle part of mature third leaf of maize genotype CE704. MC chloroplast of a leaf of control plant exhibits a well organised thylakoid membranes – appressed (*a*) and non-appressed (*na*) thylakoids, plastoglobuli (*pg*), and a small protrussion (*p*). (*B*) MC chloroplast of a plant treated with low concentration of AM (20 µM), similar to A. (*C*) MC chloroplast of a plant treated with 60 µM AM characterised by a decrease of thylakoid volume density. (*D*) A greatly damaged MC chloroplast of a plant treated with 120 µM AM. Chloroplast contains a few, irregularly distributed thylakoids which are sometimes appressed (*arrows*) and form electron dense material. Angle wise cut thylakoid membranes (*double arrows*) are also seen. An undamaged mitochondrium is also shown. (*E*) BSC chloroplast of control plant with large starch inclusions (*s*). (*F*) Decrease in volume density of starch inclusions in BSC chloroplast of a plant treated with 20 µM AM. (*G*) Increasing concentration of AM (60 µM) causes a great decrease of starch volume density. A small protrusion is also seen (*p*). (*H*) BSC chloroplast of a plant influenced by 120 µM AM shows absence of starch inclusions and very damaged thylakoids. Undamaged mitochondria (*m*) are also seen. Bars = 1 µm.

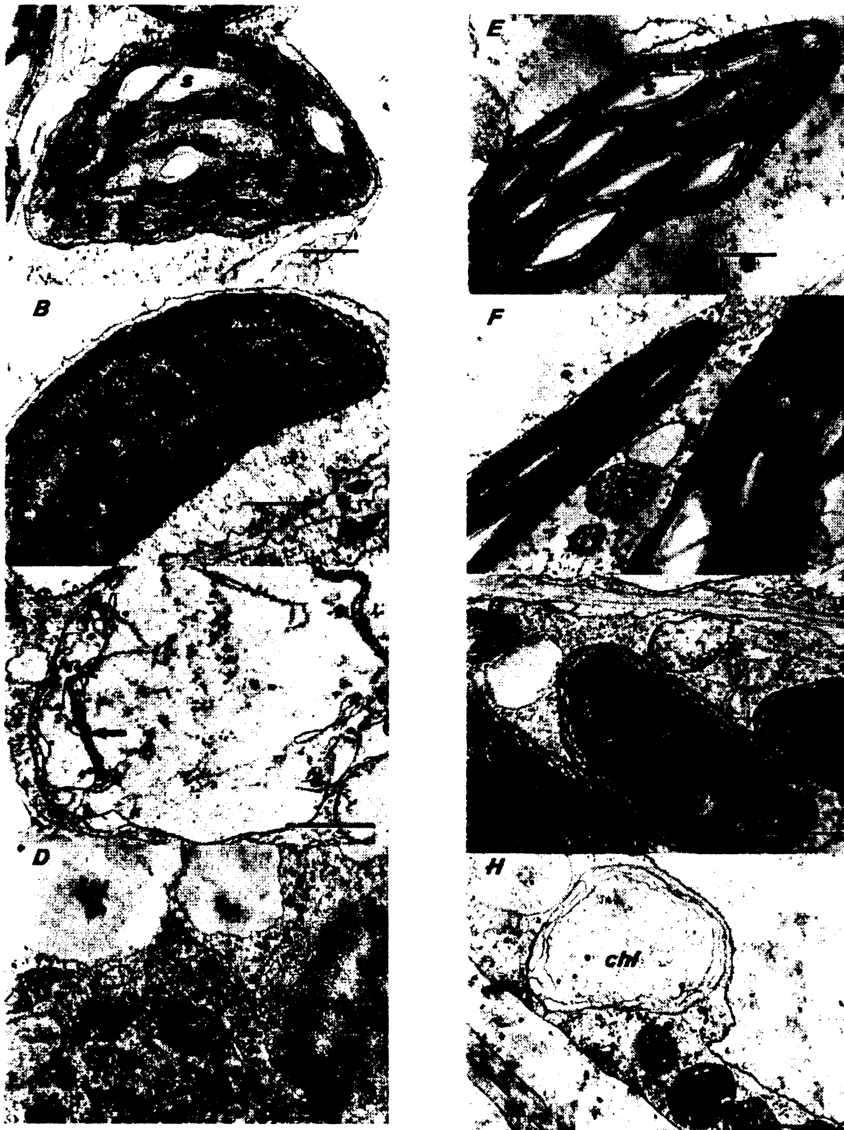


Fig. 2. Chloroplasts of mesophyll cells (MC; *A–D*) or bundle sheath cells (BSC; *E–H*) in the middle part of mature third leaf of maize genotype CE810. (*A*) MC chloroplast of a control plant exhibits a well-organised thylakoid membranes and starch inclusions (*s*). (*B*) MC chloroplast of a plant treated with low concentration of AM (20 μM) that contains no starch inclusions. (*C*) MC chloroplast of a plant treated with 60 μM AM is greatly damaged. Chloroplast contains only a few irregullarly distributed thylakoids which are appressed (*arrow*) and form electron dense material. (*D*) Greatly damaged mesophyll cell of a plant treated with 120 μM AM with mitochondria (*m*), chloroplasts devoid of any thylakoids (*chl*) or chloroplasts containing crystalline material (*ch*) resembling prolamelar bodies in etioplasts. (*E*) BSC chloroplast of control plant with large starch inclusions (s). (*F*) Decreased volume density of starch inclusions in BSC chloroplast of a plant treated with 20 μM AM. (*G*) Increasing concentration of AM (60 μM) causes a great decrease of starch volume density. (*H*) BSC chloroplast (*chl*) of a plant influenced by 120 μM AM shows absence of starch inclusions and very damaged thylakoids. Undamaged mitochondria (*m*) are also seen. Bars = 1 μm.

suggesting again a lesser sensitivity of BSC chloroplasts to AM. Loss of LHC apoproteins due to the application of high AM concentration was reported also by Dalla Vecchia *et al.* (2001) together with a loss of other Chl *a*-binding polypeptides (*e.g.* D1, D2, CP43, CP47, and 22 kDa proteins of PS2).

Fig. 3. Immunocytochemical localisation of LHC labelled by 10 nm gold particles (*black dots - arrow*) in MC chloroplast of genotype CE810 of a control plant (*A*) and of a plant treated with 120 μM AM (*B*). Bars = 0.2 μm.

Ageing chloroplasts or chloroplasts exposed to various stress conditions accumulate plastoglobuli in their stroma. Plastoglobuli contain lipids from degenerating photosynthetic membranes and some investigations performed on Car-free plants indicate that they can also contain precursors of α- and β-carotene, *i.e.* phytoene and phytofluene (Dahlin and Ryberg 1986). The results of our study of chloroplast ultrastructure in leaves of AM-treated plants suggested that the amount of plastoglobuli practically did not change with the application of different concentrations of AM. This suggests that the degree of degeneration of photosynthetic membranes is limited by the amount of photosynthetic membranes formed during chloroplast development. Lower contents of Cars and Chls, which act as stabilisers during LHC assembly and are necessary for the correct development of thylakoid membranes (Wettstein *et al.* 1995, Hoober and Eggink 1999), allowed here the formation of only a few thylakoids. The plastoglobuli in chloroplasts of AM-treated plants could therefore contain only low amount of thylakoid lipids and no increase in their volume density was needed.

Our observation that some chloroplasts formed tubular protrusions into cytoplasm, filled with peripheral reticulum and stroma and lacking thylakoids, was fairly interesting (Fig. 4). These stroma-filled tubules, recently named stromules (Gray *et al.* 2001), are usually more abundant in cells containing relatively small plastid volume. Therefore the formation of such protrusions may enable plastids to increase their surface area in order to optimise metabolic or signal-transduction processes which require movement of various molecules across the plastid envelope. Stromules also interconnect plastids (Gray *et al.* 2001). Plastids with protrusions, so-called amoeboid plastids, have been described also by other authors; they occur mainly during senescence or as a result of some stress factor (Hudák 1997). We suppose that the presence of chloroplast protrusions observed in our study could be connected with cultivation conditions (growth in nutrient solution is not optimal for maize, but was the only possible method to ensure that plants with different amount of Cars will be obtained) rather than with the AM treatment (these protrusions were found both in control and AM-treated plants).

Although the reaction of both genotypes to AM treatment was in many aspects similar, some interesting differences were also observed. The genotypes did not much differ in the content of photosynthetic pigments. CE704 contained slightly more Chls and Cars (except neoxanthin) compared to CE810, but the differences were not statistically significant. This applied both for control and AM-treated plants regardless of the concentration of AM used. The decrease in the content of photosynthetic pigments associated with the increasing concentration of AM was also similar in both genotypes. Some differences were found only for the content of β-carotene or lutein when plants were treated with 120 μM AM. In this case, CE810 was slightly more affected than the second genotype. On the other hand, the differences between both genotypes in photochemical activity of isolated MC chloroplasts were distinct. CE810 displayed higher activites of both PS1 and PS2, which applied especially for plants untreated with AM. This trend was conserved in AM treated plants as well, but the differences between both genotypes tended to diminish with increasing concentration of AM. This suggests that the PS1 complex (and to some extent the PS2 complex, too) in MC chloroplasts

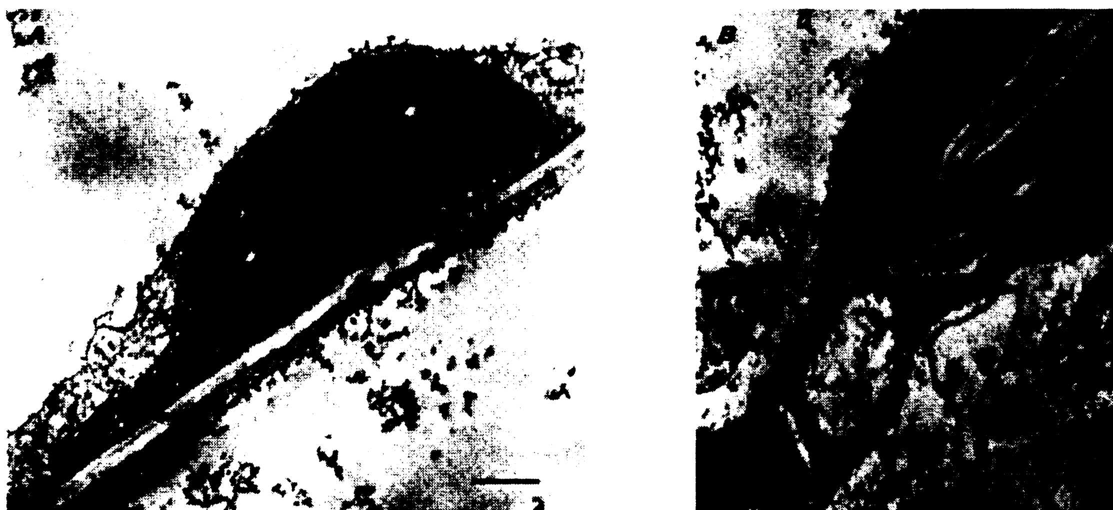


Fig. 4. (*A*) MC chloroplast with a protrussion (*arrow*) of a control plant of genotype CE704. (*B*) BSC chloroplast with a protrusion (*arrow*) of a plant of CE810 genotype treated with 20 μM AM. Bars = 1 μm.

of genotype CE810 is probably more susceptible to photooxidative damage than in CE704 genotype.

The lesser susceptibility of CE704 to such damage was further supported by the analysis of chloroplast ultrastructure. The different reaction of both genotypes to AM was clearly evident especially in MC chloroplasts of plants treated with 60 μM AM (see above). Statistical analysis of differences between both genotypes was performed only in control and 20 μM AM-treated plants. In control plants, both MC and BSC chloroplasts of genotype CE810 were characterised by significantly higher volume density of plastoglobuli compared to CE704; this difference disappeared when 20 μM AM-treated plants were analysed. As regards starch inclusions, their volume density in MC chloroplasts of both control and 20 μM AM-treated plants was significantly higher in CE810 compared to CE704, whereas in BSC chloroplasts the situation was reverse. Taken together, these results confirm our earlier observations that also suggested that CE704 is able to withstand well various stress conditions (*e.g.* low temperature, water deficit; Holá, personal communication). Finally, not only the intra-species variability in the photosynthetic apparatus can be responsible for differences between these two genotypes after the AM treatment but also differences in the rates of uptake and mobility of the herbicide or different metabolic activities relating to the herbicide effects. These features were unfortunately not studied.

Thus our study of chloroplast ultrastructure, photosynthetic pigment contents, and photochemical activity of chloroplasts in maize plants showed that various levels of Car deficiency induced by AM are reflected in various changes of chloroplast ultrastructure. The mesophyll chloroplasts were more affected compared with their counterparts in bundle sheath cells; the negative influence of the partial or total loss of Cars was obvious especially in the alterations in the amount and organisation of thylakoid membranes. These structural changes were connected also to the changes in the function of thylakoid pigment-protein complexes; of these, PS2 was more susceptible to AM-induced damage than PS1. Even the lowest concentration of the herbicide unfavourably affected successful development of chloroplasts. However, some differences between two genotypes examined in the reaction to AM-treatment were also observed, suggesting the existence of intra-specific variability in the response of the photosynthetic apparatus to development in photodamaging conditions.

References

Agnolucci, L., Dalla Vecchia, F., Barbato, R., Tassani, V., Casadoro, G., Rascio, N.: Amitrole effects on chloroplasts of barley plants grown at different temperatures. – J. Plant Physiol. **147**: 493-502, 1996.

Anderson, I.C., Robertson, D.S.: Role of carotenoids in protecting chlorophyll from photodestruction. – Plant Physiol. **35**: 531-534, 1960.

Bartoš, J., Berková, E., Šetlík, I.: A versatile chamber for gas exchange measurements in suspensions of algae and chloroplasts. – Photosynthetica **9**: 395-406, 1975.

Bolychevtseva, Y.V., Rakhimberdieva, M.G., Karapetyan, N.V., Popov, V.I., Moskalenko, A.A., Kuznetsova, N.Y.: The development of carotenoid-deficient membranes in plastids of barley seedlings treated with norflurazon. – J. Photochem.

Photobiol. B **27**: 153-160, 1995.

Dahlin, C., Ryberg, H.: Accumulation of phytoene in plastoglobuli of SAN-9789 (norflurazon)-treated dark-grown wheat. – Physiol. Plant. **68**: 39-45, 1986.

Dalla Vecchia, F., Barbato, R., La Rocca, N., Moro, I., Rascio, N.: Responses to bleaching herbicides by leaf chloroplasts of maize plants grown at different temperatures. – J. exp. Bot. **52**: 811-820, 2001.

Demmig-Adams, B., Gilmore, A.M., Adams, W.W., III: *In vivo* functions of carotenoids in higher plants. – FASEB J. **10**: 403-412, 1996.

Doulis, A.G., Debian, N., Kingston-Smith, A.H., Foyer, C.H.: Differential localisation of antioxidants in maize leaves. – Plant Physiol. **114**: 1031-1037, 1997.

Fambrini, M., Pugliesi, C., Vernieri, P., Giuliano, G., Baroncelli, S.: Characterization of a sunflower (*Helianthus annuus* L.) mutant, deficient in carotenoid synthesis and abscisic acid content, induced by *in vitro* tissue culture. – Theor. appl. Genet. **87**: 65-69, 1993.

Gray, J.C., Sullivan, J.A., Hibberd, J.M., Hansen, M.: Stromules: mobile protrusions and interconnections between plastids. – Plant Biol. 3: 223-233, 2001.

Gundersen, H.J.G., Jensen, E.B.: The efficiency of systematic sampling in stereology and its prediction. – J. Microscopy **147**: 229-263, 1987.

Hirschberg, J.: Carotenoid biosynthesis in flowering plants. – Curr. Opinion Plant Biol. 4: 210-218, 2001.

Hoober, J.K., Eggink, L.L.: Assembly of light-harvesting complex II and biogenesis of thylakoid membranes in chloroplasts. – Photosynth. Res. **61**: 197-215, 1999.

Hudák, J.: Photosynthetic apparatus. – In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 27-48. Marcel Dekker, New York – Basel – Hong Kong 1997.

Kőrnerová, M., Holá, D.: The effect of low growth temperature on Hill reaction and photosystem 1 activities and pigment contents in maize inbred lines and their F_1 hybrids. – Photosynthetica 37: 477-488, 1999.

Kushwaha, S., Bhowmik, P.C.: Inhibition of pigment biosynthesis in cucumber cotyledons by isoxaflutole. – Photosynthetica **37**: 553-558, 1999.

Kutík, J.: The development of chloroplast structure during leaf ontogeny. – Photosynthetica **35**: 481-505, 1998.

Kutík, J., Holá, D., Vičánková, A., Šmídová, M., Kočová, M., Kőrnerová, M., Kubínová, L.: The heterogeneity of structural and functional photosynthetic characteristics of mesophyll chloroplasts in various parts of mature or senescing leaf blade of two maize (*Zea mays* L.) genotypes. – Photosynthetica **39**: 497-506, 2001.

Kutík, J., Kočová, M., Holá, D., Kőrnerová, M.: The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes. – Photosynthetica **36**: 497-507, 1999.

La Rocca, N., Barbato, R., Dalla Vecchia, F., Rascio, N.: Cab gene expression in bleached leaves of carotenoid-deficient maize. – Photosynth. Res. **64**: 119-126, 2000a.

La Rocca, N., Bonora, A., Dalla Vecchia, F., Barbato, R., Rascio, N.: Effects of amitrole and norflurazon on carotenogenesis in barley plants grown at different temperatures. – In: Garab, G. (ed.): Photosynthesis: Mechanisms and Effects. Vol. V. Pp. 3865-3868. Kluwer Academic Publishers, Dordrecht – Boston – London 1998.

La Rocca, N., Dalla Vecchia, F., Barbato, R., Bonora, A., Bergantino, E., Rascio, N.: Plastid photodamage and Cab gene expression in barley leaves. – Physiol. Plant. **109**: 51-57, 2000b.

La Rocca, N., Rascio, N., Oster, U., Rüdiger, W.: Amitrole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of Lhc and RbcS genes. – Planta **213**: 101-108, 2001.

Mostowska, A.: Environmental factors affecting chloroplasts. – In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 407-426. Marcel Dekker, New York – Basel – Hong Kong 1997.

Muraja-Ljubičić, J., Wrischer, M., Ljubešić, N.: Influence of the herbicides amitrole and norflurazon on greening of illuminated potato microtubers. – Z. Naturforsch. **54c**: 333-336, 1999.

Pastori, G., Foyer, C.H., Mullineaux, P.: Low temperature induced changes in the distribution of H_2O_2 and antioxidants between the bundle sheath and mesophyll cells of maize leaves. – J. exp. Bot. **54**: 107-113, 2000.

Phillip, D., Hobes, S., Paulsen, H., Molnar, P., Hashimoto, H., Zouny, A.J.: The binding of xanthophylls to the bulk light-harvesting complex of photosystem II of higher plants. – J. biol. Chem. **277**: 25160-25169, 2002.

Rascio, N., Dalla Vecchia, F., Agnolucci, L., Barbato, R., Tassani, V., Casadoro, G.: Amitrole effects on barley etioplasts. – J. Plant Physiol. **149**: 295-300, 1996.

Sandmann, G., Böger, P.: Mode of action of herbicidal bleaching. – In: Moreland, D.E., St.John, J.B., Hess, F.D. (ed.): Biochemical Responses Induced by Herbicides. Pp. 111-130. American Chemical Society, Washington 1982.

Siefermann-Harms, D.: The light harvesting and protective functions of carotenoids in photosynthetic membranes. – Physiol. Plant. **69**: 561-568, 1987.

Wettstein, D. von, Gough, S., Kannangara, C.G.: Chlorophyll biosynthesis. – Plant Cell 7: 1039-1057, 1995.

Wetzel, C.M., Jiang, C.Z., Meehan, L.J., Voytas, D.F., Rodermel, S.R.: Nuclear organelle interactions – the immutans variegation mutant of *Arabidopsis* is plastid autonomous and impaired in carotenoid biosynthesis. – Plant J. **6**: 161-175, 1994.

Wrischer, M., Ljubešić, N., Devidé, Z.: Ultrastructural studies of degradational processes in amitrole-damaged photosynthetic membranes. – J. struct. Biol. **108**: 1-5, 1992.

Young, A.J., Phillip, D., Savill, J.: Carotenoids in higher plant photosynthesis. – In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 575-596. Marcel Dekker, New York – Basel – Hong Kong 1997.

Zito, F., Gough, S., Marcato, C., Rascio, N., Casadoro, G.: Expression of chloroplastic and cytosolic glutamine synthase in barley leaves after cold-sensitive blocking of β-carotene synthesis by amitrole or mutation. – Physiol. Plant. **94**: 535-544, 1995.

Biotechnology Letters **26:** 1549–1555, 2004.
© 2004 *Kluwer Academic Publishers. Printed in the Netherlands.*

Viability, ultrastructure and cytokinin metabolism of free and immobilized tobacco chloroplasts

Lenka Polanská[1,2]*, Anna Vičánková[1], Petre I. Dobrev[1], Ivana Macháčková[1] & Radomíra Vaňková[1]
[1] *Institute of Experimental Botany AS CR, Rozvojová 135, Prague 6 – Lysolaje, 165 02, Czech Republic*
[2] *Department of Plant Physiology, Charles University, Faculty of Science, Viničná 5, Prague 2, 128 44, Czech Republic*
**Author for correspondence (Fax: + 420 2 20390419; E-mail: polanska@ueb.cas.cz)*

Received 16 June 2004; Revisions requested 9 July 2004; Revisions received 16 August 2004; Accepted 19 August 2004

Key words: calcium alginate, chloroplast ultrastructure, cytokinin metabolism, immobilization, *Nicotiana tabacum*

Abstract

Cytokinins play a decisive role in regulation of plastid development and differentiation, but their metabolism in plastids is not known. Metabolic studies using intact chloroplasts are prevented by their instability once they are isolated from leaf cells. Chloroplasts of *Nicotiana tabacum L.* cv. Petit Havana SR1 were therefore immobilized into low-viscosity alginate. Their intactness was assessed by a glyceraldehyde-3-phosphate dehydrogenase assay which indicated that free chloroplasts totally disintegrated within 7 h, while more than 50% of immobilized chloroplasts remained intact after 24 h. The immobilization had no marked impact on ultrastructure and postponed final destruction. The metabolite profile was similar in free and immobilized chloroplasts after 4 h incubation with tritiated zeatin. Nevertheless, the yield of conversion products decreased twice in immobilized chloroplasts, which was probably the outcome of mass transfer limitations and/or the sorption to polysaccharide matrix.

Introduction

Metabolic studies on isolated organelles enable to elucidate the role of compartmentation in plant hormone metabolism and may contribute to understanding of its regulation. Cytokinins (CKs) are plant hormones which have a close relationship to plastids. They regulate their differentiation and development, especially by promoting de-etiolation (e.g. Zavaleta-Mancera *et al.* 1999, Chory *et al.* 1994). In tobacco chloroplasts, the whole spectrum of CKs was detected (Benková *et al.* 1999). Recently, the synthesis of the prenyl group of CKs in plastids and the localization of four isopentenyltransferases, CK biosynthetic proteins to plastids in *Arabidopsis*, were reported (Kasahara *et al.* 2004).

The study of metabolism in isolated chloroplasts is, however, considerably limited by their low stability at room temperature. Chloroplast viability could be positively affected by their immobilization. The entrapment into a suitable matrix may have protective effects because it enables to create a favourable microenvironment and to protect against high-shear forces. Nevertheless, the stabilizing matrix may also form diffusion barriers, e.g. for O_2 and nutrient transport (Guiseley 1989). The impact of immobilization depends on both the type and concentration of polymer and the organism used (Junter *et al.* 2002).

Photochemical activities were compared in free chloroplasts and those immobilized into agar, calcium alginate and glutaraldehyde cross-linked bovine serum albumin (Synková & Šesták 1991). Chloroplasts immobilized in calcium alginate beads were used for study of transfer of lysophosphatidylcholine from microsomes to chloroplasts, both embedded in alginate, and subsequent metabolism of lysolipids in chloroplasts (Testet *et al.* 1999). Co-immobilization of spinach chloroplasts (as a source of reducing equivalents) and yeast P450 monooxygenase was done

by Hara *et al.* (1999) who compared three methods (entrapment, adsorption and cross-linking) and seven materials. The conversion of 7-ethoxycoumarin to 7-hydroxycoumarin was highest when agarose was used.

Up to now little attention has been paid to the effect of immobilization on chloroplast physiology and ultrastructure. The immobilization into 2.4% agar gel was found to alter chloroplast ultrastructure (Šesták *et al.* 1983). Only some grana and plastoglobules were visible, even if the Hill activity remained unchanged. Another study with chloroplasts immobilized into alginate reported only 'leakage of stroma' without any image documentation (Testet *et al.* 1999). The aim of our study has been to evaluate the impact of alginate immobilization on chloroplast ultrastructure, viability and CK metabolism.

Materials and methods

Chloroplast isolation

Intact chloroplasts were isolated and purified according to Benková *et al.* (1999) from tobacco *Nicotiana tabacum* L. cv. Petit Havana SR1 which was cultivated in a growth chamber (light regime 16/8 h, 25 °C/20 °C) for 1 month and in a glasshouse at 26 °C/20 °C for another month. Leaves were deribbed, cut and mixed with a homogenization medium (0.33 M sorbitol; 50 mM Tris/HCl, pH 7.8; 0.4 mM KCl; 0.04 mM Na_2EDTA; 0.1% (w/v) bovine serum albumin; 1% (w/v) polyvinylpyrrolidone; 5 mM isoascorbic acid) in semi-frozen state, homogenized with a homogenizer and filtered through a sandwich of cotton wool between 8 layers of muslin. The chloroplast fraction was recovered by centrifugation (1000 *g*; 2 min; 4 °C), washed with a resuspension medium (RM: 0.33 M sorbitol; 2 mM Na_2EDTA; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 50 mM HEPES, pH 7.6) and resuspended in RM. This suspension was layered on a Percoll density gradient [40% and 80% (v/v) Percoll solution in RM] and centrifuged for 15 min at 1000 *g* at 4 °C. Intact chloroplasts were collected at the interface of the gradient, diluted with RM and centrifuged for 3 min at 1000 *g* at 4 °C. The pellet was resuspended in RM. The chloroplasts were immediately used. All the procedures were done at 4 °C.

Chlorophyll determination

Chlorophyll was extracted with 80% (v/v) acetone. The total chlorophyll a+b content was calculated from the absorbance at 652 nm of the clear extract after centrifugation (500 *g*, 5 min) according to Arnon (1949).

Chloroplast viability

The intactness of chloroplasts was determined by the glyceraldehyde-3-phosphate dehydrogenase (G3PD) assay (Latzko & Gibbs 1968). Free chloroplasts in RM or chloroplasts immobilized in alginate beads were incubated in a reaction mixture (0.33 M Tris/HCl, pH 8.5; 17 mM $Na_2HAsO_4 \cdot 7H_2O$; 4 mM cysteine; 20 mM NaF; 40 μM $NADP^+$). The reaction was initiated with 0.02 M glyceraldehyde 3-phosphate. Reduction of $NADP^+$ was followed at 340 nm for 5 min. The same assay was run with chloroplasts disrupted with 0.01 M $MgCl_2$. To ensure destruction of immobilized chloroplasts an additional step, sonication, was performed. The percentage of viable chloroplasts was calculated from the difference between original and disrupted sample.

Entrapment of chloroplasts in alginate

Resuspended chloroplasts (1.8 ml) were mixed with a soft paintbrush with 1.8 ml 4% (w/v) sodium alginate (low viscosity, Sigma) in RM. The mixture was extruded drop-wise at a constant rate using a syringe into continuously agitated 1% (w/v) $CaCl_2$ in RM. The beads (average diam. 2.5 mm) were stirred in the $CaCl_2$ solution for 1 h to complete the gelation process. Then they were transferred into the incubation medium (i.e. RM) containing 5 mM $CaCl_2$ and shaken at 125 rpm at room temperature.

Cytokinin metabolism determination

Substrate ([^{3}H] *trans*-zeatin, radioactively labelled on position 2 of the purine ring by Dr. Jan Hanuš, Institute of Experimental Botany, Prague, 1.3 TBq $mmol^{-1}$) was incubated with either free (incubation time 4 h) or immobilized chloroplasts (incubation time 4 or 12 h). After the incubation free chloroplasts were separated from RM by centrifugation (3000 *g*; 5 min). RM of immobilized chloroplasts was decanted. The alginate matrix was solubilized with 0.5 M sodium citrate in RM, pH 5.7. Both chloroplast samples were washed twice with RM. CKs were extracted with Bieleski

solvent (Bieleski 1964) and purified using C_{18} SPE columns (Sep-Pak Cartridge, Waters, WAT020515). After elution with 80% (v/v) methanol samples were concentrated and analyzed by HPLC using column Luna C18 (2) (150 mm/4.6 mm/3 μm, Phenomenex); flow rate: 0.6 ml min^{-1}; mobile phase: A = 40 mM acetic acid + ammonia, pH 4.1, B = methanol/acetonitrile = 1/1 (v/v); gradient: 0 min – 10% B, 2 min – 15% B, 11 min – 20% B, 11.1 min – 34% B, 19 min – 45% B, 21 min – 100% B, 23 min – 100% B, 25 min – 10% B; detection at 270 nm. Collected fractions (0.5 ml) were mixed with liquid scintillation cocktail (Elite, ICN Biomedicals) and the radioactivity was counted by Liquid Scintillation Analyser (Tri-Carb 2900TR). Media were purified directly using C_{18} columns, CKs were eluted with 80% (v/v) methanol and analyzed as chloroplast samples.

Transmission electron microscopy

Leaf blade samples, suspensions of free chloroplasts and alginate beads with immobilized chloroplasts were fixed for 2 h with 2.5% (v/v) glutaraldehyde in RM, pH 7.6, followed by 2 h in 2% (w/v) osmic acid in RM. Samples were embedded into low viscosity resin (Spurr 1969) via propylene oxide after dehydration in graded ethanol series up to 100%. The samples of suspensions of non-immobilized chloroplasts had to be treated very carefully. They were transferred on a Percoll 'cushion' [80% (v/v) Percoll solution in RM] and separated from solutions by centrifugation (1000 *g*, 5 min). The ultrastructure was evaluated on transverse ultrathin sections of embedded objects contrasted with a saturated solution of uranyl acetate in 70% (v/v) aqueous ethanol, followed by a lead citrate solution treatment according to Reynolds (1963) using a transmission electron microscope.

Results and discussion

The main goal of our study has been to study CK metabolism in chloroplasts without interference of cytoplasm and other organelles. To prolong the viability of isolated chloroplasts we encapsulated them into calcium alginate. The bioencapsulation was chosen because it represents very gentle way of immobilization with high potential protective impact (in comparison with the binding on the surface of solid carrier).

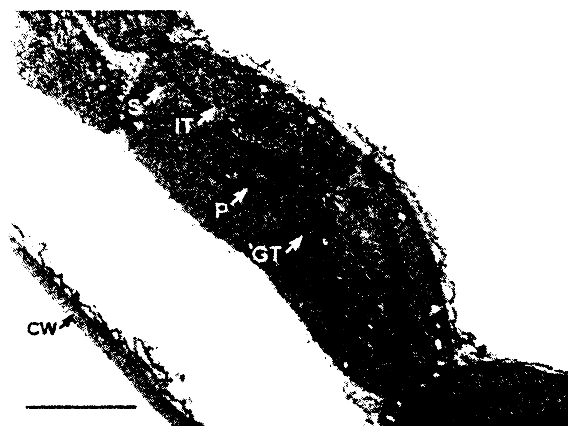


Fig. 1. Transmission electron micrograph of chloroplast cross-section taken from the intact tobacco leaves. Abbreviations used: CW – cell wall, GT – granal thylakoids, IT – intergranal thylakoids, P – plastoglobule, S – stroma. Bar = 1 μm.

As chloroplasts are very sensitive to elevated temperatures, ionotropic (low viscosity alginate) and not thermally-reversible gel (e.g. agarose) was used.

The intactness of free tobacco chloroplasts immediately after their isolation was higher than 90%, as determined by the latency of the stromal enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PD). After 4 h incubation at room temperature the viability decreased to ca. 42%. The intactness of immobilized chloroplasts decreased according to G3PD test much slower (more than 86% and 50% after 4 h and 24 h, respectively). However, to follow the reduction of $NADP^+$ approx. a six times higher amount of chloroplasts (calculated per the amount of chlorophyll) had to be used, which indicates that the low activity of G3PD might be caused by mass transfer limitations and/or the sorption on polysaccharide molecules. This is in accordance with the results of Hara *et al.* (1999) who reported difficulties in measuring the NADPH-Hill reaction in chloroplasts entrapped in gel matrices.

Taking into account the potential interference of immobilization matrix with G3PD test, the data on the chloroplast intactness were compared with their ultrastructure determined by transmission electron microscopy. The most evident change after careful chloroplast isolation was the shift of their shape from rather flat in leaves (Figure 1) to more oval or nearly round (Figures 2A and B). This was probably given by the loss of turgor pressure. Immediately after the isolation we observed damages of structure in ca. 15% of chloroplasts. During prolonged incubation vari-

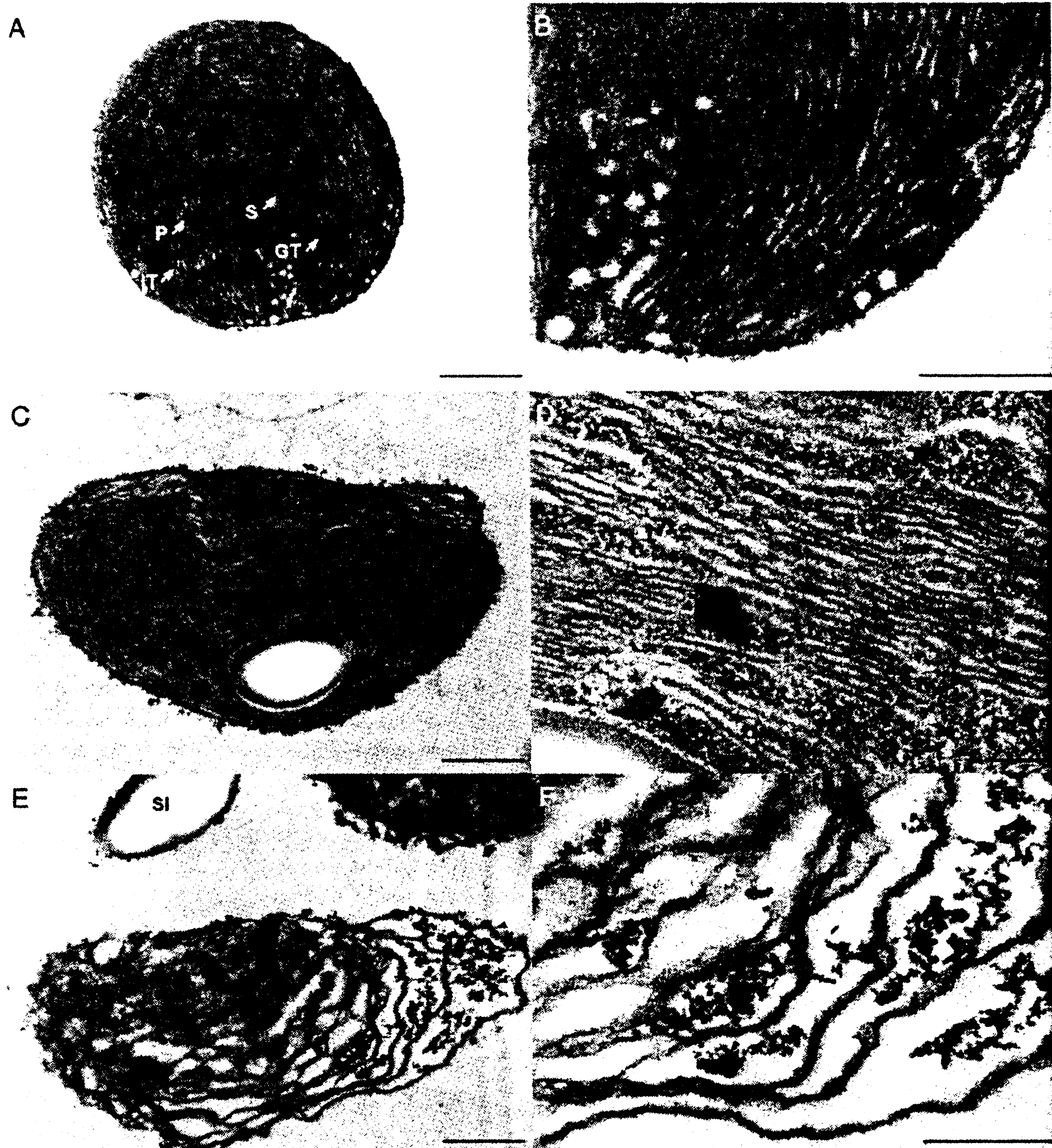


Fig. 2. Transmission electron micrographs of isolated non-immobilized tobacco chloroplast cross-sections taken immediately after isolation (A) with detail (B); taken 4 h after incubation at room temperature (C) with detail (D); and destroyed chloroplast (E) with detail (F). Abbreviations used: GT – granal thylakoids, IT – intergranal thylakoids, P - plastoglobule, S – stroma, SI – starch inclusions. Bar = 1 μm (A, C, E), 500 nm (B, F) or 200 nm (D).

ous symptoms of ultrastructure destruction occurred (thylakoid breakdown – loss of parallel arrangement and dilatation, envelope membrane ruptures). These degradation changes differ from the changes during programmed chloroplast senescence in intact leaves that are connected with the increase of the size and/or number of the lipoid waste inclusions, plastoglobuli (Kutík 1998). Negative changes were much more profound in mature chloroplasts with large starch grana, which could be the consequence of the inevitable handling during sample preparation for electron microscopy. After 4 h incubation more than 50% of

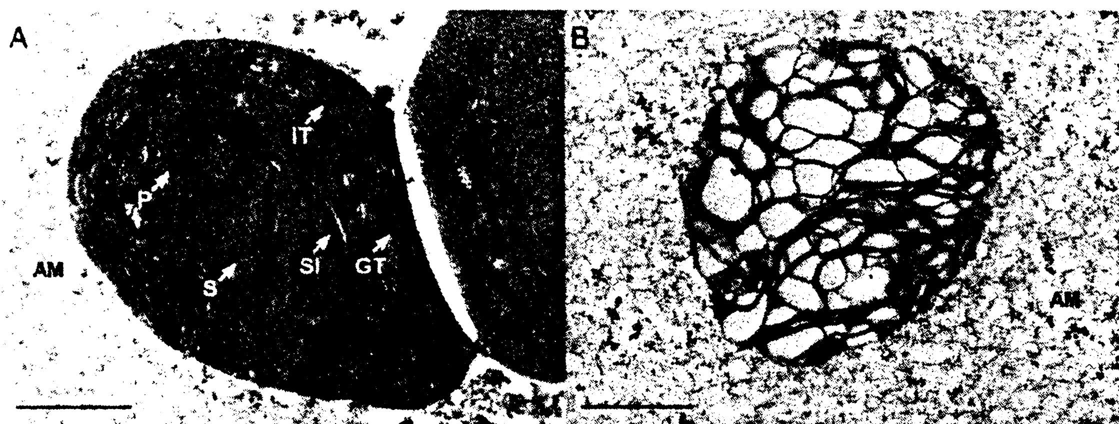


Fig. 3. Transmission electron micrographs of tobacco chloroplasts immobilized into calcium alginate beads taken 1 h (A) and 18 h (B) after immobilization. Abbreviations used: AM – alginate matrix, GT – granal thylakoids, IT – intergranal thylakoids, P – plastoglobule, S – stroma, SI – starch inclusions. Bar = 1 μm.

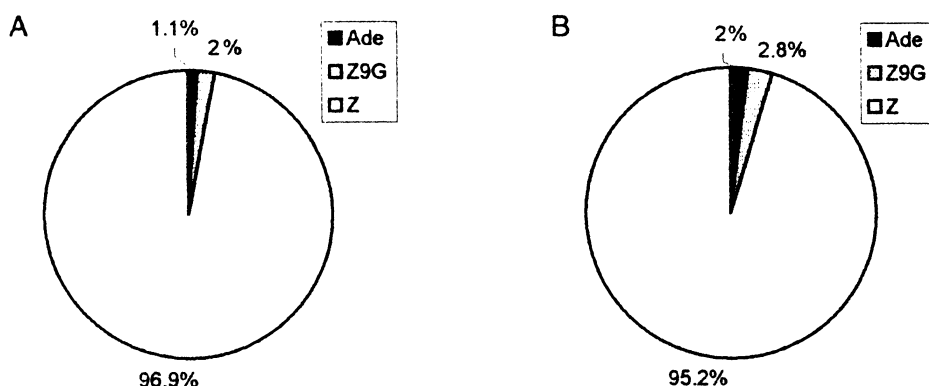


Fig. 4. Cytokinin metabolites in free chloroplasts (A) and incubation medium (B) after 4 h incubation with [^{3}H]Z. Abbreviations used: Ade – adenine, Z – *trans*-zeatin, Z9G – *trans*-zeatin-9*N*-glucoside.

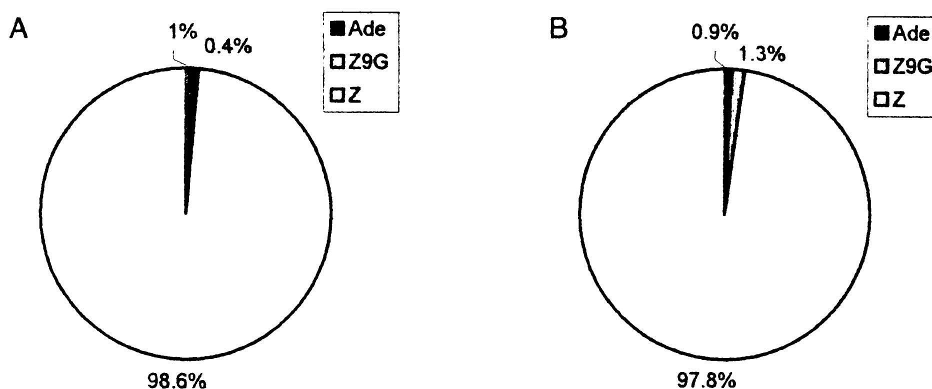


Fig. 5. Cytokinin metabolites in immobilized chloroplasts (A) and incubation medium (B) after 4 h incubation with [^{3}H]Z. Abbreviations used: Ade – adenine, Z – *trans*-zeatin, Z9G – *trans*-zeatin-9*N*-glucoside.

chloroplasts showed structure damage, e.g. disintegration of thylakoid grana (Figures 2C and 2D). The total destruction of the membrane system was found in at least 10% of free chloroplasts (Figures 2E and 2F).

The immobilization did not have significant impact on chloroplast ultrastructure (Figure 3A). It prolonged the chloroplast intactness by 2–3 h. After 5–6 h after isolation, the deterioration became accelerated. Even if the latency of the stromal enzyme G3PD was high after 18 h, we still observed destruction of inner membrane system in all immobilized chloroplasts. The thylakoid membrane system was dilated and destoyed, as well as inner and outer membranes. The disorganisation ensued with spillage of stroma in the final stage of chloroplast degradation (Figure 3B).

The additional advantage of immobilization was an easier handling during sample preparation e.g. for electron microscopy. The chloroplasts in alginate beads were more resistant and easier transferred from one solution to the following one during fixation, dehydration and embedding into the resin compared to chloroplasts in suspension that had to be centrifuged to separate them from solutions. Even if it is necessary to consider that some destructive changes might occur during preparation of samples for electron microscopy, especially in the ultrastructure of free chloroplasts, confrontation of the estimation of their viability by G3PD test and by electron microscopy clearly showed that in case of alginate immobilized chloroplasts the assayled to high overestimations.

The metabolite profile of zeatin in free chloroplasts after 4 h incubation is shown in Figure 4A. The main CK degradation product, adenine, was found which indicates that CK oxidase/dehydrogenase is active in tobacco chloroplasts. This is in accordance with our previous results (Benková *et al.* 1999). The other metabolite which correlated in its retention time with zeatin-9*N*-glucoside represents another product of CK deactivation (Mok & Martin 1994).

As the yield of zeatin conversion in isolated chloroplasts was very low, we aimed to increase it by chloroplast immobilization. The HPLC profile of CK metabolites was similar in immobilized and free chloroplasts. In case of immobilized chloroplasts, substantially lower amount of radioactivity was recovered (Figure 5A), mainly due to the losses during alginate particle solubilization and further purification. The relative yield of zeatin products also decreased compared to free chloroplasts. Prolonged incubation of immobilized chloroplasts did not significantly change the ratio of CK metabolites (data not shown). The presence of CK metabolites in RM (Figures 4B and 5B) suggests that intensive transport (and/or release from broken chloroplasts) occurs between the chloroplasts and RM. When tritiated zeatin was incubated in medium without chloroplasts no spontaneous conversion was detected. The decrease of the reaction yield of immobilized chloroplasts was caused probably by diffusion limitation and non-specific sorption. The achieved results indicate that chloroplast immobilization into alginate does not contribute to better elucidation of CK metabolism of isolated chloroplasts.

Acknowledgements

This research was supported by the grants of the Ministry of Education, Youth and Sports COST OC 840.20, LN00A081 and GACR 206/03/0369.

References

Arnon DI (1949) Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* **24**: 1–15.

Benková E, Witters E, Van Dongen W, Kolář J, Motyka V, Brzobohatý B, Van Onckelen HA, Macháčková I (1999) Cytokinins in tobacco and wheat chloroplasts, occurrence and changes due to light/dark treatment. *Plant Physiol.* **121**: 245–251.

Bieleski RL (1964) The problem of halting enzyme action when extracting plant tissues. *Anal. Biochem.* **9**: 431–442.

Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis-det* mutants have an altered response to cytokinins. *Plant Physiol.* **104**: 339–347.

Guiseley KB (1989) Chemical and physical properties of algal polysaccharides used for cell immobilization. *Enzyme Microb. Technol.* **11**: 706–716.

Hara M, Iazvovskaia S, Ohkawa H, Asada Y, Miyake J (1999) Immobilization of P450 monooxygenase and chloroplast for use in light-driven bioreactors. *J. Biosci. Bioeng.* **87**: 793–797.

Junter GA, Coquet L, Vilain S, Jouenne T (2002) Immobilized-cell physiology: current data and the potentialities of proteomics. *Enzyme Microb. Technol.* **31**: 201–212.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara S (2004) Distinct isoprenoid origins of *cis-* and *trans-*zeatin biosyntheses in *Arabidopsis*. *J. Biol. Chem.* **279**: 14049–14054.

Kutík J (1998) The development of chloroplast structure during leaf ontogeny. *Photosynthetica* **35**: 481–505.

Latzko E, Gibbs M (1968) Distribution and activity of enzymes of the reductive pentose phosphate cycle in spinach leaves and chloroplasts isolated by different methods. *Z. Pflanzenphysiol.* **59**: 184–194.

Mok DWS, Martin RC (1994) Cytokinin metabolic enzymes. In: Mok DWS, Mok MC, eds. *Cytokinins, Chemistry, Activity and Function.* Boca Raton: CRC Press, pp. 129–137. ISBN 0-8493-6252-0.

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**: 208–212.

Šesták Z, Benešová H, Zima J, Pospíšilová J, Kutík J (1983) Effects of age and water potential of leaves on photochemical activities of immobilized chloroplasts. In: Sybesma C, ed. *Proceedings of the VIth International Congress on Photosynthesis in Advances*

in *Photosynthesis Research, Brussels, Belgium*, IV.4., pp. 407–410. ISBN 90-247-2946-7.

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**: 31–43.

Synková H, Šesták Z (1991) Immobilization of chloroplasts from young and old leaves of lettuce, pea, spinach and tobacco. *Photosynthetica* **25**: 1–10.

Testet E, Verdoni N, Cassagne C, Bessoule J (1999) Transfer and subsequent metabolism of lysolipids studied by immobilizing subcellular compartments in alginate beads. *Biochim. Biophys. Acta* **1440**: 73–80.

Zavaleta-Mancera HA, Franklin KA, Ougham HJ, Thomas H, Scott IM (1999) Regreening of senescent *Nicotiana* leaves I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. *J. Exp. Bot.* **50**: 1677–1682.

Chloroplast ultrastructural development in vascular bundle sheath cells of two different maize (*Zea mays* L.) genotypes

A. Vičánková[1, 2], J. Kutík[1]

[1]*Faculty of Science, Charles University in Prague, Czech Republic*
[2]*Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

ABSTRACT

The leaves of maize have two photosynthesizing tissues with two types of chloroplasts, mesophyll cells (MC) and vascular bundle sheaths cells (BSC). The development of chloroplasts in BSC was followed by transmission electron microscopy and point counting method in the middle part of the third leaf of maize plants. From young (Y) to mature (M) leaves, volume density of photosynthetic membrane system (thylakoids) increased, to senescing (S) leaves it did not significantly change. During the whole leaf ontogeny, small thylakoid appression regions (grana) were present in BSC chloroplasts, currently assumed to be agranal. From M to S leaves, volume density of starch inclusions strongly decreased and that of plastoglobuli strongly increased.

Keywords: chloroplast; ultrastructure; C_4 photosynthesis; electron microscopy; *Zea mays* L.

Chloroplasts are cell organelles of photosynthesis. They are, therefore, the most important type of plastids. Higher plant plastids have been studied for a very long time (see e.g. Virgin and Egnéus 1983, Ryberg et al. 1993, Hudák 1997). Chloroplast ultrastructural development during leaf ontogeny was reviewed by Kutík (1998). Photosynthesis is the primary metabolic process, which is important, by fixation of inorganic carbon from CO_2, for life on our planet (e.g. Lawlor 2001). Plants with so-called C_4 photosynthesis have CO_2 fixation compartmentalized between two types of chloroplasts. Most of C_4 plants have typical Kranz (wreath) leaf anatomy. Two types of photosynthetic tissue surround vascular bundles: the bundle sheath cells (BSC) and the mesophyll cells (MC). Maize is a typical C_4 plant of so-called NADP-malic enzyme (NADP-ME) subgroup (Hudák 1997). Chloroplasts are located centrifugally in the bundle sheath cells. They have usually almost no grana (regions of appressed thylakoids) or have small grana composed of two to four thylakoids only (whereas MC chloroplasts have large grana usually from many thylakoids) but they contain usually large inclusions of reserve polysaccharide, starch (which is almost absent in MC chloroplasts). Brangeon (1973a, b) followed differentiation of dimorphic (BSC and MC) chloroplasts of young, growing maize leaves. Differentiation of MCs and BSCs was followed in maize also by molecular approaches (e.g. Furumoto et al. 2000). Kutík et al. (1999) studied stereologically the development of MC chloroplasts during ontogeny of the third leaf of maize plants in genotypes differing in photosynthetic (photochemical) activity. The aim of our work was to evaluate stereologically (for the first time, as far as we know) the development of BSC chloroplasts during ontogeny of the same leaves.

MATERIAL AND METHODS

Maize (*Zea mays* L.) plants in two parent lines, CE813 and CE829, and their hybrids, CE813×CE829 and CE829×CE813, were used in the experiments. They were cultivated in a growth chamber at temperature 25/16°C, air humidity 60 to 80%, and irradiance of 500 μmol/m^2/s. Samples for ultrastructural study were taken from the middle part of the third leaf of maize plants (coleoptile numbered as leaf zero) at the age of the plants of 13, 27, 41 days after sowing, i.e., from young (Y, growing), mature (M, not more growing) and senescing (S, yellowing) leaves. They were always taken from

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. J13/98113100004.

four plants. For each plant, five chloroplasts were evaluated. Leaf samples were double fixed with glutaraldehyde and osmic acid, then embedded into Spurr's low viscosity resin via propylene oxide after dehydration in graded ethanol series, see Kutík et al. (2001). Ultrathin sections were contrasted in saturated solution of uranyl acetate in 70% ethanol (for 20 min) and in lead citrate solution after Reynolds (1963), (for 20 min). Chloroplast ultrastructure was evaluated using transmission electron microscope PHILIPS EM 300. Quantitative evaluation of chloroplast ultrastructure was realized by point counting method, using morphometric grids laid on positive photographic images of chloroplasts. This method allows estimating three-dimensional characteristics of given body (e.g. a chloroplast) from its sections. Volume densities (relative partial volumes, if chloroplast volume = 100%, see Gundersen and Jensen 1987) of granal and intergranal thylakoids, peripheral reticulum, starch inclusions, plastoglobuli and stroma were estimated. Area, length and width of chloroplast cross sections in bundle sheaths cells were followed by the system for image processing and analysis LUCIA version 3.52 (Laboratory Imaging Ltd., Czech Republic). Statistical significance of all ontogenic and genotypic differences found was determined by ANOVA software and *t*-test.

RESULTS AND DISCUSSION

The ultrastructure and size of BSC chloroplasts (Figure 1) changed notably during the leaf ontogeny. From Y to M leaves the volume density of thylakoids increased, from M to S leaves it did not change significantly. Small grana were identifiable during whole leaf ontogeny, the lowest level of granality was reached in BSC chloroplast thylakoid system of mature leaves where compartmentalization of CO_2 fixation between MCs and BSCs worked probably best. Presence of the grana with high activity of photosystem two splitting water and producing oxygen is unfavourable for CO_2 fixation, see also Nishioka et al. (1993). From M to S leaves the amount of starch strongly decreased and the volume density of plastoglobuli increased. Both are the signs of chloroplast senescence, as was reviewed by Kutík (1998). Y and M leaves chloroplasts were larger comparing with S leaves chloroplasts. There were small differences only between BSC chloroplasts of the leaves of CE813 and CE829 inbred lines of the same age (Tables 1 and 2).

The volume density of BSC chloroplasts in the cells of mature leaves (data not shown) did not differ between both inbred lines. Comparison between BSC chloroplasts ultrastructure in mature leaves of reciprocal hybrids (Table 1) showed that CE813×CE829 chloroplasts possessed significantly less thylakoids, but at the same time much more starch than CE829×CE813 ones (determined by *t*-test).

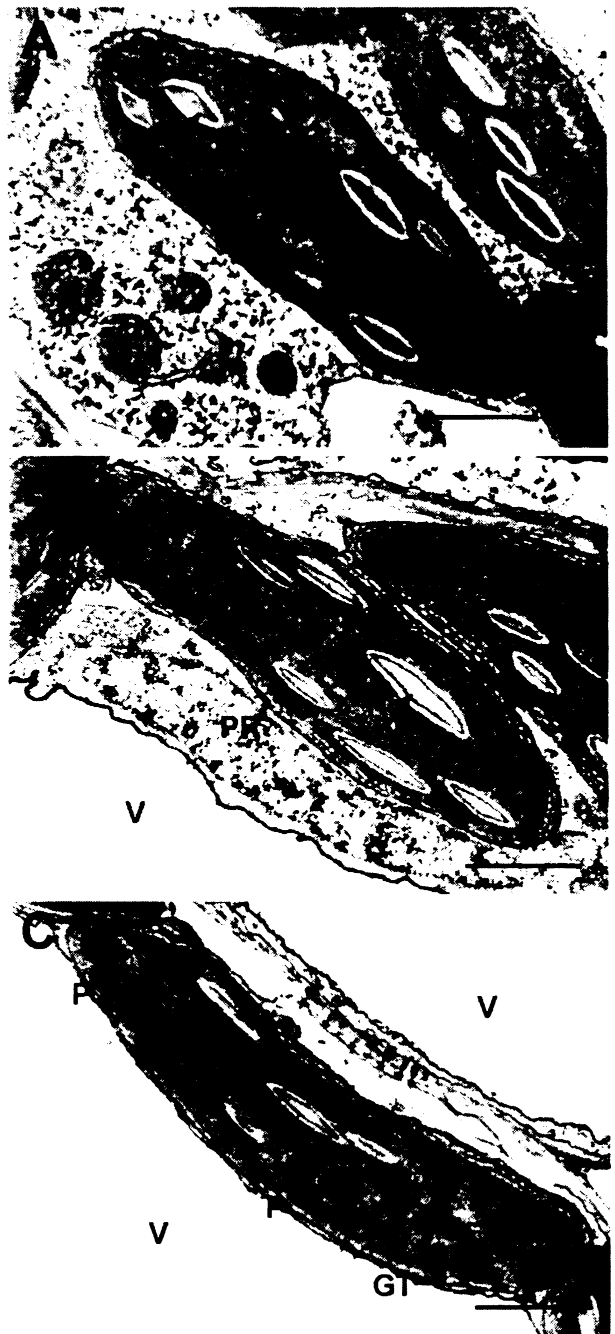


Figure 1. Transmission electron micrographs of chloroplast cross sections taken from the third leaf bundle sheath cells of maize inbred line CE813: (A) young leaf, (B) mature leaf, (C) senescing leaf; the chloroplasts contain many intergranal (nonappressed) thylakoids (IT) and several small grana (GT); peripheral reticulum (PR) is also seen in the chloroplasts; in senescing leaves the starch inclusions (SI) are smallest and plastoglobuli (P) largest; in B and C cell vacuoles (V) are also seen; bar = 1 μm

Table 1. Structural characteristics (volume densities of chloroplast compartments in % – granal thylakoids, intergranal thylakoids, all thylakoids, peripheral reticulum, plastoglobuli, starch inclusions and stroma) and dimensions of bundle sheath cell chloroplasts cross sections – chloroplast cross section area (μm²), chloroplast cross section length (μm), chloroplast cross section width (μm) – ($\bar{x} \pm s_{\bar{x}}$) in young, mature and senescing leaves of maize in inbred lines CE813, CE829 and in mature leaves of maize in hybrids CE813×CE829 and CE829×CE813

	Young leaves		Mature leaves				Senescing leaves	
	CE813	CE829	CE813	CE829	CE813× CE829	CE829× CE813	CE813	CE829
Granal thylakoids (%)	4.36 ± 0.48	4.51 ± 0.53	3.60 ± 0.32	4.58 ± 0.69	3.18 ± 0.32	5.03 ± 0.49	5.32 ± 0.88	7.36 ± 0.80
Intergranal thylakoids (%)	22.09 ± 1.31	20.43 ± 1.25	31.13 ± 1.82	30.47 ± 1.46	21.16 ± 2.60	34.65 ± 2.60	30.24 ± 1.97	30.37 ± 2.03
All thylakoids (%)	26.45 ± 1.44	24.94 ± 1.42	33.73 ± 2.00	35.04 ± 1.81	24.33 ± 1.55	40.12 ± 2.80	35.56 ± 2.38	37.73 ± 2.32
Peripheral reticulum (%)	4.77 ± 0.26	6.71 ± 0.47	6.13 ± 0.86	8.61 ± 0.86	6.12 ± 0.49	8.64 ± 2.36	6.48 ± 0.58	4.72 ± 0.87
Plastoglobuli (%)	0.19 ± 0.06	0.09 ± 0.06	0.79 ± 0.13	0.87 ± 0.21	0.69 ± 0.16	0.97 ± 0.20	2.20 ± 0.22	3.18 ± 0.43
Starch inclusions (%)	5.58 ± 0.71	6.77 ± 0.66	5.01 ± 0.60	6.89 ± 1.00	15.06 ± 1.49	2.25 ± 0.37	1.54 ± 0.29	0.54 ± 0.22
Stroma (%)	63.02 ± 1.42	61.50 ± 1.80	54.35 ± 2.00	49.99 ± 8.10	53.82 ± 2.3	48.04 ± 3.02	54.23 ± 2.29	55.36 ± 2.48
Chloroplast cross section area (μm²)	7.9 ± 0.51	6.49 ± 0.40	7.74 ± 0.45	8.93 ± 0.39	8.93 ± 0.88	6.44 ± 0.39	5.63 ± 0.28	6.81 ± 0.48
Chloroplast cross section length (μm)	5.20 ± 0.22	5.66 ± 0.31	6.08 ± 0.20	7.04 ± 0.40	6.47 ± 0.35	6.49 ± 0.30	5.30 ± 0.17	5.04 ± 0.17
Chloroplast cross section width (μm)	1.99 ± 0.09	1.49 ± 0.06	1.71 ± 0.07	1.74 ± 0.13	1.79 ± 0.10	1.27 ± 0.27	1.33 ± 0.06	1.74 ± 0.10

Dimorphic chloroplasts of maize differentiate gradually from identical plastids in very young leaves (Brangeon 1973a, b). Our results demonstrate also the presence of small thylakoid grana even in BSC chloroplasts of mature and senescing maize leaves. As in MC chloroplasts of maize (Kutík et al. 1999), BSC chloroplast ultrastructure changes substantially during whole leaf development. Steady increase of the volume density of plastoglobuli (containing probably lipoid degradation products of thylakoid membranes, see Kutík 1998) is also connected with leaf ageing. These developmental changes in BSC chloroplasts are analogical to differences found in MC chloroplasts of the same leaves of different age (Kutík et al. 1999), as well as to the differences found in MC chloroplasts of mature maize leaves in various parts of the leaf blade with the oldest cells at the top of the blade and the youngest ones at the base (Kutík et al. 2001). Stereology enables us to evaluate chloroplast ultrastructure in a simple and complex way (compared to, e.g. measuring of appressed and nonappressed thylakoid membranes length or counting grana or number of thylakoids in the grana). The results of our work confirm the dynamic nature of chloroplast dimorphism (BSC chloroplasts versus MC chloroplasts) in the leaves of maize. This dimorphism is developed best in mature leaves, which are the main source of photosynthates.

Table 2. Levels of statistical significance of differences in structural characteristics (granal thylakoids, intergranal thylakoids, all thylakoids, peripheral reticulum, starch inclusions, plastoglobuli and stroma) and dimensions (chloroplast cross section area, chloroplast cross section length and chloroplast cross section width) of BSC chloroplasts from analysis of variance followed by ANOVA test in young, mature and senescing leaves of maize of inbred lines CE813 and CE829

	CE813 (young/ mature/ senescing)	CE829 (young/ mature/ senescing)	Young (CE813/ CE829)	Mature (CE813/ CE829)	Senescing (CE813/ CE829)
Granal thylakoids	0.143	0.006	0.835	0.029	0.094
Integral thylakoids	0	0	0.365	0	0.964
All thylakoids	0.005	0	0.46	0	0.518
Peripheral reticulum	0.127	0.003	0.001	0.009	0.101
Plastoglobuli	0	0	0.246	0.72	0.05
Starch inclusions	0	0	0.227	0	0.009
Stroma	0.002	0.001	0.511	0.173	0.74
Chloroplast cross section area	0	0	0.036	0.007	0.04
Chloroplast cross section length	0.005	0	0.234	0.219	0.286
Chloroplast cross section width	0	0.138	0	0.094	0.001

Acknowledgements

Electron microscopic studies described were performed at the laboratory of the State Institute of Health in Prague, headed by Dr. D. Hulínská. The possibility to work there and kind advice given by Ing. M. Doubek are gratefully acknowledged.

REFERENCES

Brangeon J. (1973a): Compared ontogeny of the two types of chloroplasts of *Zea mays*. Journal of Microscopy, *16*: 233–242.

Brangeon J. (1973b): Effect of irradiance on granal configurations of *Zea mays* bundle sheath chloroplasts. Photosynthetica, *7*: 365–372.

Furumoto T., Hata S., Izui K. (2000): Isolation and characterization of cDNAs for differentially accumulated transcripts between mesophyll cells and bundle sheath strands of maize leaves. Plant and Cell Physiology, *41*: 1200–1209.

Gundersen H.J.G., Jensen E.B. (1987): The efficiency of systematic sampling in stereology and its prediction. Journal of Microscopy, *147*: 229–263.

Hudák J. (1997): Photosynthetic apparatus. In: Pessarakli M. (ed.): Handbook of photosynthesis. Marcel Dekker, New York, Basel, Hong Kong: 27–48.

Kutík J. (1998): The development of chloroplast structure during leaf ontogeny. Photosynthetica, *35*: 481–505.

Kutík J., Holá D., Vičánková A., Šmídová M., Kočová M., Körnerová M., Kubínová L. (2001): The heterogeneity of structural and functional photosynthetic characteristics of mesophyll chloroplasts in various parts of mature or senescing leaf blade of two maize (*Zea mays* L.) genotypes. Photosynthetica, *39*: 497–506.

Kutík J., Kočová M., Holá D., Körnerová M. (1999): The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes. Photosynthetica, *36*: 497–507.

Lawlor D.W. (2001): Photosynthesis. 3rd edition. BIOS Scientific Publishers Limited, Oxford.

Nishioka D., Brisibe E.A., Miyake H., Taniguchi T. (1993): Ultrastructural observations in the suppression of granal development in bundle sheath chloroplasts of NaDP-ME type C_4 monocot and dicot species. Japanese Journal of Crop Science, *62*: 621–627.

Reynolds E.S. (1963): The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of Cell Biology, *17*: 208–212.

Ryberg H., Ryberg M., Sundquist C. (1993): Plastid ultrastructure and development. In: Sundquist C., Ryberg M. (eds.): Pigment – Protein complexes in plastids: Synthesis and assembly. Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: 25–62.

Virgin H.I., Egnéus H.S. (1983): Control of plastid development in higher plants. In: Shropshire W., Mohr H. (eds.): Photomorphogenesis. Springer Verlag, Berlin, Heidelberg, New York, Tokyo: 289–311.

Recieved on June 7, 2004

ABSTRAKT

Vývoj ultrastruktury chloroplastů v buňkách pochev cévních svazků dvou různých genotypů kukuřice (*Zea mays* L.)

Listy kukuřice tvoří dvě fotosyntetická pletiva s dvěma typy chloroplastů, mezofylové buňky a buňky pochev cévních svazků. Vývoj chloroplastů v buňkách pochev cévních svazků byl sledován pomocí transmisní elektronové mikroskopie a bodové stereologické metody ve střední části čepele třetího listu rostlin kukuřice. Objemová hustota tylakoidů (fotosyntetického membránového systému) stoupla v průběhu dospívání listů, v průběhu stárnutí listů se průkazně nezměnila. Během celé listové ontogeneze jsme sledovali přítomnost gran (na sebe přitisklých tylakoidů) v chloroplastech v buňkách pochev cévních svazků, považovaných běžně za agranální. Objemová hustota škrobových inkluzí v chloroplastech výrazně klesla a objemová hustota plastoglobulů výrazně stoupla v průběhu senescence listů.

Klíčová slova: chloroplast; ultrastruktura; C_4 fotosyntéza; elektronová mikroskopie; *Zea mays* L.

Corresponding author:

Mgr. Anna Vičánková, Ústav experimentální botaniky, Akademie věd České republiky, Rozvojová 135, 165 00 Praha 6, Česká republika
phone: + 420 220 390 402, fax: + 420 220 390 419, e-mail: vicankova@ueb.cas.cz

---

Maize F_1 hybrid differs from its maternal parent in the development of bundle sheath, but not mesophyll, cells´ chloroplasts: the quantitative analysis of chloroplast ultrastructure and dimensions in different parts of leaf blade at the beginning of its senescence

A. VIČÁNKOVÁ*, D. HOLÁ**,†, J. KUTÍK*

Charles University in Prague, Faculty of Science, Department of Plant Physiology and Department of Genetics and Microbiology**, Viničná 5, CZ-128 43 Praha 2, Czech Republic*

Abstract

The quantitative changes of chloroplast ultrastructure and dimensions in mesophyll (MC) and bundle sheath (BSC) cells, associated with the onset of leaf senescence, were followed along the developmental leaf blade gradient of the third leaf of maize (*Zea mays* L.). To ascertain whether the rapidity of structural changes associated with the transition of chloroplasts from mature to senescent state is a heritable trait, the parental and the first filial generations of plants were used. The heterogeneity of leaf blade, associated with the development of maize leaf (with the oldest regions at the apex and the youngest ones at the base) was clearly discernible in the ultrastructure and dimensions of chloroplasts; however, there were differences in the actual pattern of chloroplast development between both genotypes as well as between both cell types examined. While the course of MC chloroplasts´ development at the onset of leaf senescence in maize hybrid followed that of its parent rather well, this did not apply for the BSC chloroplasts. In this case, each genotype was characterized by its own distinguishable developmental pattern, particularly as regards the accumulation of starch inclusions and the associated changes of BSC chloroplasts´ size and shape.

Additional key words: chloroplast development; genetic variability; electron microscopy; granal thylakoids; leaf blade heterogeneity; peripheral reticulum; plastoglobuli; starch inclusions; *Zea mays* L.

Introduction

A characteristic feature of plants with NADP-ME type of C_4 photosynthesis is a spatial compartmentation of various processes linked to the photosynthetic carbon metabolism: the initial fixation of atmospheric CO_2 by PEP carboxylase, its conversion into C_4 organic acids, its subsequent release from these acids to provide higher CO_2 concentrations for RuBisCO (in order to increase the carboxylation efficiency of this enzyme at the expense of its oxygenation activity), and the regeneration of PEP as the primary acceptor of CO_2. These processes take place in two different photosynthetic cell

---

Received: 27 March2006, a*ccepted:*

† Author for correspondence; *e-mail: danahola@natur.cuni.cz*

Abbreviations: BSC – bundle sheath cell; F_1 – the first filial generation; L/W – the ratio of chloroplast´s cross-section length to width; MC – mesophyll cell; ME – malic enzyme; PEP – phosphoenolpyruvate; PS2 – Photosystem 2; RuBisCO – ribulose-1,5-bisphosphate carboxylase/oxygenase

Acknowledgements: The results are part of the research supported by grant No. 103/1998 of the Grant Agency of the Charles University. The authors are grateful to Dr. J. Poruba of the Maize Breeding Station CEZEA, Čejč, for supply of maize caryopses, to Dr. D. Hulínská and Ing. M. Doubek of the State Institute of Health, Prague, for the facilitation of transmission electron microscopy studies, to Dr. J. Opatrná and Dr. J. Petrášek of the Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, for their assistance with the image analysis, and to Dr. I. Macháčková of the same institute for her personal help and encouragement during whole work.

types, *i.e.* the bundle sheath cells (BSCs) surrounding the vascular bundles, and the mesophyll cells (MCs), which in turn surround the BSC ones. The arrangement of these cells in leaves of NADP-ME plants follows the pattern reffered to as the Kranz anatomy (Kranz is the German word for "wreath"), where the two cell layers give the appearance of wreath surrounding each vein (Furbank and Foyer 1988, Nelson and Langdale 1989, Nelson and Dengler 1992, Edwards *et al.* 2001, Leegood 2002, Brown *et al.* 2005). The BSCs and MCs differ in their structural properties; the most conspicuous feature of BSCs being their larger size compared to MCs, their thick cell walls and the centrifugal or centripetal arrangement of chloroplasts (Nelson and Langdale 1989, Dengler *et al.* 1994, Leegood 2002, Brown *et al.* 2005).

The BSCs and the MCs show various differences in the ultrastructure of chloroplasts, related to their different role in photosynthetic carbon fixation processes. The high demand for NADPH and ATP in MCs (where it is needed for the conversion of oxaloacetate to malate and of pyruvate to PEP, as well as for other non-photosynthetic processes demanding high supply of reducing equivalents and energy, *e.g.* nitrogen assimilation, biosynthesis of lipids) necessitates the preference for a linear photosynthetic electron transport in thylakoid membranes of these cells, and, thus, for a high amount and photochemical activity of PS2, associated with high granality of MC chloroplasts´ thylakoids (Chow *et al.* 2005, Majeran *et al.* 2005). On the other hand, the biosynthesis and the accumulation of starch takes place preferentially (but not exclusively) in the BSCs, which are often quite filled up with starch inclusions (Spilatro and Preiss 1987, Nelson and Langdale 1989, Lunn and Hatch 1995, Majeran *et al.* 2005).

The distinctive role of BSC and MC chloroplasts in photosynthetic processes of NADP-ME type C_4 plants is further reflected in the cell-specific expression of genes coding for various chloroplast proteins (Sheen and Bogorad 1987, 1988, Kubicki *et al.* 1994, Furumoto *et al.* 2000, Edwards *et al.* 2001, Hahnen *et al.* 2003, Majeran *et al.* 2005). The regulation of this cell-specific expression occurs at both transcriptional and post-transcriptional levels and is closely linked to the existence of Kranz anatomy. It seems that the default pattern of MC photosynthesis even in C_4 plants is a C_3 mechanism and that the switch of MCs to C_4 photosynthesis depends on their close proximity to BSCs and on the availability of light (Nelson and Langdale 1989, Nelson and Dengler 1992, Cousins *et al.* 2003, Hahnen *et al.* 2003, Majeran *et al.* 2005). The C_4 specialization of BSCs is similarly light-dependent, requires their adjacent position to veins and is influenced also by their procambial / meristematic origin (Nelson and Dengler 1992, Jankovski *et al.* 2001, Majeran *et al.* 2005).

The whole process of the differentiation of BSC and MC chloroplasts during the development of photosynthetic tissue in C_4 plants has been rather extensively studied using various methods of plant anatomy, biochemistry and molecular biology (Nelson and Langdale 1989, Nelson and Dengler 1992, Langdale and Kidner 1994, Edwards *et al.* 2001, Majeran *et al.* 2005). Many of these studies were made on maize (*Zea mays* L.) and took the advantage of the unique characteristics of this grass species. Leaves of plants belonging to the grass family have been for a long time ideal model objects for the study of chloroplast development, as they show a distinctive, well-defined developmental gradient from base to tip during their growth. This gradient can be observed for the content of photosynthetic pigments, the activity of photosynthetic and other metabolic processes, the amount of mRNA, proteins and lipids, the content and organisation of chloroplast DNA *etc.* (*e.g.* Leech *et al.* 1973, Kirchanski 1975, Perchorowicz and Gibbs 1980, Miranda *et al.* 1981 a, b, Martineau and Taylor 1985, Langdale *et al.* 1987, 1988, Nelson and Langdale 1989, Nelson and Dengler 1992,

Nishioka *et al.* 1993, Williams *et al.* 1993, Šesták and Šiffel 1997, Oldenburg and Bendich 2004), as well as for chloroplast ultrastructure and dimensions (Kirchanski 1975, Rascio *et al.* 1980, Wellburn *et al.* 1982, Kutík 1985, 1992, 1998, Nishioka *et al.* 1993).

The developmental pattern of chloroplasts along the grass leaf blade is partly discernible in mature, fully developed leaves as well (Kutík *et al.* 2001, Kołodziejek *et al.* 2003) and becomes again more pronounced with the onset of leaf senescence (Martinoia *et al.* 1983, Biswal and Biswal 1988, Chonan *et al.* 1991, Kutík 1985, 1998, Matile 1992, Sakai *et al.* 1999, Kutík *et al.* 2001, Biswal *et al.* 2003, Kołodziejek *et al.* 2003). However, not only there can be differences in this respect among various grass species (Kołodziejek *et al.* 2003), but such variability can be observed also within individual species (different genotypes can display different rates of chloroplast development) (Kutík and Kočová 1996, Kutík *et al.* 1999, 2001, Vičánková and Kutík 2005). Whether the rapidity of structural changes associated with the transition of chloroplasts from mature to senescent state is a heritable trait, is not known. Thus, we had decided to make a quantitative study of the ultrastructural heterogeneity of chloroplasts in various parts of leaf at the beginning of its senescence in two generations of maize (parental and F_1). Our main purpose was to ascertain whether the developmental pattern showed by the filial generation follows that of its maternal parent, and whether there are any differences in this respect between MC and BSC chloroplasts.

Material and Methods

The ultrastructure and dimensions of chloroplasts were evaluated in mesophyll and bundle sheath cells of three different parts of the third leaf of maize. Plants of two maize genotypes (the inbred line 2023 and its F_1 hybrid 2023×CE810; 2023 being the maternal parent of this hybrid) were grown in planting dishes filled with garden soil and placed in the growth chamber (*Klimabox RK1-007, Kovodružstvo Slaný*, Czech Republic) under conditions of 16/8 h day/night period, 25/16 °C temperature, 70/80 % relative air humidity and 230-470 μmol m^{-2} s^{-1} photon flux density. They were allowed to grow under these conditions till the maturity of the third leaf was reached. Small pieces of leaf blade for the evaluation of chloroplast ultrastructural and dimensional parameters were taken from three parts of leaf blade – the basal one (at approx. one quarter of the entire leaf blade length, measured from the base), the middle one, and the apical one (at approx. three quarters of the entire leaf blade length) – 24 d after the date of sowing. In relation to leaf width, the position of the samples was always midway between the central rib and the leaf margin. The collection of samples was repeated again four days later, *i.e.* on 28-d old plants, when the tip of the third leaf begun to display senescence symptoms.

The leaf blade samples were first fixed with glutaraldehyde followed by osmic acid treatment, dehydrated through ethanol series, and embedded into Spurr´s low viscosity resin (see Kutík *et al.* 1999). Transverse ultrathin sections were then prepared from the embedded objects that were first contrasted with a saturated uranyl acetate solution followed by the lead citrate solution (Reynolds 1963). The microphotographs were taken using the transmission electron microscope *Phillips EM 300* (the Netherlands) (primary magnifications 7,000×, final magnifications 30,000×), scanned and imported into Lucia image analysis system for the quantification of selected ultrastructural and dimensional parameters of MC and BSC chloroplasts. The volume densities of granal thylakoids, plastoglobuli, peripheral reticulum and starch inclusions

were determined, together with the total area, the length (L) and the width (W) of chloroplast cross sections. The shape of the chloroplasts was inferred from the L/W ratio.

The ultrastructural and dimensional parameters of chloroplasts were evaluated in four plants of each genotype for each sampling date, each plant being represented by five randomly selected chloroplasts from the respective leaf part and cell type. Data from both sampling dates were pooled together for the purposes of statistical analysis. The differences between both genotypes, between individual leaf parts, and between MC and BSC chloroplasts were determined by the analysis of variance accompanied by Tukey-Kramer´s tests; all 20 values of each parameter representing each respective sample were used as the source data for this analysis. The mutual relationships between the parameters examined were investigated by correlation analysis (Pearson´s correlation coefficient). Each sample (representing the respective cell type / leaf part / plant age / genotype) was represented here by four values (averaged from five chloroplasts for each individual plant) of each ultrastructural or dimensional parameter. Similar analysis was used to ascertain whether there exists any parallelism in the development of MC or BSC chloroplast ultrastructure in the inbred line and its hybrid; the initial data for this analysis were the means of each parameter, calculated from all 20 values evaluated for the respective cell type / leaf part / plant age / genotype. All statistical evaluation was made with the *CoStat (Version 6.204)* programme (CoHort Software, Monterey, CA, the U.S.A.).

Results

The representative samples of chloroplast cross sections from mesophyll and bundle sheath cells of the basal, middle and apical parts of leaf blade are shown in Fig. 1. The average area of these cross sections in the youngest leaf part examined (*i.e.* the basal part of leaf of 24-d old plants) was rather similar in both cell types, but the hybrid was at this stage characterized by larger BSC chloroplasts compared to the inbred line, and the area and the width of BSC chloroplasts´ cross-sections in this genotype were significantly higher compared to the MC chloroplasts´ ones. In almost every other case the size of BSC chloroplasts highly and significantly exceeded that of the MC ones (Table 1).

The average area of MC chloroplasts´ cross-sections slightly increased in both genotypes with the increasing distance from the leaf base and with the advancement of leaf age, and the genotypes did not differ much in this parameter. This increase in the total area of chloroplast cross sections was related more to the increase of chloroplast width (or, more precisely, height) than chloroplast length. However, there was a significant drop in the size of MC chloroplasts´ cross sections in the apical part of leaf of 28-d old plants (this leaf part already displayed the external symptoms of senescence, *i.e.* yellow color, dry tissue at the extreme leaf tip), that was caused by the diminution of the length of chloroplasts´ cross-sections. Thus, the shape of MC chloroplasts gradually changed from rather flat one observed in the youngest tissue (the basal part of leaf of 24-d old plants) to the more rounded one in the oldest tissue (the apical part of leaf of 28-d old plants) (Fig. 1). The relationship between the inbred line and the hybrid as regards the values of the L/W ratio calculated for MC chloroplasts also changed with the increasing age of leaf blade tissue, with the hybrid genotype exceeding the inbred one in younger leaf parts, and the reverse situation in more aged leaf parts (Table 1).

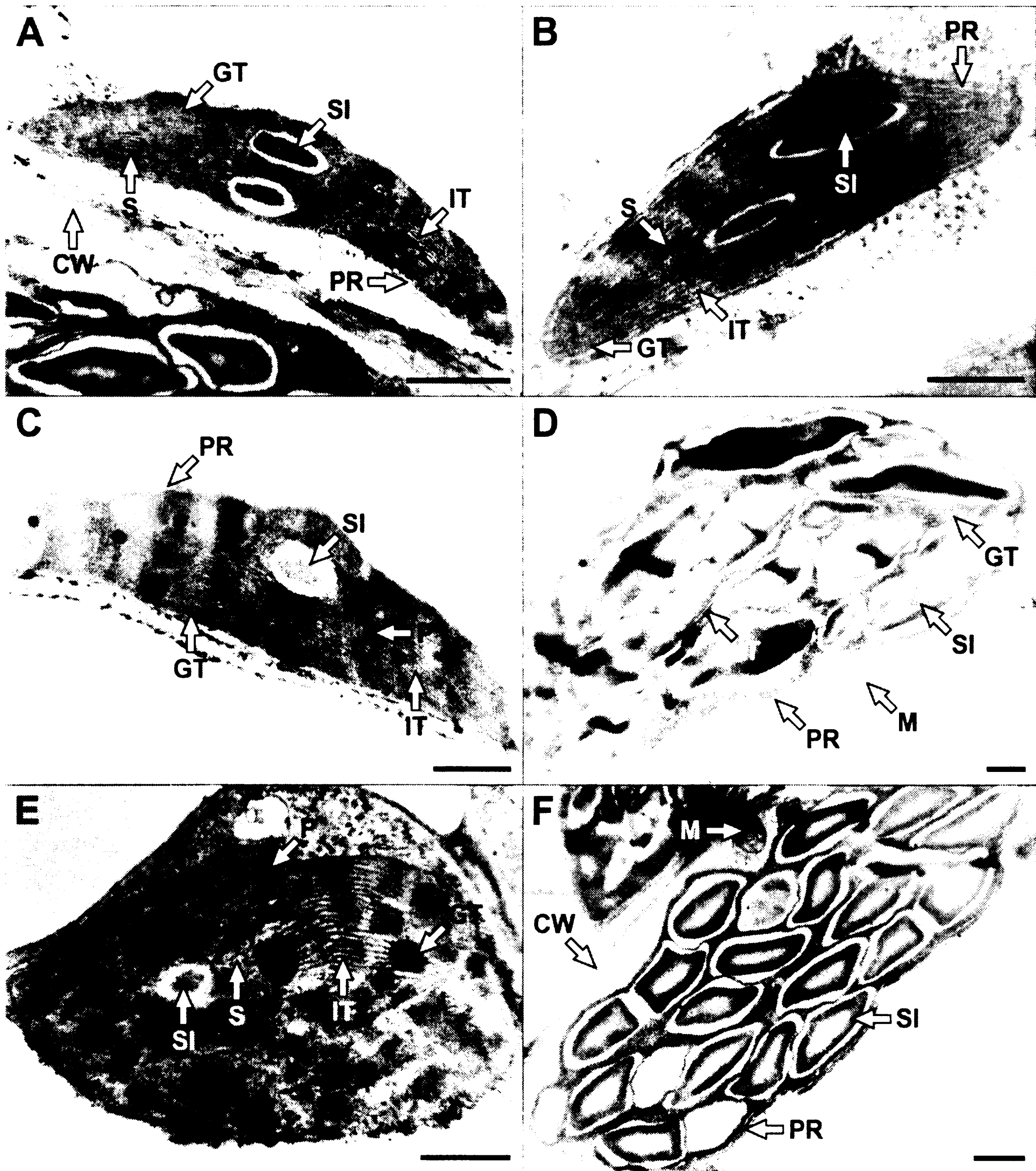


Fig. 1. Transmission electron micrographs of chloroplast cross sections taken from mesophyll (MC) and bundle sheath (BSC) cells in three parts of the third leaf of maize F_1 hybrid 2023×CE810. The cross-sections of MC (A, C, E) and BSC (B, D, F) chloroplasts in the basal (A, B), middle (C, D) and apical (E, F) part of leaf are shown. CW – cell wall, GT – granal thylakoids, IT – intergranal thylakoids, M – mitochondrion, P – plastoglobulus, PR – peripheral reticulum, S – stroma, SI – starch inclusion. Bar – 1 μm.

The BSC chloroplasts of the inbred line were always less rounded than those of the hybrid and these differences between genotypes in the L/W ratio were usually statistically significant. The increasing trend, observed for the average area of chloroplast cross sections in mesophyll cells, was partly discernible in the bundle sheath cells as well, but there were two great deviations: the middle and the apical parts of leaf of 24-d old hybrid plants were characterized by extremely large chloroplasts (this applied both for the length and the width of the chloroplast cross sections). No drop in the BSC chloroplasts´ dimensions was observed for the already senescing leaf apex of 28-d old plants (Table 1).

As expected, the thylakoid membranes constituted the largest proportion of MC chloroplasts´ inner volume, and the volume density of thylakoid grana in this type of chloroplasts was approximately 40 %. This applied for all three leaf parts and both plant ages, as well as for both genotypes examined, the only exception being the apical part of leaf of 28-d old plants. The relative partial volume of granal thylakoids in this part of leaf significantly decreased both in the inbred line and in the hybrid; however, the decrease observed in the hybrid was much more pronounced compared to the inbred line. The BSC chloroplasts also contained some grana, but their amount was very low compared to the MC chloroplasts. The basal part of leaf of 24-d old plants, as well as the leaf apex of 28-d old plants, was characterized by comparatively high relative partial volume of granal thylakoids in both genotypes examined. Otherwise, no specific trend was observed for this chloroplast compartment in the BSC chloroplasts. The inbred line generally showed higher grana volume density compared to the hybrid; in some cases, the differences between genotypes were statistically significant (Table 2).

The BSC chloroplasts of the hybrid were rather filled up with starch and the relative partial volume of starch inclusions varied between 25 and 40 % (with the exception of the middle part of leaf of 24-d old plants, where it was even higher). The values of the volume density of starch inclusions in the inbred line were similar, but this applied only for 28-d old plants; all leaf parts examined in the younger plants showed significantly lower amount of starch (between 5 and 10 %) and the inbred thus significantly differed from the hybrid in these cases. The amount of starch in MC chloroplasts was extremely small; however, some starch inclusions were usually found in all leaf parts examined (more suspiciously in the hybrid) and there were no statistically significant differences among various leaf parts regardless of plant age (Fig. 1, Table 2).

The number and size of plastoglobuli increased with the developmental stage of chloroplasts, both in MCs and BSCs. Although this increase was rather gradual in MC chloroplasts of both genotypes, the individual leaf parts did not significantly differ in this parameter, with the exception of the leaf apex of 28-d old plants, that was characterized by the highest relative partial volume of plastoglobuli. The same applied for the BSC chloroplasts of the hybrid. As regards the inbred line, we found several statistically significant differences between various parts of leaf blade in the volume density of plastoglobuli, and the inbred line also showed significantly higher values of this parameter compared to the hybrid. In all cases, the MC chloroplasts contained significantly more plastoglobuli than the BSC ones (Table 2).

*Table 1. Dimensions of the mesophyll cells´ (MC) and bundle sheath cells´ (BSC) chloroplasts in basal, middle and apical parts of the third leaf of two maize genotypes (I … inbred line 2023, H … its F_1 hybrid 2023×CE810). Means ± standard errors of mean (SEM) are shown, together with the statistical significance of the differences between genotypes (** … significant with $p \leq 0.01$, * … significant with $p \leq 0.05$, ns … non-significant), between individual leaf parts (the letters ABCD denoting the differences for the particular row of the table; only those parts marked with different letters significantly differ at $p \leq 0.05$), and between MC and BSC chloroplasts (** … significant with $p \leq 0.01$, * … significant with $p \leq 0.05$, ns … non-significant).*

Parameter / Genotype		Base				Middle				Apex			
		24-d old plants		28-d old plants		24-d old plants		28-d old plants		24-d old plants		28-d old plants	
		MC chloroplasts											
Area (µm²)	I	ns	9.01 ± 0.55^{BCD}	ns	8.56 ± 1.26^{CD}	ns	12.68 ± 0.99^{AB}	ns	11.34 ± 1.21^{ABC}	ns	14.40 ± 0.78^{A}	*	7.33 ± 0.74^{D}
	H		8.27 ± 0.77^{CD}		10.07 ± 1.19^{BC}		13.05 ± 1.05^{AB}		10.26 ± 1.30^{BC}		16.96 ± 1.13^{A}		5.11 ± 0.41^{D}
Length (µm)	I	ns	6.72 ± 0.27^{AB}	ns	5.51 ± 0.40^{BC}	ns	6.68 ± 0.33^{AB}	ns	6.17 ± 0.39^{B}	ns	8.08 ± 0.43^{A}	*	4.09 ± 0.24^{C}
	H		7.13 ± 0.40^{AB}		6.05 ± 0.51^{AB}		7.28 ± 0.30^{A}		5.52 ± 0.52^{B}		7.53 ± 0.51^{A}		3.17 ± 0.25^{C}
Width (µm)	I	*	1.57 ± 0.07^{B}	ns	1.86 ± 0.15^{AB}	ns	2.34 ± 0.12^{A}	ns	2.07 ± 0.21^{AB}	ns	2.24 ± 0.12^{A}	ns	2.00 ± 0.11^{AB}
	H		1.35 ± 0.07^{B}		1.91 ± 0.18^{AB}		2.20 ± 0.12^{A}		2.07 ± 0.21^{A}		2.50 ± 0.12^{A}		2.00 ± 0.11^{A}
Length/width ratio	I	**	4.35 ± 0.18^{A}	ns	3.23 ± 0.30^{A}	ns	2.97 ± 0.18^{A}	ns	4.98 ± 1.86^{A}	ns	3.66 ± 0.18^{A}	*	2.12 ± 0.15^{A}
	H		5.38 ± 0.28^{A}		3.39 ± 0.30^{B}		3.41 ± 0.18^{B}		2.70 ± 0.21^{B}		3.12 ± 0.24^{B}		1.64 ± 0.14^{C}
		BSC chloroplasts											
Area (µm²)	I	**	8.32 ± 0.73^{D}	ns	18.60 ± 2.12^{BC}	**	16.90 ± 0.70^{BCD}	*	25.79 ± 3.07^{AB}	**	13.67 ± 0.74^{CD}	ns	32.62 ± 4.27^{A}
	H		14.28 ± 1.44^{C}		19.40 ± 1.74^{C}		87.89 ± 4.59^{A}		17.88 ± 1.02^{C}		61.71 ± 9.01^{B}		24.24 ± 6.41^{C}
Length (µm)	I	ns	6.78 ± 0.40^{B}	ns	8.54 ± 0.66^{AB}	**	8.63 ± 0.28^{AB}	**	10.93 ± 1.19^{A}	**	8.80 ± 0.40^{AB}	*	11.29 ± 1.43^{A}
	H		7.79 ± 0.47^{B}		8.15 ± 0.33^{B}		14.13 ± 0.48^{A}		7.29 ± 0.29^{B}		12.05 ± 0.75^{A}		7.24 ± 1.18^{B}
Width (µm)	I	**	1.50 ± 0.07^{D}	ns	2.62 ± 0.16^{ABC}	**	2.57 ± 0.14^{BC}	ns	3.48 ± 0.37^{AB}	**	1.86 ± 0.07^{CD}	ns	3.56 ± 0.38^{A}
	H		2.41 ± 0.17^{B}		2.97 ± 0.16^{B}		6.72 ± 0.28^{A}		3.24 ± 0.20^{B}		6.02 ± 0.72^{A}		3.08 ± 0.49^{B}
Length/width ratio	I	**	4.56 ± 0.22^{A}	ns	3.37 ± 0.26^{A}	**	3.57 ± 0.24^{A}	ns	3.81 ± 0.77^{A}	*	4.79 ± 0.22^{A}	*	3.38 ± 0.39^{A}
	H		3.41 ± 0.23^{A}		2.83 ± 0.14^{AB}		2.14 ± 0.08^{B}		2.40 ± 0.17^{AB}		2.84 ± 0.86^{AB}		2.42 ± 0.16^{AB}
		Statistical significance of the differences between MC and BSC chloroplasts											
Area	I	ns		**		**		**		ns		**	
	H	**		**		**		**		**		**	
Length	I	ns		**		**		**		ns		**	
	H	ns		**		**		**		**		**	
Width	I	ns		**		ns		**		**		**	
	H	**		**		**		**		**		*	
Length/width ratio	I	ns		ns		ns		ns		**		**	
	H	**		ns		**		ns		ns		**	

*Table 2. Ultrastructural parameters of the mesophyll cells´ (MC) and bundle sheath cells´ (BSC) chloroplasts in basal, middle and apical parts of the third leaf blade of two maize genotypes (I … inbred line 2023, H … its F_1 hybrid 2023×CE810). Means ± standard errors of mean (SEM) of volume densities of the respective chloroplast compartments are shown, together with the statistical significance of the differences between genotypes (** … significant with $p \leq 0.01$, * … significant with $p \leq 0.05$, ns … non-significant, nd … non-determined), between individual leaf parts (the letters ABCD denoting the differences for the particular row of the table; only those parts marked with different letters significantly differ at $p \leq 0.05$), and between MC and BSC chloroplasts (** … significant with $p \leq 0.01$, * … significant with $p \leq 0.05$, ns … non-significant)*

Parameter / Genotype		Base				Middle				Apex			
		24-d old plants		28-d old plants		24-d old plants		28-d old plants		24-d old plants		28-d old plants	
		MC chloroplasts											
Granal thylakoids (%)	I	ns	41.98±2.56 ^{A}	ns	34.91±3.01 ^{AB}	ns	39.57±2.44 ^{A}	ns	41.44±2.03 ^{A}	ns	37.63±1.76 ^{A}	**	27.60±2.52 ^{B}
	H		38.37±2.65 ^{A}		41.83±2.84 ^{A}		42.45±2.12 ^{A}		41.71±1.92 ^{A}		40.63±1.63 ^{A}		16.96±2.27 ^{B}
Plastoglobuli (%)	I	ns	0.15±0.05 ^{B}	ns	2.30±0.48 ^{B}	ns	0.45±0.08 ^{B}	ns	3.00±0.42 ^{B}	*	4.56±1.28 ^{B}	**	11.28±2.28 ^{A}
	H		0.21±0.06 ^{B}		1.34±0.31 ^{B}		0.64±0.15 ^{B}		2.75±0.45 ^{B}		1.72±0.23 ^{B}		19.52±1.75 ^{A}
Peripheral reticulum (%)	I	*	3.23±0.32 ^{A}	ns	2.04±0.64 ^{A}	ns	1.77±0.21 ^{A}	**	3.47±0.73 ^{A}	ns	2.41±0.68 ^{A}	*	2.95±0.46 ^{A}
	H		2.05±0.30 ^{A}		1.66±0.35 ^{A}		1.81±0.20 ^{A}		1.31±0.24 ^{A}		1.45±0.14 ^{A}		1.75±0.24 ^{A}
Starch inclusions (%)	I	*	0±0.00 ^{A}	ns	0.24±0.23 ^{A}	*	0.04±0.03 ^{A}	ns	1.86±1.02 ^{A}	ns	0.83±0.23 ^{A}	ns	1.28±0.50 ^{A}
	H		0.64±0.29 ^{A}		0.87±0.31 ^{A}		1.65±0.76 ^{A}		3.57±1.58 ^{A}		0.99±0.29 ^{A}		0.99±0.35 ^{A}
		BSC chloroplasts											
Granal thylakoids (%)	I	**	9.66±1.10 ^{A}	ns	2.74±0.58 ^{D}	**	8.38±0.92 ^{AB}	ns	3.18±0.42 ^{CD}	**	4.84±0.53 ^{BCD}	ns	7.77±2.31 ^{ABC}
	H		4.84±0.40 ^{AB}		1.84±0.19 ^{B}		0.89±0.19 ^{B}		3.13±0.41 ^{AB}		1.59±0.27 ^{B}		6.99±2.81 ^{A}
Plastoglobuli (%)	I	nd	0±0.00 ^{D}	*	0.50±0.24 ^{BCD}	**	0.18±0.06 ^{CD}	*	1.00±0.23 ^{B}	**	0.74±0.14 ^{BC}	ns	1.78±0.23 ^{A}
	H		0±0.00 ^{B}		0.01±0.01 ^{B}		0±0.00 ^{B}		0.32±0.18 ^{B}		0.07±0.02 ^{B}		1.57±0.32 ^{A}
Peripheral reticulum (%)	I	**	3.51±0.23 ^{A}	ns	1.42±0.26 ^{BC}	ns	1.02±0.24 ^{C}	ns	1.02±0.25 ^{C}	**	2.30±0.18 ^{B}	ns	1.60±0.35 ^{BC}
	H		2.00±0.21 ^{A}		2.46±1.41 ^{A}		0.65±0.13 ^{A}		1.11±0.23 ^{A}		0.78±0.14 ^{A}		1.81±0.49 ^{A}
Starch inclusions (%)	I	**	5.85±1.59 ^{B}	ns	31.08±3.07 ^{A}	**	8.82±1.69 ^{B}	ns	34.20±2.97 ^{A}	**	5.98±0.77 ^{B}	ns	32.96±3.52 ^{A}
	H		26.97±2.71 ^{BC}		33.56±2.45 ^{BC}		57.40±1.96 ^{A}		32.35±1.73 ^{BC}		38.67±1.95 ^{B}		24.80±4.66 ^{C}
		Statistical significance of the differences between MC and BSC chloroplasts											
Granal thylakoids	I	**		**		**		**		**		**	
	H	**		**		**		**		**		**	
Plastoglobuli	I	**		**		*		**		**		**	
	H	**		**		**		**		**		**	
Peripheral reticulum	I	ns		ns		*		**		ns		*	
	H	ns		ns		**		ns		ns		*	
Starch inclusions	I	**		**		**		**		**		**	
	H	**		**		**		**		**		**	

The percentage of MC chloroplast cross sections constituted by the peripheral reticulum varied between 1 and 2 in the hybrid, and between 1.8 and 3.5 in the inbred line. Similar values of this parameter were observed in the BSC chloroplasts, but the variability between individual leaf parts was somewhat higher. When there were some statistically significant differences between both types of chloroplasts, the volume density od peripheral reticulum in the MC ones invariably exceeded that of the BSC ones. However, with the exception of BSC chloroplasts of the inbred line, we did not find any statistically significant differences among leaf parts and there was also no specific trend as regards the differences between genotypes (Table 2).

The relationship between various ultrastructural and dimensional parameters of chloroplasts in mesophyll or bundle sheath cells, as well as between both cell types, was examined using the correlation analysis. The relative partial volume of granal thylakoids in MC chloroplasts significantly and positively correlated with the area of chloroplast cross sections both in the inbred and in the hybrid. On the other hand, the volume density of plastoglobuli significantly decreased with increasing length of MC chloroplasts´ cross-sections, and the correlation between the volume densities of granal thylakoids and plastoglobuli was negative as well (again in both genotypes). The hybrid also showed significant negative relationship between the volume density of plastoglobuli and the area of chloroplasts´ cross-sections or the L/W ratio; this type of correlation was not found for the inbred line. All other possible relationships between MC chloroplasts´ parameters were statistically non-significant (as regards the Pearson´s correlation coefficient) (Table 3).

Other types of ultrastructural relationships were found for the BSC chloroplasts. The relative partial volume of the starch inclusions significantly and negatively correlated with the volume densities of granal thylakoids. The amount of starch was positively related to the chloroplast size, while the reverse was true for the volume density of peripheral reticulum or granal thylakoids and the BSC chloroplasts´ width. The width of the BSC chloroplasts´ cross-sections significantly increased with their length. All these relationships were found in both genotypes, but there were also several other statistically significant correlations observed either only in the inbred line or in its hybrid. The positive correlation between the plastoglobuli volume density and the dimensional parameters of BSC chloroplasts was found in the inbred line only, whereas in the hybrid this relationship was either negative or statistically non-significant. The inbred L/W ratio was related to the relative partial volume of starch inclusions (negatively) and peripheral reticulum (positively). On the other hand, the statistically significant correlations observed only in the hybrid included the inverse relationship between the length (or the area) of BSC chloroplasts´ cross-sections and the volume density of peripheral reticulum or granal thylakoids, and between volume densities of plastoglobuli and starch inclusions (Table 3).

We found also some mutual interdependence of the ultrastructural (or dimensional) parameters of chloroplasts in MCs and BSCs. In this case, the differences between both genotypes were even more marked. The relative partial volume of granal thylakoids in MC chloroplasts was negatively correlated to the relative partial volume of plastoglobuli in BSC chloroplasts in both the inbred line and its hybrid, the interrelationship between the plastoglobuli volume densities in both types of chloroplasts was a positive one, and the relative partial volume of plastoglobuli in BSC chloroplasts was negatively related to the length of MC chloroplasts´ cross-sections. Other statistically significant values of Pearson´s correlation coefficient were unique either for one or the other genotype. The inbred line was characterized by several positive relationships between the volume density of plastoglobuli or starch inclusions

in MC chloroplasts and some dimensional parameters of BSC chloroplasts. The length of MC chloroplasts´ cross-sections was also negatively related to the area, the width or the starch amount of BSC chloroplasts in this genotype. The hybrid showed negative correlations between the relative partial volumes of granal thylakoids of BSC chloroplasts and the MC chloroplasts dimensions, the same applied for the relationship between BSC and MC chloroplasts´ granal thylakoids, while the volume density of plastoglobuli in MC chloroplasts was inversely related to the volume density of BSC chloroplasts´ grana. There were also several positive interrelationships between MC and BSC chloroplasts dimensions in this genotype, as well as the positive correlation between the area of MC chloroplasts´ cross-sections and the amount of starch in BSC chloroplasts (Table 3).

*Table 3. Relationships between individual ultrastructural and/or dimensional parameters of the mesophyll cells´ (MC) and bundle sheath cells´ (BSC) chloroplasts evaluated as the correlations between the respective parameters in maize inbred 2023 and its F_1 hybrid 2023×CE810. Only those correlations that were statistically significant for at least one genotype are shown as the values of Pearson´s correlation coefficient (r) ± standard errors (SE), together with the level of statistical significance (** ... significant with $p \leq 0.01$, * ... significant with $p \leq 0.05$, ns ... non-significant). TG ... the volume density of granal thylakoids, PG ... the volume density of plastoglobuli, PR ... the volume density of peripheral reticulum, SI ... the volume density of starch inclusions, A ... the total area of chloroplasts´ cross-sections, L ... the length of chloroplasts´ cross-sections, W ... the width of chloroplasts´ cross-sections.*

Relationship	r ± SE(r) Inbred	r ± SE(r) Hybrid	Relationship	r ± SE(r) Inbred	r ± SE(r) Hybrid
MC chloroplasts			MC – BSC chloroplasts		
TG – PG	-0.503±0.184 *	-0.772±0.136 **	TG (MC) – TG (BSC)	ns	-0.565±0.176 **
TG – A	0.422±0.193 *	0.510±0.183 *	TG (MC) – PG (BSC)	-0.422±0.193 *	-0.615±0.168 **
PG – A	ns	-0.446±0.191 *	TG (BSC) – PG (MC)	ns	0.623±0.167 **
PG – L	-0.519±0.182 **	-0.701±0.152 **	TG (BSC) – A (MC)	ns	-0.431±0.192 *
PG – L W	ns	-0.606±0.170 **	TG (BSC) – L (MC)	ns	-0.418±0.194 *
			PG (MC) – PG (BSC)	0.786±0.132 **	0.802±0.128 **
BSC chloroplasts			PG (MC) – A (BSC)	0.551±0.178 **	ns
			PG (MC) – L (BSC)	0.418±0.194 *	ns
TG – SI	-0.483±0.187 *	-0.455±0.190 *	PG (MC) – W (BSC)	0.408±0.195 *	ns
TG – L	ns	-0.459±0.189 *	PG (BSC) – SI (MC)	0.493±0.186 *	ns
TG – W	-0.406±0.195 *	-0.434±0.192 *	PG (BSC) – L (MC)	-0.562±0.176 **	-0.530±0.181 **
PG – SI	ns	-0.465±0.189 *	PG (BSC) – L W (MC)	ns	-0.464±0.189 *
PG – A	0.529±0.181 **	ns	SI (MC) – A (BSC)	0.546±0.179 **	ns
PG – L	0.449±0.191 *	-0.438±0.192 *	SI (MC) – L (BSC)	0.642±0.163 **	ns
PG – W	0.434±0.192 *	ns	SI (MC) – W (BSC)	0.450±0.190 *	ns
PR – A	ns	-0.478±0.187 *	SI (BSC) – A (MC)	ns	0.413±0.194 *
PR – L	ns	-0.473±0.188 *	SI (BSC) – L (MC)	-0.558±0.177 **	ns
PR – W	-0.580±0.138 **	-0.501±0.185 *	A (MC) – A (BSC)	ns	0.506±0.184 *
PR – L W	0.421±0.193 *	ns	A (MC) – L (BSC)	ns	0.554±0.178 **
SI – A	0.631±0.166 **	0.748±0.142 **	A (MC) – W (BSC)	ns	0.584±0.173 **
SI – L	0.425±0.193 *	0.838±0.117 **	A (BSC) – L (MC)	-0.511±0.183 *	ns
SI – W	0.747±0.142 **	0.826±0.120 **	L (MC) – L (BSC)	ns	0.426±0.193 *
SI – L W	-0.448±0.191 *	ns	L (MC) – W (BSC)	-0.466±0.189 *	ns
L – W	0.712±0.150 **	0.907±0.090 **	W (MC) – W (BSC)	ns	0.465±0.189 *

The examination of the relationship between the inbred line and its hybrid, as regards various ultrastructural and dimensional parameters of MC or BSC chloroplasts, revealed that the development of MC chloroplasts (as inferred from the examination of chloroplasts in three different parts of leaf blade) in leaves of the hybrid followed the behaviour of its parent rather well (the only non-significant correlations were found for the volume density of peripheral reticulum and the L/W ratio). On the other hand, no such similarity was found for the BSC chloroplasts, with the exception of significant correlation in the volume density of plastoglobuli (Table 4).

*Table 4. Relationship between maize inbred line 2023 and its F_1 hybrid 2023×CE810 evaluated as the correlations between the average values of selected ultrastructural or dimensional parameters of the mesophyll cells´ (MC) or bundle sheath cells´ (BSC) chloroplasts, determined for three different parts of the third leaf blade in 24-d old and 28-d old plants. The values of Pearson´s correlation coefficient (r) ± standard errors (SE) are shown, together with the level of statistical significance (** ... significant with p ≤ 0.01, * ... significant with p ≤ 0.05, ns ... non-significant).*

Parameter	MC chloroplasts r ± SE(r)	BSC chloroplasts r ± SE(r)
Granal thylakoids	0.835±0.275 *	0.409±0.456 ns
Plastoglobuli	0.946±0.162 **	0.900±0.219 *
Peripheral reticulum	-0.138±0.495 ns	0.300±0.478 ns
Starch inclusions	0.922±0.193 **	-0.193±0.491 ns
Area	0.941±0.169 **	-0.224±0.487 ns
Length	0.914±0.202 *	-0.294±0.478 ns
Width	0.922±0.193 **	-0.193±0.491 ns
Length width ratio	0.492±0.435 ns	0.634±0.387 ns

Discussion

For our study of the changes in chloroplast ultrastructure and dimensions during the beginning of leaf senescence, we choose two genotypes of maize: the inbred line 2023 and its direct descendant, F_1 hybrid 2023xCE810, which inherited its chloroplasts maternally from the 2023 inbred. As this hybrid showed somewhat faster development compared to its parent, during the first sampling date, when the third leaves of the inbred line have just completed their transition into the maturity stage, the corresponding leaves of the hybrid were already fully mature. Four days later, at the second sampling date, the apical parts of leaves of hybrid plants showed full symptoms of senescence (yellow color, dry tip), the middle part of leaf was light-green and the basal one was fully green, whereas the entire length of 2023 leaves displayed green color and only the extreme tip of leaves has begun to get yellow. Thus, we could compare the course of chloroplast development in relation to the different onset of leaf senescence in both genotypes.

From the beginning of leaf maturity, MC chloroplasts in our samples of leaf blade were characterized by well-developed thylakoid grana that usually contained between 15 and 30 thylakoids. Their volume density stayed the same in almost all leaf parts examined (so that, presumably, they have already reached their full extent at the first sampling date) and, despite the developmental lead of hybrid genotype over its parent (and despite its different activity of PS2 compared to the inbred line, see Kutík *et al.* 2001), there were no differences between both genotypes in this parameter. Only at the senescing apical part of leaf of 28-d old plants we found a significant drop in the volume densities of granal thylakoids, and the difference between both genotypes manifested itself, as this drop was more pronounced in the hybrid. The decay of thylakoid membranes, and particularly the diminishing of the grana, is a typical phenomenon associated with the senescence of chloroplasts (Naito *et al.* 1981, Biswal and Biswal 1988, Matile 1992, Kutík *et al.* 1999, 2001, Zavaleta-Mancera *et al.* 1999, Prakash *et al.* 2001, Biswal *et al.* 2003, Kołodziejek *et al.* 2003). Similar sign of chloroplast senescence is the increase in the number and size of plastoglobuli, small particles accumulating the products of thylakoid lipids and chlorophyll breakdown, carotenoids and carotenoid esters (Naito *et al.* 1981, Tevini and Steinmuller 1985, Biswal and Biswal 1988, Matile 1992, Kutík *et al.* 1993, Biswal 1995, Kutík 1998, Matile *et al.* 1989, Biswal *et al.* 2003). Such increase was clearly discernible in the senescing apical part of leaves at the second sampling date, and, again, the hybrid

exceeded its parent in this parameter. The association between the degradation of thylakoid membranes and the accumulation of the products of their breakdown in plastoglobuli was thus obvious and was confirmed also by the results of the correlation analysis.

However, the inverse relationship between thylakoids and plastoglobuli volume densities applied only for the MC chloroplasts. Besides the fact that the BSC chloroplasts had much less thylakoids (which is one of their well-known characteristics, see *e.g.* Kirchanski 1975, Nishioka *et al.* 1993, Vičánková and Kutík 2005) compared to the MC ones, and the relative volume density of their plastoglobuli was also lower than that of the plastoglobuli in MC chloroplasts, no direct correlation between these two chloroplast compartments was found in this case. The reason for this lies probably in the different pattern of leaf blade heterogeneity observed for the plastoglobuli and grana volume densities in BSC chloroplasts. Whereas an increasing trend from the youngest leaf base to the oldest leaf apex, similar to the one observed in MC chloroplasts and concordant with the gradual advancement of chloroplast senescence, was discernible for the plastoglobuli volume density, the BSC grana behaved differently. The high volume density of thylakoid grana found in the basal part of leaves is in good agreement with findings of other authors, who observed that the amount of granal thylakoids of the BSC chloroplasts decreases during their development and that this decrease is light-dependent (Brangeon 1973, Kirchanski 1975, Nishioka *et al.* 1993). As the leaf base of maize leaves is more shielded from light by leaves of higher insertions, larger grana are only to be expected here. However, surprisingly high volume density of BSC chloroplasts´ grana was found also in the senescing leaf apex. We observed similar results when comparing BSC chloroplasts in the middle third of leaf blade of young, mature and senescing maize leaves (Vičánková and Kutík 2005). The stacking of thylakoid membranes could be in this case rather secondary, due to the fact that BSC chloroplasts at this developmental stage were fully packed with starch granules and there was thus less possibility of thylakoid spacing. The superiority of the inbred line over its progeny, shown for the granal thylakoids´ volume density in BSC chloroplasts, could be possibly associated with its slightly lagging development (more discernible in younger plants), but similar superiority was observed also for plastoglobuli (*i.e.* symptom of senescence), and this discrepancy must be therefore caused by some unknown factors related to genetic differences between both genotypes.

Peripheral reticulum is a rather elusive chloroplast compartment: its role in the chloroplast function is still far from being clear. It is typical for C_4 plants and probably participates in the transport of primary assimilates (malate, aspartate) from mesophyll to bundle sheath cells (Laetsch and Kortschak 1972, Chapman *et al.* 1975, Kirchanski 1975, Hudák 1997, Kratsch and Wise 2000). This agrees well with our findings that the volume density of this compartment in MC chloroplasts of mature leaf blade was mostly higher than in the BSC ones. The inbred line usually showed greater size of peripheral reticulum in MC chloroplasts compared to the hybrid (in younger plants this applied for BSC chloroplasts as well); this was perhaps also related to the delay in chloroplast development displayed by the parental line (*i.e.* the transport processes between MCs and BSCs were fully active even in the oldest part of 28-d old inbred plants).

The size and shape of chloroplasts also changes with their development (Naito *et al.* 1981, Kutík 1985, Kura-Hotta *et al.* 1990, Kutík and Kočová 1996, Kutík *et al.* 1999, 2001, Zavaleta-Mancera *et al.* 1999, Biswal *et al.* 2003) and we observed slight gradual enlargement of MC chloroplasts until the stage of senescence was reached by leaf tissue. This increase in the size was caused by the rise of chloroplast height (*i.e.* width of their cross-sections), so that the chloroplasts gradually acquired more rounded

shape. In the senescing part of leaf apex, this rounding of MC chloroplasts was very suspicious, and at the same time there was a sharp drop in their size as well – another evidence of their transition to senescing state (Kura-Hotta *et al.* 1990, Kutík *et al.* 1999, Sakai *et al.* 1999). Again, the slight delay in the development of the inbred genotype after its hybrid could be seen here. The BSC chloroplasts, which are generally larger than those of MCs (Kirchanski 1975), also displayed this increasing trend but their shape stayed more-or-less the same in all parts of leaf tissue examined and no decrease in the BSC chloroplasts´ size was observed in the senescing leaf apex. The MC and BSC chloroplasts thus differ in the course of their development. The inbred line was characterized by more flat BSC chloroplasts compared to its progeny; this difference could be partly caused by the larger amount of starch inclusions that filled up the BSC chloroplasts of hybrid and caused their „swelling“.

As regards the accumulation of starch in the BSC chloroplasts, there were interesting differences between both genotypes. The leaves of 24-d old plants of the inbred line, which were at the beginning of their maturity, did not yet contain much starch inclusions, probably due to the fact that the production of 3-phosphoglycerate by the Calvin-Benson cycle (followed by its transport to MC chloroplasts, reduction to triosophosphates and return to BSC chloroplasts, where it is used for starch synthesis) had not yet reached its full efficiency. On the other hand, the hybrid, which was ahead of its parent in the development of leaves, had already sufficient level of photosynthetic metabolism and its synthesis and accumulation of starch in BSC chloroplasts was well advanced. Four days later, both genotypes showed similar amounts of starch, and with the advancement of leaf tissue senescence, the parental genotype even slightly gained over its progeny. The assessment of the changes in volume density of starch inclusions in BSC chloroplasts (and, consequently, of BSC chloroplasts´ shape and dimensions), brought actually the most interesting piece of information about the genotypic differences in chloroplast ultrastructure, and about the possible heritability of the changes associated with chloroplast development. Whereas the development of MC chloroplasts during the onset of senescence of maize leaves occured with about the same rapidity (judging from the changes of almost all ultrastructural and dimensional parameters examined) in the inbred line as in its hybrid, this did not apply for the BSC chloroplasts. Here we observed significant correlations between parent and its progeny only for the volume density of plastoglobuli, but the synthesis of starch and its accumulation in the starch inclusions of BSC chloroplasts followed different course in each genotype. Thus, the different nuclear genetic background of hybrid asserted itself in this case.

Other differences between both genotypes examined involved mostly the correlations among various ultrastructural and/or dimensional characters of chloroplasts. Thus, for example, the increase of plastoglobuli with the increasing BSC chloroplast´ size (*i.e.* the symptoms of advancing development of chloroplasts) observed in the inbred line was not followed by similar trend in the hybrid; on the contrary, the reverse was true. The enlargement of MC chloroplasts was accompanied by similar enlargement of BSC chloroplasts in the hybrid but not in its parent (again, probably due to the differences in the advancement of starch accumulation and the related „swelling“ of chloroplasts). We can thus conclude that although several parameters related to the arrangement of chloroplast inner structure indeed seem to be directly inherited from parent to its progeny, this does not apply generally. Moreover, there are differences in the inheritance of chloroplast ultrastructural or dimensional parameters between two basic chloroplast types found in the photosynthetic tissue of NADP-ME type of C_4 plants. Whereas the course of MC chloroplast development at the onset of leaf

senescence in maize hybrid simply follows that of its parent, this is not true for the BSC chloroplasts, where each genotype has its own distinguishable pattern of chloroplast development.

References

Biswal, U.C., Biswal, B., Raval, M.K.: Chloroplast Biogenesis: From Proplastid to Gerontoplast. Kluwer Academic Publishers, Dordrecht-Boston-London 2003.

Biswal, U.C., Biswal, B.: Ultrastructural modifications and biochemical changes during senescence of chloroplasts. – Int. Rev. Cytol. **113**: 271-321, 1988.

Biswal, B.: Carotenoid catabolism during leaf senescence and its control by light. – J. Photochem. Photobiol. B: Biol. **30**: 3-13, 1995.

Brangeon, J.: Compared ontogeny of the two types of chloroplasts of *Zea mays*. – J. Microsc. **16**: 233-242, 1973.

Brown, N.J., Parsley, K., Hibberd, J.M.: The future of C4 research – maize, *Flaveria*, or *Cleome* ? – Trends Plant. Sci. **10**: 215-221, 2005.

Chapman, E.A., Bain, J.M., Gove, D.W.: Mitochondria and chloroplast peripheral reticulum in the C_4 plants *Amaranthus edulis* and *Atriplex spongiosa*. – Aust. J. Plant Physiol. **2**: 207-223, 1975.

Chonan, N., Matsuda, T., Tsuchiya, T.: [Ultrastructural changes in chloroplast and phloem of rice leaves during senescence under different nitrogen supply.] – Jap. J. Crop Sci. **60**: 247-254, 1991. [In Jap.].

Chow, W.S., Kim, E.H., Horton, P., Anderson, J.M.: Granal stacking of thylakoid membranes in higher plant chloroplasts: the physicochemical forces at work and the functional consequences that ensue. – Photochem. Photobiol. Sci. **4**: 1081-1090, 2005.

Cousins, A.B., Adam, N.R., Wall, G.W., Kimball, B.A., Pinter, P.J., Jr., Ottman, M.J., Leavitt, S.W., Webber, A.N.: Development of C_4 photosynthesis in sorghum leaves grown under free-air CO_2 enrichment (FACE). – J. exp. Bot. **54**: 1969-1975, 2003.

Dengler, N.G., Dengler, R.E., Donnely, P.M., Hattersley, P.W.: Quantitative leaf anatomy of C_3 and C_4 grasses (Poaceae): Bundle sheath and mesophyll surface area relationships. – Ann. Bot. **73**: 241-255, 1994.

Edwards, G.E., Franceschi, V.R., Ku, M.S.B., Voznesenskaya, E.V., Pyankov, V.I., Andreo, C.S.: Compartmentation of photosynthesis in cells and tissues of C_4 plants. – J. exp. Bot. **52**: 577-590, 2001.

Furbank, R.T., Foyer, C.H.: C_4 plants as valuable model experimental systems for the study of photosynthesis. – New. Phytol. **109**: 265-277, 1988.

Furumoto, T., Hata, S., Izui, K.: Isolation and characterization of cDNAs for differentially accumulated transcripts between mesophyll cells and bundle sheath strands of maize leaves. – Plant Cell Physiol. **41**: 1200-1209, 2000.

Hahnen, S., Joeris, T., Kreuzaler, F., Peterhänsel, C. : Quantification of photosynthetic gene expression in maize C_3 and C_4 tissues by real-time PCR. – Photosynth. Res. **75**: 183-192, 2003.

Hudák, J.: Photosynthetic apparatus. – In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 27-48. Marcel Dekker, New York-Basel-Hong Kong 1997.

Jankovski, J.P., Smith, L.G., Nelson, T.: Specification of bundle sheath cell fates during maize leaf development: Roles of lineage and positional information evaluated through analysis of the *tangled1* mutant. – Development **128**: 2747-2753, 2001.

Kirchanski, S.J.: The ultrastructural development of the dimorphic plastids of *Zea mays* L. – Amer. J. Bot. **62**: 695-705, 1975.

Kołodziejek, I., Kozioł, J., Wałęza, M., Mostowska, A.: Ultrastructure of mesophyll cells and pigment content in senescing leaves of maize and barley. – J. Plant Growth Regul. **22**: 213-227, 2003.

Kratsch, H.A., Wise, R.R.: The ultrastructure of chilling stress. – Plant Cell Environ. **23**: 337–350, 2000.

Kubicki, A., Steinmüller, K., Westhoff, P.: Differential transcription of plastome-encoded genes in the mesophyll and bundle-sheath chloroplasts of the monocotyledonous NADP-malic enzyme-type C_4 plants maize and *Sorghum*. – Plant mol. Biol. **25**: 669-679, 1994.

Kura-Hotta, M., Hashimoto, H., Satoh, K., Katoh, S.: Quantitative determination of changes in the number and size of chloroplasts in naturally senescing leaves of rice seedlings. – Plant Cell Physiol. **31**: 33-38, 1990.

Kutík, J.: Chloroplast development. – In: Šesták Z. (ed.): Photosynthesis During Leaf Development. Pp. 51-75. Academia, Praha;

Dr W. Junk Publishers, Dordrecht-Boston-Lancaster 1985.
Kutík, J.: Microtubule-like structures in developing wheat-leaf chloroplasts. – Biol. Plant. **34**: 355-357, 1992.
Kutík, J.: The development of chloroplast structure during leaf ontogeny. – Photosynthetica **35**: 481-505, 1998.
Kutík, J., Čincerová, A., Dvořák, M.: Chloroplast ultrastructural development during the ontogeny of the second leaf of wheat under nitrogen deficiency. – Photosynthetica **28**: 447-453, 1993.
Kutík, J., Holá, D., Vičánková, A., Šmídová, M., Kočová, M., Körnerová, M., Kubínová, L.: The heterogeneity of structural and functional photosynthetic characteristics of mesophyll chloroplasts in various parts of mature or senescing leaf blade of two maize (*Zea mays* L.) genotypes. – Photosynthetica **39**: 497-506, 2001.
Kutík, J., Kočová, M., Holá, D., Körnerová, M.: The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes. – Photosynthetica **36**: 497-507, 1999.
Kutík, J., Kočová, M.: The development of chloroplast ultrastructure and photochemical activity during leaf ontogeny in maize (*Zea mays* L.). – Biológia (Bratislava) **51**: 59, 1996.
Laetsch, W.M., Kortschak, H.P.: Chloroplast structure and function in tissue cultures of a C_4 plant. – Plant Physiol. **49**: 1021-1023, 1972.
Langdale, J.A., Kidner, C.A.: *bundle sheath defective*, a mutation that disrupts cellular differentiation in maize leaves. – Development **120**: 673-681, 1994.
Langdale, J.A., Metzler, M.C., Nelson, T.: The *argentia* mutation delays normal development of photosynthetic cell types in *Zea mays*. – Dev. Biol. **122**: 243-255, 1987.
Langdale, J.A., Rothermel, B., Nelson, T.: Cellular pattern of photosynthetic gene expression in developing maize leaves. – Genes Dev. **2**: 106-115, 1988.
Leech, R.M., Rumsby, M.G., Thomson, W.W.: Plastid differentiation, acyl lipid and fatty acid changes in developing green maize leaves. – Plant Physiol. **52**: 240-245, 1973.
Leegood, R.C.: C_4 photosynthesis: principles of CO_2 concentration and prospects for its introduction into C_3 plants. – J. exp. Bot. **53**: 581-590, 2002.
Lunn, J.E., Hatch, M.D.: Primary partitioning and storage of photosynthate in sucrose and starch in leaves of C_4 plants. – Planta **197**: 385-391, 1995.
Majeran, W., Cai, Y., Sun, Q., van Wijk, K.J.: Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. – Plant Cell **17**: 3111-3140, 2005.
Martineau, B., Taylor, W.C.: Photosynthetic gene expression and cellular differentiation in developing maize leaves. – Plant Physiol. **78**: 399-404, 1985.
Martinoia E., Heck, U., Dalling, M.J., Matile, P.: Changes in chloroplast number and chloroplast constituents in senescing barley leaves. – Biochem. Physiol. Pflanz. **178**: 147-155, 1983.
Matile, P., Düggelin, T., Schellenberg, M., Rentsch, D., Bortlik, K., Peisker, C., Thomas, H.: How and why is chlorophyll broken down in senescent leaves. – Plant Physiol. Biochem. **27**: 595-604, 1989.
Matile, P.: Chloroplast senescence. – In: Baker, N.R., Thomas, H. (eds.): Crop Photosynthesis: Spatial and Temporal Determinants. Pp. 413-440. Elsevier, Amsterdam-London 1992.
Miranda, V., Baker, N.R., Long, S.P.: Anatomical variation along the length of the *Zea mays* leaf in relation to photosynthesis. – New Phytol. **88**: 595-605, 1981a.
Miranda, V., Baker, N.R., Long, S.P.: Limitations of photosynthesis in different regions of the *Zea mays* leaf. – New Phytol. **89**: 179-190, 1981b.
Naito, K., Ueda, K., Tsuji, H.: Differential effects of benzyladenine on the ultrastructure of chloroplasts in intact bean leaves according to their age. – Protoplasma **105**: 293-306, 1981.
Nelson, T., Dengler, N.G.: Photosynthetic tissue differentiation in C_4 plants. – Int. J. Plant Sci. **153**: S93-S105, 1992.
Nelson, T., Langdale, J.A.: Patterns of leaf development in C_4 plants. –Plant Cell **1**: 3-13, 1989.
Nishioka, D., Brisibe, E.A., Miyake, H., Taniguchi, T.: Ultrastructural observations in the suppression of granal development in bundle sheath chloroplasts of NADP-ME type C_4 monocot and dicot species. – Jap. J. Crop Sci. **62**: 621-627, 1993.
Oldenburg, D.J., Bendich, A.J.: Changes in the structure of DNA molecules and the amount of DNA per plastid during chloroplast development in maize. – J. mol. Biol. **344**: 1311-1330, 2004.
Perchorowicz, J.T., Gibbs, M.: Carbon dioxide fixation and related properties in sections of the developing green maize leaf. – Plant Physiol. **65**: 802-809, 1980.
Prakash, J.S.S., Baig, M.A., Mohanty, P.: Senescence induced structural reorganization

of thylakoid membranes in *Cucumis sativus* cotyledons: LHC II involvement in reorganization of thylakoid membranes. – Photosynth. Res. **68**: 153-161, 2001.

Rascio, N., Colombo, P.M., Orsenigo, M.: The ultrastructural development of plastids in leaves of maize plants exposed to continuous illumination. – Protoplasma **102**: 131-139, 1980.

Reynolds, E.S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. – J. Cell Biol. **17**: 208-214, 1963.

Sakai, A., Kuroiwa, H., Kuroiwa, T.: Senescence program in rice (*Oryza sativa* L.) leaves: Analysis of the blade of the second leaf at the tissue and cellular levels. – Protoplasma **207**: 222-232, 1999.

Šesták Z., Šiffel, P.: Leaf-age related differences in chlorophyll fluorescence. – Photosynthetica **33**: 347-369, 1997.

Sheen, J.Y., Bogorad, L.: Regulation of levels of nuclear transcripts for C_4 photosynthesis in bundle sheath and mesophyll cells of maize leaves. – Plant mol. Biol. **8**: 227-238, 1987.

Sheen. J.Y., Bogorad, L.: Differential expression in bundle sheath and mesophyll cells of maize of genes for Photosystem II components encoded by the plastid genome. – Plant Physiol. **86**: 1020-1026, 1988.

Spilatro, S.R., Preiss, J.: Regulation of starch synthesis in the bundle sheath and mesophyll of *Zea mays* L. – Plant Physiol. **83**: 621-627, 1987.

Tevini, M., Steinmuller, D.: Composition and function of plastoglobuli. II. Lipid composition of leaves and plastoglobuli during beech leaf senescence. – Planta **163**: 91-96, 1985.

Vičánková, A., Kutík, J.: Chloroplast ultrastructural development in vascular bundle sheath cells of two different maize (*Zea mays* L.) genotypes. – Plant Soil Environ. **51**: 491-495, 2005.

Wellburn, A.R., Robinson, D.C., Wellburn, F.A.M.: Chloroplast development in low light-grown barley seedlings. – Planta **154**: 259-265, 1982.

Williams, J.H.H., Collis, B.E., Pollock, C.J., Williams, M.L., Farrar, J.F.: Variability in the distribution of photoassimilates along leaves of temperate Gramineae. – New Phytol. **123**: 699-703, 1993.

Zavaleta-Mancera, H.A., Thomas, B.J., Thomas, H., Scott, I.M.: Regreening of senescent *Nicotiana* leaves. II. Redifferentiation of plastids. – J. exp. Bot. **50**: 1683-1689, 1999.

Altered cytokinin metabolism affects cytokinin, auxin and abscisic acid contents in leaves and chloroplasts and chloroplast ultrastructure in transgenic tobacco

Lenka Polanská[1,2*], Anna Vičánková[1,2], Marie Nováková[1,3], Jiří Malbeck[1], Petre I. Dobrev[1], Břetislav Brzobohatý[4], Radomíra Vaňková[1] and Ivana Macháčková [1]

[1]*Institute of Experimental Botany AS CR, Rozvojová 135, 165 02 Prague 6 – Lysolaje, Czech Republic*

[2]*Charles University in Prague, Faculty of Science, Department of Plant Physiology, Viničná 5, 128 44 Prague 2, Czech Republic*

[3]*Charles University in Prague, Faculty of Science, Department of Biochemistry, Hlavova 2030/8, 128 43 Prague 2, Czech Republic*

[4]*Institute of Biophysics AS CR, Královopolská 135, 612 65 Brno, Czech Republic*

*polanska@ueb.cas.cz

Key words: Abscisic acid, auxin, β-glucosidase, chloroplast ultrastructure, cytokinin metabolism, cytokinin oxidase/dehydrogenase, isopentenyltransferase, *Nicotiana tabacum*, *Sho*, zeatin *O*-glucosyltransferase

Abstract

Cytokinins are hormones that are involved in regulation of many aspects of plant growth and development including plastid differentiation and functions. The occurrence of cytokinins in chloroplasts as well as formation of a prenyl side-chain of isoprenoid cytokinins and the location of four isopentenyltransferases, cytokinin biosynthetic enzymes, in plastids indicates importance of cytokinins for chloroplast development. The impact of modified cytokinin metabolism on the changes in endogenous cytokinin, indolacetic and abscisic acid contents in leaves and in isolated intact chloroplasts was determined by LCMS and 2D HPLC, and the alterations in chloroplast ultrastructure by electron microscopy. Cytokinin overproduction was achieved by the expression of a *Sho* gene, encoding an isopentenyltransferase homologue from *Petunia hybrida*, which resulted in leaves in an increase of the levels of isopentenyladenine type of cytokinins, especially of *N*-glucosides. The extent of the cytokinin content increase was lower in isolated chloroplasts than in leaves. Cytokinin levels were reduced in leaves of tobacco

harbouring a cytokinin oxidase/dehydrogenase (*AtCKX3*) gene from *Arabidopsis thaliana*. The total cytokinin content in chloroplasts isolated from *AtCKX3* plants was decreased as well, though the levels of cytokinin nucleotides were higher than in the corresponding wild type. In transformant overexpressing a maize β-glucosidase *Zm-p60.1,* naturally targeted to plastids, which is capable of releasing biologically active cytokinins from their *O*- and *N3*-glucosides, a decrease of *O*-glucosides in chloroplasts was found. In leaves, the changes in cytokinin *O*-glucosides were not significant, though an increase of free bases and ribosides was apparent. *O*-glucosides were enormously accumulated in leaves, but not in isolated chloroplasts, of plants overexpressing a *ZOG1* gene encoding zeatin *O*-glucosyltransferase from *Phaseolus lunatus*, indicating that the cytokinin conjugates are accumulated primarily in another compartment. Due to intense cross talk among plant hormones, levels of free indolacetic and abscisic acid were affected by manipulation of cytokinin content. Chloroplasts of cytokinin overproducing plants constitutively overexpressing *Sho* gene displayed ultrastructure alterations including the occasional occurrence of discernible crystalloid structures and increased number of plastoglobuli compared with the wild type. When an inducible promoter was used, no crystalloids were found, but after induction of the expression of *Sho* gene an increased thylakoid membrane stacking was observed. All the other transformants did not display any striking differences in chloroplast ultrastructure. Taken together, our results suggest that the compartmentation of plant hormones plays an important role in the fine-tuning of homeostasis of hormone concentration in plant cells and that chloroplasts are rather independent organelles in respect to regulation of cytokinin metabolism, which reflects the importance of cytokinins for plastid function.

Introduction

Plant hormones cytokinins (CKs) are known to be involved in many processes related to plastid development and functions. Already 10 years after the discovery of kinetin (6-furfurylaminopurine) as a plant growth regulator CK (Miller *et al.*, 1955), it was shown that kinetin could stimulate chloroplast differentiation from proplastids, including grana formation, in tobacco tissue culture (Stetler and Laetsch, 1965). Kinetin was also found to almost double chloroplast number per cell in etiolated tobacco leaf discs incubated in the light (Boasson and Laetsch, 1969).

It has been demonstrated that exogenously applied CKs induce partial development of chloroplasts from proplastids, amyloplasts and etioplasts, especially in darkness (e.g.: Chory *et al.*, 1994, Kusnetsov *et al.*, 1994). For example, benzyladenine (BA) treatment of excised watermelon cotyledons accelerated degradation of reserve material and differentiation of plastids with a more developed inner membrane system as well as increased levels of plastid pigments and enzymes (Rubisco and NADPH-glyoxylate reductase) (Longo *et al.*, 1979). Moreover, applied BA accelerated the redevelopment of grana and stroma in regreening plastids, the increase of reappeared NADPH-protochlorophyllide oxidoreductase, contents of chlorophyll and proteins in regreening tobacco leaves (Zavaleta-Mancera *et al.*, 1999 a, b). CK mode of action in plastids seems to be mediated by the stimulation of the expression of several plastid-related genes, both of nuclear and plastid origin, in particular of nuclear encoded gene for chlorophyll *a/b*-binding polypeptide of the light-harvesting complex and gene coding for the small subunit of Rubisco in *Nicotiana tabacum* (Abdelghani *et al.*, 1991), *Arabidopsis* (Chory *et al.*, 1994) or *Dianthus caryophyllus* (Winiarska *et al.*, 1994). For extensive reviews see Schmülling *et al.* (1997) or Parthier (1989).
Recently, Brenner *et al.* (2005) identified through genome-wide expression profiling five rapidly CK induced plastid transcripts in *Arabidopsis* seedlings indicating a fast transfer of the CK signal to plastids or its direct perception there. CK effect on gene expression may be mediated via the hormone interaction with specific proteins. In the presence of *trans*-zeatin, 64 kDa chloroplast zeatin-binding protein was found to activate chloroplast but not nuclear genome (Lyukevich *et al.*, 2002).
The occurrence of endogenous CKs in plastids was proved. CKs were present in chloroplast tRNAs (e.g. Vreman *et al.,* 1978). Zeatin-type CKs were detected in isolated spinach chloroplasts by paper chromatography and soybean callus bioassay (Davey and Van Staden, 1981). Immunolocalization of CKs revealed low labeling in the stroma of plastids of *Tilia cordata* embryo cotyledons and roots (Kärkönen and Simola, 1999). We performed detailed analysis of the level of a whole range of CK metabolites (free bases, ribosides, glucosides and ribotides) in isolated intact chloroplasts using LCMS (Benková *et al.*, 1999).
The importance of CKs for plastid development and function may be deduced from the partial localization of CK biosynthetic pathway to this compartment. Kasahara *et al.* (2004) showed that the prenyl group of CKs is synthesised in plastids by the methylerythritol phosphate pathway and that four isopentenyltransferases (AtIPT1,

AtIPT3, AtIPT5 and AtIPT8) are localised to plastids in *Arabidopsis* cells. Unexpectedly, Sakakibara *et al.* (2005) have found that *Agrobacterium* IPT modifies CK biosynthesis in infected plant cells, as it is targeted to and functions in plastids, even though it lacks typical plastid-targeting sequence, and it prefers another substrate than plant own IPTs. Enormous increase of CK content in tobacco, which resulted from the expression of *Agrobacterium tumefaciens isopentenyltransferase* gene leading to anomalies in chloroplast ultrastructure and enhanced CK content in chloroplasts (Synková *et al.*, 2006).

In the present study, we evaluated the impact of manipulation of CK metabolism on endogenous CK pool in leaves and isolated intact chloroplasts and chloroplast ultrastructure of tobacco (*Nicotiana tabacum* L.). We compared CK overproducing plants (expressing isopentenyltransferase *Sho),* plants with decreased CK levels (overexpressing CK oxidase/dehydrogenase *AtCKX3)* and plants with altered CK glucoconjugation (overexpressing β-glucosidase *Zm-p60.1* or zeatin *O*-glucosyltransferase *ZOG1).* Taking into account the immense cross-talk among plant hormones, we determined as well the contents of auxin (IAA) and abscisic acid (ABA).

Materials and methods

Plant materials and growth conditions

Four different types of transgenic tobacco plants with altered CK metabolism and the corresponding wild types (WT) in vegetative stage were used:

1) tobacco *in vitro* lines expressing an isopentenyltransferase *Sho* (*Shooting*) gene from *Petunia hybrida* under the control of a constitutive CaMV 35S promoter or construct with four 35S enhancer elements (Zubko *et al.*, 2002) and WT *Nicotiana tabacum* L. cv. Petit Havana SR1 cultivated on solid hormone-free MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}) and 8-9-week-old transgenic plants harbouring the *Sho* gene under a dexamethasone inducible promoter. *Sho* expression was induced in transgenic plants with 20 μM dexamethasone (in 0.05% DMSO, 5 x 50 ml per plant during 13 days). As controls transgenic plants treated with water or 0.05% DMSO and WT SR1 plants treated with water, 0.05% DMSO or 20μM dexamethasone in 0.05% DMSO were used. The plants were cultivated in growth chamber (16/8 hr photoperiod at 130 μmol m^{-2} s^{-1}, day/night temperature of 25/23°C and relative humidity ca 80%) for 5 weeks and then transferred to a greenhouse.

All the other plants were cultivated in a soil substrate in a growth chamber (16/8 hr photoperiod at 150 μmol photons m^{-2} s^{-1}, 26°C/20°C and relative humidity ca 80%):

2) 11-week-old tobacco plants (*35S:AtCKX3*) overexpressing a gene for CK oxidase/dehydrogenase from *Arabidopsis thaliana* under a constitutive CaMV 35S promoter (Werner *et al.*, 2001) and 9-week-old WT *Nicotiana tabacum* L. cv. Samsun NN.
3) 8-9-week-old tobacco plants (*35S:ZOG1*) harbouring a zeatin *O*-glucosyltransferase *ZOG1* gene from *Phaseolus lunatus* under a constitutive CaMV 35S promoter line 7-20 (Martin *et al.*, 2001) and WT *Nicotiana tabacum* L. cv. Wisconsin 38.
4) 8-9-week-old tobacco plants (*35S:P60*) overexpressing a maize ß-glucosidase *Zm-p60.1* naturally targeted to plastids under a CaMV 35S promoter (Kiran *et al.*, 2006) and WT *Nicotiana tabacum* L. cv. Petit Havana SR1. Transgenic plants were selected with methotrexate (0.5 mg l^{-1}) added to solid MS medium supplemented with sucrose (15 g l^{-1}).

Chloroplast isolation

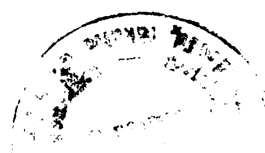
Intact chloroplasts were isolated and purified as described in Benková *et al.* (1999) and in Kiran *et al.* (2006). The chloroplast fraction was recovered from the homogenate of deribbed leaves in isotonic medium by centrifugation, layered on a Percoll density gradient (40% and 80% (v/v) Percoll solution in medium) and centrifuged. Intact chloroplasts were collected at the interface of the gradient, diluted with medium and centrifuged. The pellet was resuspended in medium. All the procedures were done at 4°C.

Chlorophyll determination

Chlorophyll was extracted into 80% (v/v) acetone. The total chlorophyll a+b content was calculated from the absorbance at 652 nm of the clear extract after centrifugation (500×g, 5 min) according to Arnon (1949).

Chloroplast intactness

The intactness of chloroplasts was determined by the latency of glyceraldehyde-3-phosphate dehydrogenase according to Latzko and Gibbs (1968). Chloroplasts were incubated in a reaction mixture (0.33M Tris/HCl, pH 8.5; 17mM $Na_2HAsO_4 \cdot 7H_2O$; 4mM cysteine; 20mM NaF; 40 μM $NADP^+$). The reaction was initiated with 0.02 M



glyceraldehyde-3-phosphate. Reduction of $NADP^+$ was followed at 340 nm for 5 min. The same assay was run with chloroplasts disrupted with 0.01 M $MgCl_2$. The percentage of intact chloroplasts was calculated from the difference between the original and disrupted sample.

Extraction and purification of IAA, ABA and CK

Detailed procedure of hormone extraction, purification and quantification has been described in Kiran *et al.* (2006). IAA, ABA and CKs were extracted overnight at –20 °C with Bieleski solvent (Bieleski 1964). [^{3}H] IAA and [^{3}H] ABA (Sigma, USA) and 12 deuterium-labeled CKs ([$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z-7G, [$^2H_5$]Z-9G, [$^2H_5$]Z-OG, [$^2H_5$]ZR-OG, [$^2H_3$]DZ, [$^2H_3$]DZR, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP-7G, [$^2H_6$]iP-9G; Apex Organics, UK) were added as internal standards. The extracts were purified using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) and Oasis MCX mixed mode, cation exchange, reverse-phase column (150 mg, Waters) (Dobrev and Kamínek 2002). After a wash with 1M HCOOH, IAA and ABA were eluted with 100% MeOH and evaporated to dryness. Further, CK phosphates (CK nucleotides) were eluted with 0.34 M NH_4OH in water and CK bases, ribosides, and glucosides were eluted with 0.34 M NH_4OH in 60% (v/v) MeOH. Nucleotides were converted to nucleosides with alkaline phosphatase. IAA and ABA were separated and quantified by 2D-HPLC according to Dobrev *et al.* (2005). Purified CK samples were analyzed by LC-MS system consisting of HTS PAL autosampler (CTC Analytics, Switzerland), Rheos 2000 quaternary pump (FLUX, Switzerland) with Csi 6200 Series HPLC Oven (Cambridge Scientific Instruments, England) and LCQ Ion Trap mass spectrometer (Finnigan, USA) equipped with an electrospray. 10 μL of sample were injected onto a C18 column (AQUA, 2 mm × 250 mm × 5 μm, Phenomenex, USA) and eluted with 0.0005% acetic acid (A) and acetonitrile (B). The HPLC gradient profile was as following: 5 min 10% B, then increasing to 17% within 10 min, and to 46% within further 10 min at a flow rate of 0.2 ml min^{-1}. Column temperature was kept at 30°C. The effluent was introduced in mass spectrometer being operated in the positive ion, full-scan MS/MS mode. Quantification was performed using a multilevel calibration graph with deuterated CKs as internal standards.

Transmission electron microscopy

Leaf blade samples were fixed for 2 h with 2.5% (v/v) glutaraldehyde in RM, pH 7.6, followed by 2 h in 2% (w/v) osmic acid in RM. After dehydration in graded ethanol series up to 100% samples were via propylene oxide embedded into low viscosity resin (Spurr 1969). The ultrastructure was evaluated on transverse ultrathin sections of embedded objects contrasted with a saturated solution of uranyl acetate in 70% (v/v) aqueous ethanol, followed by a lead citrate solution treatment according to Reynolds (1963) using a transmission electron microscope.

Results

Hormone analyses

To investigate the role of hormone compartmentation and to study the interactions among plant hormones we determined the contents of CKs, free IAA and ABA in leaves and in isolated intact chloroplasts of four different types of transgenic tobacco with altered CK metabolism and corresponding wild types at vegetative stage of plant development. The intactness of analysed chloroplasts immediately after their isolation was over 90% checked by the latency of the activity of the stromal enzyme glyceraldehyde-3-phosphate dehydrogenase. The plant hormone content is most frequently presented in picomoles per gram fresh weight of plant tissue. Here we also show hormone quantification expressed on chlorophyll content allowing the confrontation with hormone levels in isolated chloroplasts.

Plants expressing isopentenyltransferase Sho

The total CK content was elevated in mature leaves of tobacco plants after the induction of the expression of the gene for CK biosynthetic enzyme isopentenyltransferase *Sho* by dexamethasone almost 9 times in comparison with corresponding WT (SR1) (Fig. 1A). The level of physiologically active CK bases and ribosides (especially of iP and iPR) increased almost 4 times, content of CK *N*-glucosides (predominantly of iP7G) 8 times and CK phosphate level even more than 40 times (iPMP). No significant difference in the content of CK *O*-glucosides was observed. The CK levels did not significantly differ among SR1 plants treated with water, with dexamethasone in DMSO or with DMSO solution or non-induced *Sho* gene carrying tobacco plants (data not shown). The elevation of CKs in induced *Sho* transformed leaves was lower when CK content was expressed per mg of chlorophyll, as the transgenics had higher chlorophyll content than

WT (Fig. 1B). The difference between *Sho* transformants and WT was much lower in isolated chloroplasts (Fig. 1C) than in leaves. Nevertheless, CK content in chloroplasts from plants expressing *Sho* was more than twice as high as that of the WT. As in leaves, CK phosphate fraction of transgenics was represented predominantly by iPMP, in contrast to chloroplasts of WT.

Higher CK content was accompanied with slightly higher free IAA and ABA levels in induced *Sho* leaves in comparison with the WT when expressed on fresh weight (Table 1). These differences were not significant when expressed on chlorophyll content. In chloroplasts of induced *Sho* plants lower IAA, but higher ABA content compared with chloroplasts of WT was found.

Plants expressing CK oxidase/dehydrogenase AtCKX3

The constitutive overexpression of CK catabolic enzyme CK oxidase/dehydrogenase *AtCKX3* significantly delayed development of transgenic plants, thus the hormone contents and chloroplast ultrastructure were analysed in older transgenic plants compared with WT plants. The total CK content was reduced in leaves (Fig. 2A) to about one-half of that in the WT. Particularly altered were the levels of CK *N*-glucosides and to a lesser extent free bases and ribosides. The chlorophyll content was reduced in transgenic plants, thus the differences between transgenic and control tobacco are lowered when CK values are presented in picomoles per mg chlorophyll (Fig. 2B). The CK content was changed to a lesser extent in isolated intact chloroplasts (Fig. 2C). Moreover, the values of CK nucleotides were even higher in chloroplasts of *AtCKX3* than in chloroplasts of control tobacco.

The content of free IAA was not significantly changed in *AtCKX3* leaves (Table 2) compared with control plants when expressed on fresh weight, but it was higher when expressed on chlorophyll content. ABA level was lower in leaves of transgenic tobacco. We did not observed any significant changes of IAA or ABA levels in chloroplasts (Table 2).

Plants expressing β-glucosidase Zm-p60.1

Trend towards an increase in CK free bases and ribosides was observed in leaves (Fig. 3A and B) of tobacco overexpressing maize β-glucosidase *Zm-p60.1* compared with leaves of WT. Changes in the content of CK glucosides in leaves were not significant,

although it decreased in chloroplasts (Fig. 3C). No changes in CK nucleotides or *N*-glucosides were found.

The IAA measurements revealed significantly lower level of IAA in leaves as well in chloroplasts of transgenic plants compared with the WT level of IAA (Table 3). ABA content was reduced significantly in *Zm-p60.1* leaves as compared with control plants (Table 3). In chloroplasts the difference in ABA level was less pronounced.

Plants expressing zeatin O-glucosyltransferase ZOG1

When gene encoding zeatin *O*-glucosyltransferase (*ZOG1*) was constitutively expressed in tobacco, CK *O*-glucosides accumulated in leaves (Fig. 4A). The level of ZOG was two orders of magnitude larger in leaves of transformants than in controls. No significant difference in the content of *N*-glucosides was observed. The contents of free bases, ribosides and nucleotides were only marginally decreased in leaves of *ZOG1* tobacco compared with that of the WT. These differences diminished when the CK content is expressed on chlorophyll (Fig. 4B), while that of *O*-glucosides is more profound, as the chlorophyll content is lower in *ZOG1* than in WT. In contrast, in chloroplasts very low levels of CK *O*-glucosides were found (Fig. 4C). The predominant CK forms were free bases and ribosides, which content as well as content of CK nucleotides were higher in *ZOG1* chloroplasts compared with that of the WT.

In leaves of transgenic plants twofold higher IAA content was detected (Table 4). ABA level was not significantly changed in *ZOG1* leaves when expressed on fresh weight. Nevertheless, it was higher when expressed on chlorophyll. IAA and ABA contents were only slightly higher in *ZOG1* chloroplasts than in chloroplasts of controls.

Ultrastructure observations

The most striking anomaly in chloroplast ultrastructure was the occasional occurrence of crystalloid structures in chloroplasts of *in vitro* cultivated CK overproducing plants harbouring an isopentenyltransferase *Sho* gene under four 35S enhancers (Fig. 5A, detailed view 5B) as well as 35S promoter. However, some chloroplasts from these plants did not show apparent changes in the structure compared to control (Figure 5C). Chloroplasts from *Sho* overexpressing plants contained more plastoglobuli than chloroplasts of control plants (Fig. 5D). The crystalloids were never present in chloroplasts of control SR1 plants cultivated *in vitro*. After induction of the *Sho* gene expression no crystalloids were found, only an increased grana stacking was observed

(Fig. 5E). The crystalloids did not appear in chloroplasts of either SR1 plants treated with water (Fig. 5F), with dexamethasone in DMSO or with DMSO solution, respectively, or in chloroplasts of non-induced *Sho* gene carrying tobacco plants.

The representative chloroplasts from the other types of transgenic tobacco and corresponding controls are shown in figure 6. The most obvious feature observed was the different starch accumulation. The chloroplasts of tobacco overexpressing CK oxidase/dehydrogenase *AtCKX3* (Fig. 6A) and β-glucosidase *Zm-p60.1* (Fig. 6C) have less starch inclusions than chloroplasts of control SNN (Fig. 6B) and SR1 (Fig. 6D), respectively. In contrast, the chloroplasts of zeatin *O*-glucosyltransferase *ZOG1* transformants (Fig. 6E) contained increased number of starch inclusions than control W38 (Fig. 6F). With increasing starch content the shape of chloroplasts altered from lens-shaped to more loaf-like.

Discussion

Hormone analyses

In order to elucidate the extent of chloroplast CK autonomy, we compared the impact of altered CK metabolism on CK pool in isolated intact chloroplasts and in whole leaf tissue. Considering hormone cross-talk the effect of altered CK metabolism on IAA and ABA levels was followed, too. The presence of these hormones in chloroplasts was demonstrated in earlier studies by using immunocytochemical (e.g. Sossountzov *et al.*, 1986; Ohmiya and Hayashi, 1992; Kärkönen and Simola, 1999; Pastor *et al.,* 1999) or fractionation technique followed by HPLC or LCMS analysis (e.g. Fregeau and Wightman, 1983; Sandberg *et al.*, 1990; Benková *et al.*, 1999). Chloroplasts were found to be the compartment where most of the ABA in leaf tissue is formed by the methyl-erythritol-phosphate pathway (Millborrow and Lee 1998). This pathway also mainly provides the prenyl group of CKs (Kasahara *et al.*, 2004). Nordström *et al.* (2004) demonstrated *de novo* CK synthesis in tobacco leaves and suggested that the presence of chloroplasts might be a prerequisite for the iPMP-independent pathway of CK biosynthesis.

Plants expressing isopentenyltransferase Sho

Numerous studies of plants transformed with the *ipt* gene, encoding an isopentenyltransferase from *Agrobacterium tumefaciens*, demonstrated enhanced biosynthesis of endogenous CK, especially of Z-type (e.g. Redig *et al.* 1996, Motyka *et*

al. 2003). Recently, plant own isopentenyltransferase genes *(AtIPT1-9)* were identified in *Arabidopsis thaliana* genome (Takei *et al.* 2001; Kakimoto 2001) and their homologue *Sho* in *Petunia hybrida* (Zubko *et al.*, 2002). In contrast to bacterial *ipt*, the overexpression of plant homologue *AtIPT8* (Sun *et al.*, 2003) as well as *Sho* (Zubko *et al.*, 2002) leads to profound accumulation of iP-type of CKs. We used tobacco plants with dexamethasone-inducible *Sho* expression and we found about 9 times higher total CK content in leaf tissue compared with WT. The CK content was increased in isolated intact chloroplasts as well, though to a relatively lower extent (about 2 times). This is in accordance with our data obtained when CK content increased in tobacco overexpressing *isopentenyltransferase* gene from *Agrobacterium tumefaciens* under the control of the light-inducible promoter for the *Pisum sativum* small subunit of Rubisco (P*ssu-ipt*) (Synková *et al.*, 2006). In contrary to CK levels in whole leaf tissue, CK elevation was relatively lower in chloroplasts, especially no dramatic accumulation of CK glucosides was observed. This indicates that the main pool of CKs is accumulated outside chloroplasts in CK-overproducing plants overexpressing P*ssu-ipt* as well as *Sho* gene.

We found a slightly increased content of free IAA in leaves when *Sho* expression was induced compared with WT whereas the isolated chloroplasts contained lower level of IAA than WT chloroplasts. Macháčková *et al.* (1997) found increased IAA level in *in vitro* cultivated potato plants carrying the *Agrobacterium ipt* gene. In contrast, Eklöf *et al.* (1997, 2000) found in CK-overproducing *ipt* tobacco lower levels of free IAA and reduced rates of IAA synthesis and turnover. Thus, it has been suggested that CKs might downregulate IAA levels. Recently, in *Arabidopsis* plants with glucocorticoid-inducible *ipt* expression CK long-term effect on decreasing auxin biosynthesis rate and pool size was observed, probably mediated through an altered development (Nordström *et al.*, 2004).

We determined higher ABA content in chloroplasts and in leaves of plants overexpressing *Sho* than in WT. However, the difference in ABA levels was in leaves not significant when expressed on chlorophyll content. After overexpression of bacterial *ipt* both a decrease of ABA content, e.g. in leaves of P*ssu-ipt* tobacco (Synková *et al.*, 1999) and in petunia corollas with delayed senescence expressing *ipt* under the control of the promoter from a senescence associated gene of *Arabidopsis*, SAG 12 (Chang *et al.*, 2003), as well as an increase, e.g. in potato transformed by a vector plasmid containing the *Agrobacterium ipt* gene Macháčková *et al.* (1997), was reported.

Plants expressing CK oxidase/dehydrogenase AtCKX3

The CK oxidase/dehydrogenase selectively cleaves unsaturated N^6 side chains from Z, iP and their corresponding ribosides, while CK nucleotides, *O*-glucosides and CKs with saturated side chains are not the CKX substrates (Armstrong, 1994). It was shown that overexpression of *AtCKX* genes in tobacco and *Arabidopsis* plants resulted in reduced content of endogenous CKs and strongly altered phenotype with dwarfed shoot habit, enhanced root growth and delayed flowering and senescence (Werner *et al.*, 2001 and 2003). In 2-week-old tobacco seedlings overexpressing *35S:AtCKX1* the total CK content was reduced to 61 - 63% of WT total CK content. In *35S:AtCKX2* the decrease was even more profound (31 to 48%) (Werner *et al.*, 2001). Transgenic *Arabidopsis* plants overexpressing *35S:AtCKX1* and *35S:AtCKX2* had increased CK breakdown (30 to 45% of WT total CK content) (Werner *et al.*, 2003). In shoots harvested 14 days after germination more abundant Z-derived CKs were more strongly reduced (20 to 41% of WT) than were iP-derivatives (44 to 58% of WT), in contrast to tobacco where the more profound changes were in iP-type CKs. To our knowledge, the CK content has never been measured in any *35S:AtCKX3* transgenic plant. These plants are phenotypically identical to *35S:AtCKX1* and the enzyme is ultimately targeted to the same compartment, vacuole, proved by *in planta* experiments by fusing the C terminus of AtCKX3 to green fluorescent protein and expressing this construct under 35S promoter (Werner *et al.*, 2003). Nevertheless, the apparent K_m value for AtCKX3 against iP (7 μM) was significantly higher (14 times) than for AtCKX1 (0.5 μM) and the maximum velocity values of CKX extracts of *Arabidopsis* callus derived from root tissue overexpressing the *AtCKX* genes V_{max} even 77fold higher (Werner *et al.*, 2003). We have showed that the ectopic expression of *AtCKX3* reduced the CK levels in tobacco leaves of adult plants and as well influenced the CK levels in another compartment than to which the enzyme has been targeted, in chloroplasts. In leaves, the most strongly reduced CK metabolites were *N*-glucosides that are believed to be the physiologically inactive irreversible conjugates of CKs. However, Werner *et al.* (2001) determined very low concentrations of *N*-glucosides in tobacco WT seedlings that were only marginally changed in transgenic plants. This discrepancy could be explained by different developmental stage of analysed tobacco plants. In *Arabidopsis* seedlings the Z9G and iPG were measurable and were reduced in *35S:AtCKX1* and *35S:AtCKX2* plants compared with WT plants. The *N*-glucosides are supposed not to be degraded by CKX,

as zeatin-9-glucoside was not substrate for maize CKX (Bilyeu *et al., 2001*), even though this is in contrast with results of an earlier study (McGaw and Horgan, 1983).

Werner *et al.* (2003) found reduced levels of IAA in *Arabidopsis* seedlings overexpressing *35S:AtCKX1* (53-66% of WT content) and *35S:AtCKX2* (73-76% of WT content). The authors speculate that there need not have been direct regulation of IAA metabolism by CKs, but the different tissue composition, i.e. reduced size of shoot apical meristem with fewer meristematic cells, reduced cell production in leaves, in transgenic plants might lead to a lowering of IAA-producing shoot tissue. Nevertheless, we did not observe any significant differences in IAA levels of leaves of *AtCKX3* and control adult tobacco when expressed on fresh weight. Moreover, the IAA content was higher in leaves of transgenic plants when expressed on chlorophyll content.

Brugière *et al.* (2003) demonstrated induction of maize *CKX1* gene expression in leaf discs by ABA suggesting a role for this hormone in lowering CK concentrations under different abiotic stresses. Setter *et al.* (2001) observed a dramatic ABA level increase in kernels and a concomitant Z-type CK decrease in pedicels of drought-stressed maize during early kernel development. Using massive parallel signature sequencing gene expression technology only the up-regulation of *AtCKX7* by ABA application in *Arabidopsis* seedlings was identified (Hoth *et al.,* 2002). We have found lower ABA level in leaves of transgenic *AtCKX3* tobacco compared with control plants, which might be linked with prolonged life span and retarded senescence of transgenic plants.

Plants expressing β-glucosidase Zm-p60.1

Maize β-glucosidase Zm-p60.1 was shown to be capable of releasing active CKs from *O-* and *N3*-glucosides in tobacco protoplasts (Brzobohatý *et al.,* 1993) and to be located in plastids (Kristoffersen et al., 2000). We have recently thoroughly analysed the transgenic tobacco overexpressing *Zm-p60.1* (Kiran *et al.,* 2006). We have demonstrated that these plants are perturbed in CK metabolism, as seedlings grown on exogenous zeatin accumulated CKs to higher levels than WT and formed ectopic structures at the base of hypocotyls at lower zeatin concentration than WT seedlings. Nevertheless, the adult plants grown in the absence of exogenous zeatin displayed no phenotype alterations, although changes in hormone content were found. In upper leaves and internodes higher content of CK free bases and ribosides than in corresponding parts of WT was determined (Kiran *et al.*, 2006). We have observed tendency to higher accumulation of free bases and ribosides in leaves as well as in

chloroplasts of transgenic plants. The level of CK *O*-glucosides decreased in chloroplasts isolated from transgenic plants. We previously performed kinetic analysis of Zm-p60.1. We determined the apparent K_m value for Zm-p60.1 in chloroplasts isolated from transgenic tobacco with ^{3}H-labelled ZOG as a substrate and we found that it is 10-fold lower than the apparent K_m determined *in vitro* for the purified enzyme against the same substrate (Kiran *et al.*, 2006).

We reported steeper fall of IAA gradient from high to low from youngest leaves downward and lower IAA content in apex and first internodes in Zm-p60.1 tobacco than in corresponding parts of control plants (Kiran *et al.*, 2006). Here we show that IAA levels decreased in leaves as well as in chloroplasts of plants overexpressing *Zm-p60.1.*

ABA measurements revealed lower ABA content in mature leaves of transgenic plants than in leaves of WT plants. This finding is in contrast to our previous study, when tendency of increased ABA accumulation especially in older leaves was observed. In chloroplasts, however, the ABA level did not significantly differ between transgenics and WT.

Plants expressing zeatin O-glucosyltransferase ZOG1

Martin *et al.* (2001) constructed transgenic tobacco carrying zeatin *O*-glucosyltransferase (*ZOG1*) gene from *Phaseolus lunatus* under the control of a constitutive CaMV 35S promoter. They found ZOG content elevated to 26 pmol per fresh weight in leaves from less than 1 pmol in control plants and no substantial changes in the level of other CK metabolites. We detected even one order of magnitude higher accumulation of ZOG in leaves of transgenic tobacco, which could be caused by older analysed material in our case and different cultivation conditions. In the previous study the tobacco plants were grown hydroponically. The elevated CK *O*-glucoside concentrations in whole leaves did not significantly influence the content of these metabolites in isolated intact chloroplasts of transgenic plants. This indicates that CK *O*-glucosides are accumulated particularly outside chloroplasts, probably within vacuoles that are supposed to serve as the main storage compartment especially for CK *O*-glucosides (Fusseder and Ziegler 1988).

Martin *et al* (2001) reported that IAA does not serve as a substrate for zeatin *O*-glucosyltransferase and therefore excluded changes in IAA glucosylation to trigger the observed variations in morphology of transformants (e.g. aerial roots, multiple shoots, more compact stature than WT). Interestingly, we found twofold higher IAA content in

leaves of transgenic plants. ABA level was remarkably higher only when expressed on chlorophyll in *ZOG1* leaves. Both IAA and ABA contents were higher in *ZOG1* chloroplasts than in chloroplasts of WT.

Ultrastructure observations

We have observed considerable changes in the ultrastructure of CK overproducing tobacco constitutively expressing *Sho* gene, including increased number of plastoglobuli and appearance of crystalloids in chloroplasts of plants grown *in vitro*. These crystalloids were not found in chloroplasts of plants either with dexamethasone induced *Sho* expression or other CK metabolism transformants analyzed in this work or their corresponding controls. In another type of transgenic tobacco with elevated endogenous CKs, carrying *Agrobacterium tumefaciens isopentenyltransferase* gene under the control of the light-inducible promoter for the *Pisum sativum* small subunit of Rubisco (P*ssu-ipt*), anomalies in chloroplast ultrastructure were found too (Synková *et al.*, 1997 and 2003). Irregular or amoeboid shape of plastids often accompanied with „tubular clusters" resembling peripheral reticulum, sometimes swollen thylakoid membranes, larger light plastoglobuli and protein crystals with a fine membrane like structure were observed in a significant part of plastids from P*ssu-ipt* tobacco. Anomalous vesicles and large amount of swollen thylakoids were also found in P*ssu-ipt* tobacco calli (Beinsberger *et al.*, 1991). The distinct chloroplast crystalline structures occupying up to 16% of chloroplast volume in transgenic P*ssu-ipt* plants were further studied and it was suggested that they are probably formed by light-harvesting complex proteins, which aggregate in the form of 2D crystals that constitute membrane stacks (Synková *et al.*, 2006). Smaller crystals were also present in mitochondria and peroxisomes. Nevertheless, no significant differences in chloroplast cross section length, starch content, grana width and number of thylakoids per granum were reported between chloroplasts of transgenic and control tobacco during plant ontogeny (Synková *et al.*, 2003). The number of plastids per mesophyll cell exhibited the peak at the onset of flowering in control plants, whereas the chloroplast number was stable in transgenic tobacco throughout the ontogeny. We recently found that CK levels in chloroplasts of P*ssu-ipt* plants were elevated (Synková *et al.*, 2006). We have suggested that the ultrastructure anomalies might be the consequence of CK overproduction creating long-lasting unfavourable conditions within the cell (Synková *et al.*, 2006). Here we report only twice higher CK content in chloroplasts of plants with dexamethasone-induced *Sho*

expression, where no crystalloids were present. Unfortunately, because of lack of material we were not able to assess the CK content in chloroplasts of *Sho* overexpressing tobacco cultivated *in vitro*, and thus we could not confirm the correlation between the occurrence of crystalloids and increased CK content in these organelles as in the case of P*ssu-ipt* plants.

When dexamethasone-inducible *Sho* plants were analysed, increased grana stacking was noticed. Likewise, an increase in grana stacking was observed in chloroplasts of transgenic tobacco carrying *ipt* from *Agrobacterium* (Čatský *et al.*, 1993) and in tobacco plantlets cultivated *in vitro* when CK was applied exogenously (Wilhelmová and Kutík, 1995). Besides, an elevated concentration of BA in the medium induced profound starch accumulation and lowering of chlorophyll a/b ratio in chloroplasts. The chloroplasts of CK treated plantlets were smaller and more flattened than chloroplasts of control plantlets. An increased chloroplast number per cell, fewer plastoglobuli and a higher degree of grana stacking than in controls was noted in chloroplasts of sepals of *Helleborus niger* depistillated flowers after CK treatment (Salopek-Sondi *et al.*, 2002). In contrast, aberrant chloroplast development (irregular-shaped chloroplasts with little thylakoid membrane stacking) was observed among other developmental abnormalities in shooty seedlings and callus of tobacco with 3-fold higher CK levels than in controls where *CHRK1* (a chitinase-related receptor-like kinase) expression was suppressed (Lee *et al.*, 2003).

It has been demonstrated that the effect of CKs on plastid structure depends on the stage of plant development. In early phase of development of *Zea mays* seedlings, pre-treatment with BA in the dark stimulated photosynthetic activity and chlorophyll accumulation. After light exposure BA did not increase photosynthetic activity nor chlorophyll accumulation, accelerated grana stacking in mesophyll cell chloroplasts and grana loss in chloroplasts of bundle sheath cells, whereas later it stimulated plastid multiplication and does not affect the ultrastructure of mature chloroplasts (Caers and Vendrig, 1986).

Except of *Sho* tobacco, we did not reveal any considerable changes in the ultrastructure of chloroplasts of other CK transformants analysed. Only a difference in starch accumulation associated with a change in chloroplast shape was perceived. Thus, chloroplasts of *AtCKX3* and *Zm-p60.1* tobacco contained less starch and were more flattened than the chloroplasts of WT, whereas chloroplasts of *ZOG1* tobacco with more starch inclusions were relatively thicker than WT chloroplasts. Stoynova *et al.* (1996)

reported increased starch content in chloroplasts of radish plants after treatment with phenylurea CK . It should be noted that starch grains are formed especially in cells of intensively photosynthesising plants and that starch accumulation is influenced by many factors, especially by light conditions (Lloyd *et al.*, 2005).
The involvement of endogenous CKs in starch formation and amyloplast development by application of lovastatin, an inhibitor of HMG-CoA reductase (key enzyme in mevalonic acid synthesis) to CK-autonomous tobacco BY-2 cells was studied (Miyazawa *et al.*, 2002). After addition of lovastatin to amyloplast inducing, auxin-depleted, medium, the content of endogenous *trans*-zeatin riboside decreased, which led to the reduction of starch accumulation and to the decrease of the transcriptional level of the ADP-glucose pyrophosphorylase small subunit gene (encoding a protein indispensable for starch biogenesis). These changes were restored by application of mevalonic acid or different CKs.
Further, Ananieva *et al.* (2004) showed that the dark-induced chloroplast destruction in intact zuccini (*Cucurbita pepo)* cotyledons during senescence correlated with a decline in the content of endogenous both physiologically active CK bases, ribosides and CK nucleotides, whereas the recovery of the normal chloroplast ultrastructure was accompanied by an enhancement of active CKs and CK nucleotides.

In conclusion, we found a highly non-uniform distribution of CKs between chloroplasts and the rest of tobacco leaf cells in transgenic lines with several distinct alterations of CK metabolism. Thus, it is evident that the estimation of overall hormone content in plant tissue is not sufficient to assess its level at a particular site of its action. Our results suggest that chloroplasts are relatively independent organelles in respect to regulation of CK metabolism, which reflects the importance of CKs for their development and function.

Acknowledgements
We are very grateful to Prof. Peter Meyer (*Sho*), Prof. Thomas Schmülling (*AtCKX3*) and Prof. Machteld Mok (*ZOG1*) for kind donation of transgenic material. This work was supported by grants nos 206/03/0369 (Grant Agency of the Czech Republic) and IAA600040612 (Grant Agency of the Academy of Sciences of the Czech Republic).

References:

Abdelghani MO, Suty L, Chen JN, Renaudin JP, Teyssendier de la Serve B (1991): Cytokinins modulate the steady-state levels of light-dependent and light-independent proteins and mRNAs in tobacco cell suspensions. Plant Sci 77: 29-40

Ananieva K, Malbeck J, Kamínek M, Staden JV (2004): Changes in endogenous cytokinin levels in cotyledons of *Cucurbita pepo* (zucchini) during natural and dark-induced senescence. Phys Plant 122: 133-142

Armstrong DJ (1994): Cytokinin oxidase and the regulation of cytokinin degradation. In DWS Mok, MC Mok, eds, Cytokinins: Chemistry, Activity, and Function. CRC Press, Boca Raton, FL, pp 139–154

Arnon DI (1949): Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1–15

Beinsberger SEI, Valcke RLM, Deblaere RY, Clijster HMM, De Greef JA, Van Onckelen HA (1991): Effects of the introduction of *Agrobacterium tumefaciens* T-DNA *ipt* gene in *Nicotiana tabacum* L. cv. Petit Havana SR1 plant cells. Plant Cell Physiol 32: 489-496

Benková E, Witters E, Van Dongen W, Kolář J, Motyka V, Brzobohatý B, Van Onckelen HA, Macháčková I (1999): Cytokinins in tobacco and wheat chloroplasts. Occurrence and changes due to light/dark treatment. Plant Physiol 121: 245-251

Bieleski RL (1964) The problem of halting enzyme action when extracting plant tissues. Anal Biochem 9:431–442

Bilyeu KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, Kramer MD, Morris RO (2001): Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol 125: 378-386

Boasson R, Laetsch WM (1969): Chloroplast replication and growth in tobacco. Science 166: 749-751

Brenner WG, Romanov GA, Köllmer I, Bürkle L, Schmülling T (2005): Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. The Plant Journal 44: 314–333

Brugière N, Jiao S, Hantke S, Zinselmeier Ch, Roessler JA, Niu X, Jones RJ, Habben JE (2003): Cytokinin oxidase gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic stress. Plant Physiol 132: 1228-1240

Brzobohatý B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K (1993): Release of active cytokinin by a β-glucosidase localized to the maize root meristem. Science 262: 1052–1054

Caers M, Vendrig JC (1986): Benzyladenine effects on the development of the photosynthetic apparatus in *Zea mays*: Studies on photosynthetic activity, enzymes and (etio)chloroplast ultrastructure. Physiol Plant 66: 685-691

Čatský J, Pospíšilová J, Macháčková I, Wilhelmová N, Šesták Z (1993): Photosynthesis and water relations in transgenic tobacco plants with T-DNA carrying gene 4 for cytokinin synthesis. Biologia Plantarum 35: 393-399

Chang H, Jones ML, Banowetz GM, Clark DG (2003): Overproduction of cytokinins in petunia flowers transformed with P_{SAG12}-IPT delays corolla senescence and decreases sensitivity to ethylene. Plant Physiology 132: 2174–2183

Chory J, Reinecke D, Sim S, Wasburn T, Brenner M (1994): A role for cytokinins in de-etiolation in *Arabidopsis.* Plant Physiol 104: 339-347

Davey JE, Van Staden J (1981): Cytokinins in spinach chloroplasts. Ann Bot 48: 243-246

Dobrev PI, Kamínek M (2002): Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J Chromatogr 950: 21–29

Dobrev PI, Havlíček L, Vágner M, Malbeck J, Kamínek M (2005): Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography. Journal of Chromatography A 1075: 159-166

Eklöf S, Åstot C, Moritz T, Blackwell J, Olsson O, Sandberg G (1997): Auxin-cytokinin interactions in wild-type and transgenic tobacco. Plant and Cell Physiology 33: 225-235

Eklöf S, Åstot C, Sitbon F, Moritz T, Olsson O, Blackwell J, Sandberg G (2000): Transgenic tobacco plants co-expressing *Agrobacterium iaa* and *ipt* genes have wild-type hormone levels but display both auxin- and cytokinin-overexpressing phenotypes. Plant J 23: 279-284

Fregeau JA, Wightman F (1983): Natural occurrence and biosynthesis of auxins in chloroplast and mitochondrial fractions from sun flower leaves. Plant Sci Lett 32: 23-34

Fusseder A, Ziegler P (1988): Metabolism and compartmentation of dihydrozeatin exogenously supplied to photoautotrophic suspension cultures of *Chenopodium rubrum*. Planta 173: 104–109

Hoth S, Morgante M, Sanchez J-P, Hanafey MK, Tingey SV, Chua N-H (2002): Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant. J Cell Sci 115: 4891-4900

Kakimoto T (2001): Identification of plant cytokinin biosynthesis enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. Plant Cell Physiol 42: 677-685

Kärkönen A, Simola LK (1999): Localization of cytokinins in somatic and zygotic embryos of *Tilia cordata* using immunocytochemistry. Physiol. Plant. 105: 356-366

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara S (2004): Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. J Biol Chem 279: 14049–14054

Kiran NS, Polanská L, Fohlerová R, Mazura P, Válková M, Šmeral M, Zouhar J, Malbeck J, Dobrev PI, Macháčková I, Brzobohatý B (2006): Ectopic over-expression of the maize β-glucosidase Zm-p60.1 perturbs cytokinin homeostasis in transgenic tobacco. J Exp Bot 57: 985-996

Kristoffersen P, Brzobohatý B, Hohfeld I, Bako L, Melkonian M, Palme K (2000): Developmental regulation of the maize Zm-p60.1 gene encoding a β-glucosidase localized to plastids. Planta 210: 407–415

Kusnetsov VV, Oelmüller R, Sarwatt MI, Porfirova SA, Cherepneva GN, Herrmann RG, Kulaeva ON (1994): Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons without notable effect on steady-state mRNA levels. Planta 194: 318-327

Latzko E, Gibbs M (1968): Distribution and activity of enzymes of the reductive pentose phosphate cycle in spinach leaves and chloroplasts isolated by different methods. Z Pflanzenphysiol 59: 184–194

Lee HJ, Takei K, Sakakibara H, Cho HS, Kim DM, Kim YS, Min SR, Kim WT, Sohn DY, Lim YP, Pai HS (2003): CHRK1, a chitinase-related receptor-like kinase, plays a role in plant development and cytokinin homeostasis in tobacco. Plant Mol Biol 53: 877-890

Lloyd JR, Kossmann J, Ritte G (2005): Leaf starch degradation comes out of the shadows. Trends in Plant Science 10: 130-137

Longo GP, Pedretti M, Rossi G, Longo CP (1979): Effect of benzyladenine on the development of plastids and microbodies in excised watermelon cotyledons. Planta 145: 209-217

Lyukevich TV, Kusnetsov VV, Karavaiko NN, Kulaeva ON, Selivankina SY (2002): The involvement of the chloroplast zeatin-binding protein in hormone-dependent transcriptional control of chloroplast genome. Russ J Plant Phys 49: 92-98

Macháčková I, Sergeeva L, Ondřej M, Zaltsman O, Konstantinova T, Eder J, Ovesná J, Golyanovskaya S, Rakitin Y, Aksenova N 1997: Growth pattern, tuber formation and hormonal balance in *in vitro* potato plants carrying *ipt* gene. Plant growth regulation 21: 27-36

Martin RC, Mok DWS, Smets R, Van Onckelen HA, Mok MC (2001): Development of transgenic tobacco harbouring a zeatin *O*-glucosyltransferase gene from *Phaseolus*. In Vitro Cell Dev. Biol-Plant 37: 354-360

Mc Gaw BA, Horgan R (1983): Cytokinin oxidase from *Zea mays* kernels and *Vinca rosea* crown-gall tissue. Planta 159: 30-37

Milborrow BV, Lee H-S (1998): Endogenous biosynthetic precursors of (+)-abscisic acid. VI. Carotenoids and ABA are formed by the "non-mevalonate" triose-pyruvate pathway in chloroplasts. Austr. J. Plant Physiol. 25: 507-512

Miller CO, Skoog F, von Saltza MH, Strong F (1955): Isolation, structure and synthesis of kinetin, a substance promoting cell division. J Am Chem Soc 78: 1375-1380

Miyazawa Y, Kato H, Muranaka T, Yoshida S (2002): Amyloplast formation in cultured tobacco BY-2 cells requires a high cytokinin content. Plant Cell Physiol 43: 1534-1541

Motyka V, Vaňková R, Čapková V, Petrášek J, Kamínek M, Schmülling T (2003): Cytokinin induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. Physiol Plant 117 (1): 11-21

Murashige T, Skoog F (1962): A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15: 473–497

Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Åstot C, Doležal K, Sandberg G (2004): Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. Proceedings of the National Academy of Sciences, USA 101: 8039–8044

Ohmiya A, Hayashi T (1992): Immuno-gold localization of IAA in leaf cells of *Prunus persica* at different stages of development. Physiol Plant 85: 439-445

Parthier B (1989): Hormone-induced alterations in plant gene expression. Biochem Physiol Pflanz 185: 289-314

Pastor A, López-Carbonell M, Alegre L (1999): Abscisic acid immunolocalization and ultrastructural changes in water-stressed lavender (*Lavandula stoechas* L.) plants. Physiol Plant 105: 272-279

Redig P, Schmülling T, Van Onckelen H (1996): Analysis of cytokinin metabolism in *ipt* transgenic tobacco by liquid chromatography-tandem mass spectrometry. Plant Physiol 112: 141-148

Reynolds ES (1963): The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208-212

Sakakibara H, Kasahara H, Ueda N, Kojima M, Takei K, Hishiyama S, Asami T, Okada K, Kamiya Y, Yamaya T, Yamaguchi S (2005): *Agrobacterium tumefaciens* increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. PNAS 102: 9972–9977

Salopek-Sondi B, Kovač M, Prebeg T, Magnus V (2002): Developing fruit direct post-floral morphogenesis in *Helleborus niger* L. J Exp Bot 53: 1949-1957

Sandberg G, Gardeström P, Sitbon F, Olsson O (1990): Presence of indole-3-acetic acid in chloroplasts of *Nicotiana tabacum* and *Pinus sylvetris*. Planta 180: 562-568

Schmülling T, Schäfer S, Romanov G (1997): Cytokinins as regulators of gene expression. Phys Plant 100: 505-519

Setter TL, Flanningan BA, Melkonian J (2001): Loss of kernel set due to water deficit and shade in maize: carbohydrate supplies, abscisic acid, and cytokinins. Crop Sci 41: 1530-1540

Sossountzov L, Sotta B, Maldiney R, Sabbagh I, Miginiac E (1986): Immunoelectron-microscopy localization of abscisic acid with colloidal gold on Lowicryl-embeded tissues of *Chenopodium polyspermum* L. Planta 168: 471-481

Spurr AR (1969): A low viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31-43

Stetler DA, Laetsch WM (1965): Kinetin-induced chloroplast maturation in cultures of tobacco tissue. Science 149: 1387-1388

Stoynova EZ, Iliev LK, Georgiev GT (1996): Structural and functional alterations in radish plants induced by phenylurea cytokinin 4-PU-30. Biologia Plantarum 38: 237-244

Sun J, Niu Q-W, Tarkowski P, Zheng B, Tarkowska D, Sandberg G, Chua N-H, Zuo J (2003): The Arabidopsis *AtIPT8/PGA22* gene encodes an isopentenyl transferase that is involved in de novo cytokinin biosynthesis. Plant Physiol 131: 167-176

Synková H, Van Loven K, Valcke R (1997): Increased content of endogenous cytokinins does not delay degradation of photosynthetic apparatus in tobacco. Photosynthetica 33: 595-608

Synková H, Van Loven K, Pospíšilová J, Valcke R (1999): Photosynthesis of transgenic P*ssu-ipt* tobacco. J Plant Physiol 155:173-182

Synková H, Pechová R, Valcke R (2003): Changes in chloroplast ultrastructure in P*ssu-ipt* tobacco during plant ontogeny. Photosynthetica 41: 117-126

Synková H, Schnablová R, Polanská L, Hušák M, Šiffel P, Vácha F, Malbeck J, Macháčková I, Nebesářová J (2006): Three-dimensional reconstruction of anomalous chloroplasts in transgenic *ipt*. Planta (in press)

Takei K, Sakakibara H, Sugiyama, T (2001): Identification of genes encoding adenylate isopentenyltranferase, a cytokinin biosynthesis enzyme in *Arabidopsis thaliana*. J. Biol. Chem. 276: 26405-26410

Vreman HJ, Thomas R, Corse J, Swaminathan S, Murai N (1978): Cytokinins in tRNA obtained from *Spinacia oleracea* L. leaves and isolated chloroplasts. Plant Physiol 61: 296-306

Werner T, Motyka V, Strnad M, Schmülling T (2001): Regulation of plant growth by cytokinin. Proc Natl Acad Sci USA 98: 10487-10492

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003): Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15: 2532-2550

Wilhelmová N, Kutík J (1995): Influence of exogenously applied 6-benzylaminopurine on the structure of chloroplasts and arrangement of their membranes. Photosynthetica 31: 559-570

Winiarska G, Legocka J, Schneider J, Deckert J (1994): Cytokinin-controlled expression of the apoprotein of the light-harvesting complex of photosystem II in the tissue culture of *Dianthus caryophyllus*. III. Effect of benzyladenine on the protein synthesis *in vivo* and on the level of translatable LHCP mRNA. Acta Physiologiae Plantarum 16: 203-210

Zavaleta-Mancera HA, Franklin KA, Ougham HJ, Thomas H, Scott IM (1999a): Regreening of senescent *Nicotiana* leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll *a/b*-binding protein. J Exp Bot 50: 1677-1682

Zavaleta-Mancera HA, Thomas BJ, Thomas H, Scott IM (1999b): Regreening of senescent *Nicotiana* leaves. II. Redifferentiation of plastids. J Exp Bot 50: 1683-1689

Zubko E, Adams CJ, Macháčková I, Malbeck J, Scollan C, Meyer P (2002): Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. The Plant Journal 29: 797-808

Table 1

Endogenous contents of IAA and ABA in leaves and in isolated intact chloroplasts of transgenic dexamethasone-inducible *Sho* and control SR1 tobacco. Values represent the mean of two replicates (individual measurements are shown in parentheses).

IAA	***Sho***	**SR1**
Leaves [pmol g^{-1} FW]	90.70 (117.89; 63.52)	71.17 (81.05; 61.30)
Leaves [pmol mg^{-1} chlorophyll]	73.74 (95.84; 51.64)	74.14 (84.43; 63.85)
Chloroplasts [pmol mg^{-1} chlorophyll]	2.47 (2.79; 2.15)	3.93 (2.76; 3.95)
ABA		
Leaves [pmol g^{-1} FW]	453.46 (749.12; 157.81)	359.98 (628.10; 91.85)
Leaves [pmol mg^{-1} chlorophyll]	368.67 (609.04; 128.30)	374.98 (654.27; 95.68)
Chloroplasts [pmol mg^{-1} chlorophyll]	16.80 (18.72; 14.88)	11.71 (13.34; 12.24)

Table 2

Endogenous contents of IAA and ABA in leaves and in isolated intact chloroplasts of transgenic *35S:AtCKX3* and control SNN tobacco. Values represent the mean of two replicates (individual measurements are shown in parentheses).

IAA	***35S:AtCKX3***	**SNN**
Leaves [pmol g^{-1} FW]	125.0 (177.4; 72.6)	122.1 (147.3; 96.9)
Leaves [pmol mg^{-1} chlorophyll]	200.4 (279.1; 121.8)	114.4 (151.6; 77.2)
Chloroplasts [pmol mg^{-1} chlorophyll]	5.2 (4.3; 6.1)	5.2 (9.9; 0.4)
ABA		
Leaves [pmol g^{-1} FW]	359.5 (447.7; 271.3)	1102.3 (822.9; 1381.6)
Leaves [pmol mg^{-1} chlorophyll]	579.9 (704.4; 455.4)	974.0 (847.2; 1100.9)
Chloroplasts [pmol mg^{-1} chlorophyll]	68.0 (44.0; 92.0)	58.7 (106.6; 10.9)

Table 3

Endogenous contents of IAA and ABA in leaves and in isolated intact chloroplasts of transgenic *35S:Zm-p60.1* and control SR1 tobacco. Values represent the mean of two replicates (individual measurements are shown in parentheses).

IAA	***35S:Zm-p60.1***	**SR1**
Leaves [pmol g^{-1} FW]	85.6 (95.1; 76.2)	144.0 (146.5; 141.5)
Leaves [pmol mg^{-1} chlorophyll]	60.2 (66.9; 53.6)	135.2 (137.5; 132.8)
Chloroplasts [pmol mg^{-1} chlorophyll]	0.8 (1.4; 0.2)	1.2 (2.1; 0.3)
ABA		
Leaves [pmol g^{-1} FW]	481.6 (486.9; 476.3)	839 (785.9; 892.1)
Leaves [pmol mg^{-1} chlorophyll]	338.7 (342.4; 334.9)	787.5 (737.6; 837.4)
Chloroplasts [pmol mg^{-1} chlorophyll]	23.6 (21.5; 25.8)	26.2 (40.4; 12.1)

Table 4

Endogenous contents of IAA and ABA in leaves and in isolated intact chloroplasts of transgenic *35S:ZOG1* and control W38 tobacco. Values represent the mean of two replicates (individual measurements are shown in parentheses).

IAA	***35S:ZOG1***	**W38**
Leaves [pmol g^{-1} FW]	88.8 (102.1; 75.5)	44.2 (38.4; 50.0)
Leaves [pmol mg^{-1} chlorophyll]	77.2 (88.8; 65.6)	24.0 (20.8; 27.1)
Chloroplasts [pmol mg^{-1} chlorophyll]	1.4 (1.8; 1.1)	0.9 (0.5; 1.4)
ABA		
Leaves [pmol g^{-1} FW]	528.1 (662.4; 393.8)	523.2 (566.9; 479.6)
Leaves [pmol mg^{-1} chlorophyll]	459.1 (575.9; 342.4)	284.0 (307.7; 260.3)
Chloroplasts [pmol mg^{-1} chlorophyll]	13.1 (20.9; 5.2)	10.1 (15.0; 5.2)

Figure legends

Figure 1: Cytokinin content (B+R = free bases and ribosides, NG = *N*-glucosides, OG = *O*-glucosides, P = phosphates) in leaves (per fresh weight 1A and per chlorophyll 1B) and in isolated intact chloroplasts (1C) of transgenic dexamethasone-inducible *Sho* (SHO, black) and control SR1 (WT, grey) tobacco. Error bars display SD.

Figure 2: Cytokinin content (B+R = free bases and ribosides, NG = *N*-glucosides, OG = *O*-glucosides, P = phosphates) in leaves (per fresh weight 2A and per chlorophyll 2B) and in isolated intact chloroplasts (2C) of transgenic *35S:AtCKX3* (CKX, black) and control SNN (WT, grey) plants. Error bars display SD.

Figure 3: Cytokinin content (B+R = free bases and ribosides, NG = *N*-glucosides, OG = *O*-glucosides, P = phosphates) in leaves (per fresh weight 3A and per chlorophyll 3B) and in isolated intact chloroplasts (3C) of transgenic *35S:Zm-p60.1* (P60, black) and control SR1 (WT, grey) tobacco. Error bars display SD.

Figure 4: Cytokinin content (B+R = free bases and ribosides, NG = *N*-glucosides, OG = *O*-glucosides, P = phosphates) in leaves (per fresh weight 4A and per chlorophyll 4B) and in isolated intact chloroplasts (4C) of transgenic *35S:ZOG1* (ZOG, black) and control W38 (WT, grey) tobacco. Error bars display SD.

Figure 5: Transmission electron micrographs of representative chloroplast cross-sections taken from the intact leaves of tobacco expressing *Sho* gene and four 35S enhancer elements (A, C) with detail of crystalloid (B), and control SR1 (D) tobacco growing under sterile conditions; and chloroplast cross-section detail taken from leaf of transgenic dexamethasone-inducible *Sho* (E) and control SR1 (F) tobacco cultivated in greenhouse. Abbreviations used: C- crystalloid, CW – cell wall, GT – granal thylakoids, IT – intergranal thylakoids, M- mitochondrion, P – plastoglobulus, S – stroma, SI – starch inclusion. Bar = 2 µm (A, C and D), 500 nm (B, E and F).

Figure 6: Transmission electron micrographs of representative chloroplast cross-section taken from the intact leaves of transgenic *35S:AtCKX3* (A) and control SNN (B), transgenic *35S:Zm-p60.1* (C) and control SR1 (D), transgenic *35S:ZOG1* (E) and

control W38 (F) tobacco. Abbreviations used: CW – cell wall, GT – granal thylakoids, IT – intergranal thylakoids, M- mitochondrion, P – plastoglobulus, S – stroma, SI – starch inclusion. Bar = 2 μm.

Figure 1

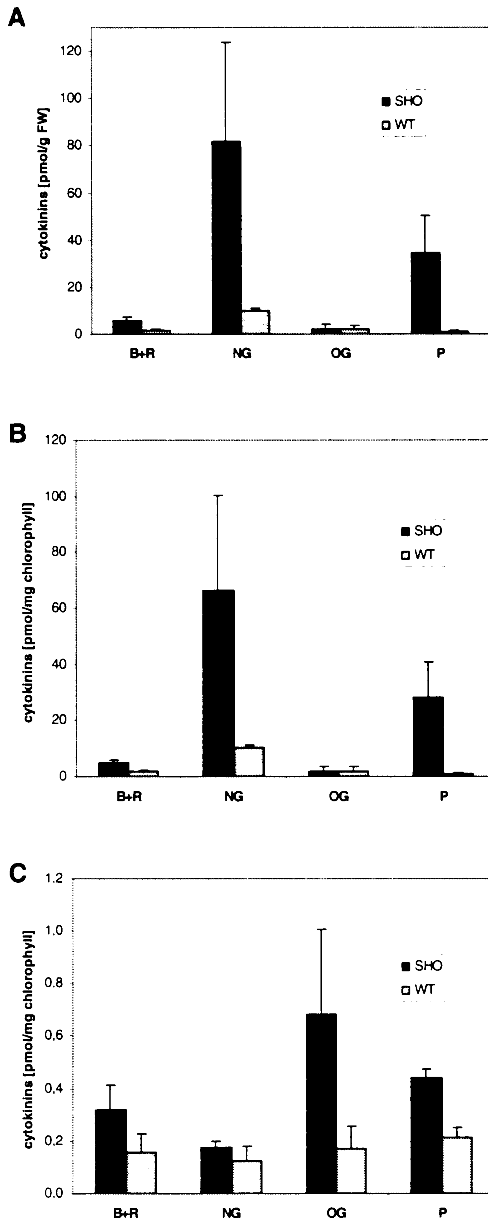


Figure 2

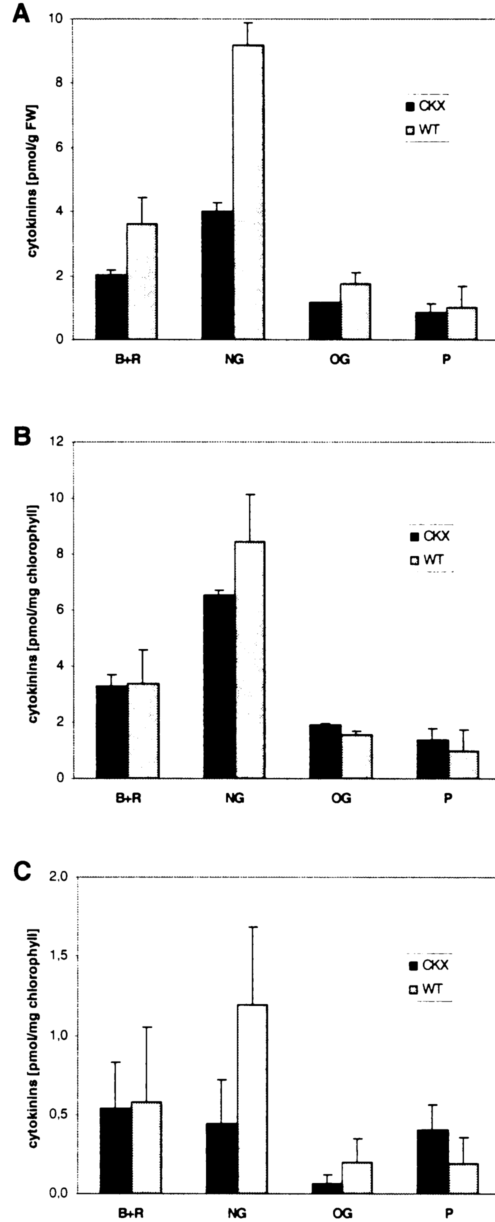


Figure 3

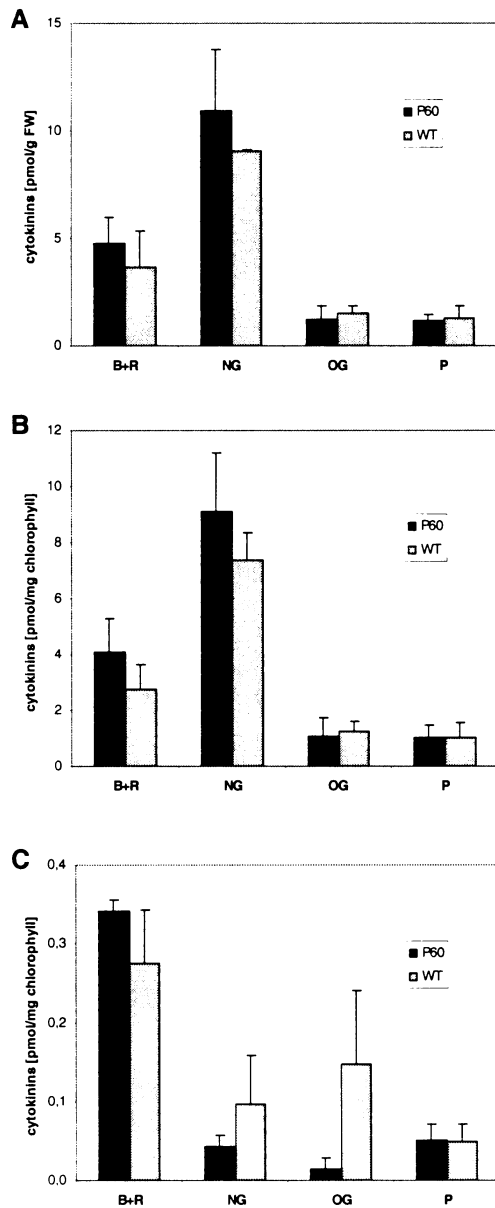


Figure 4

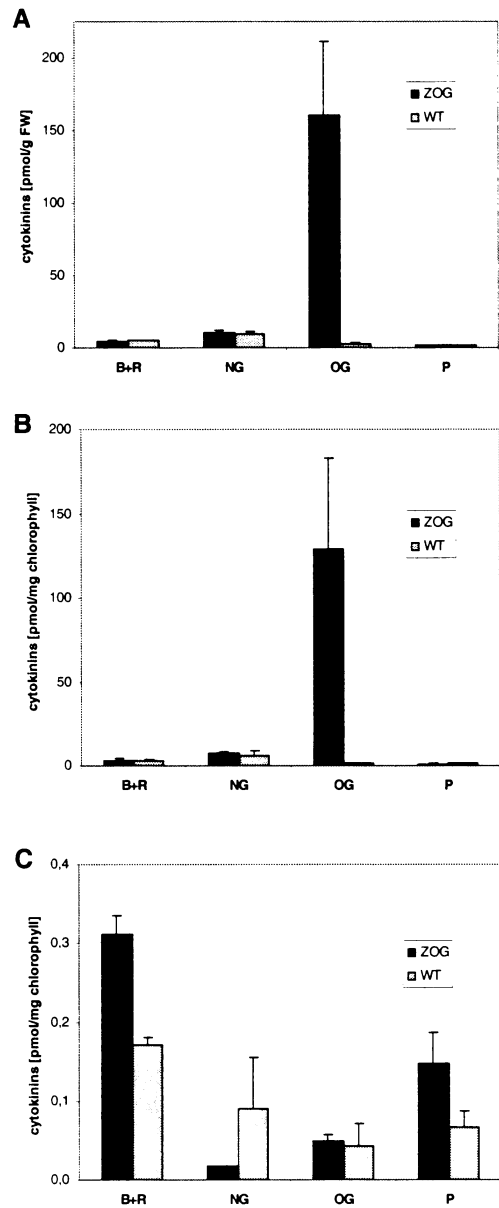


Figure 5

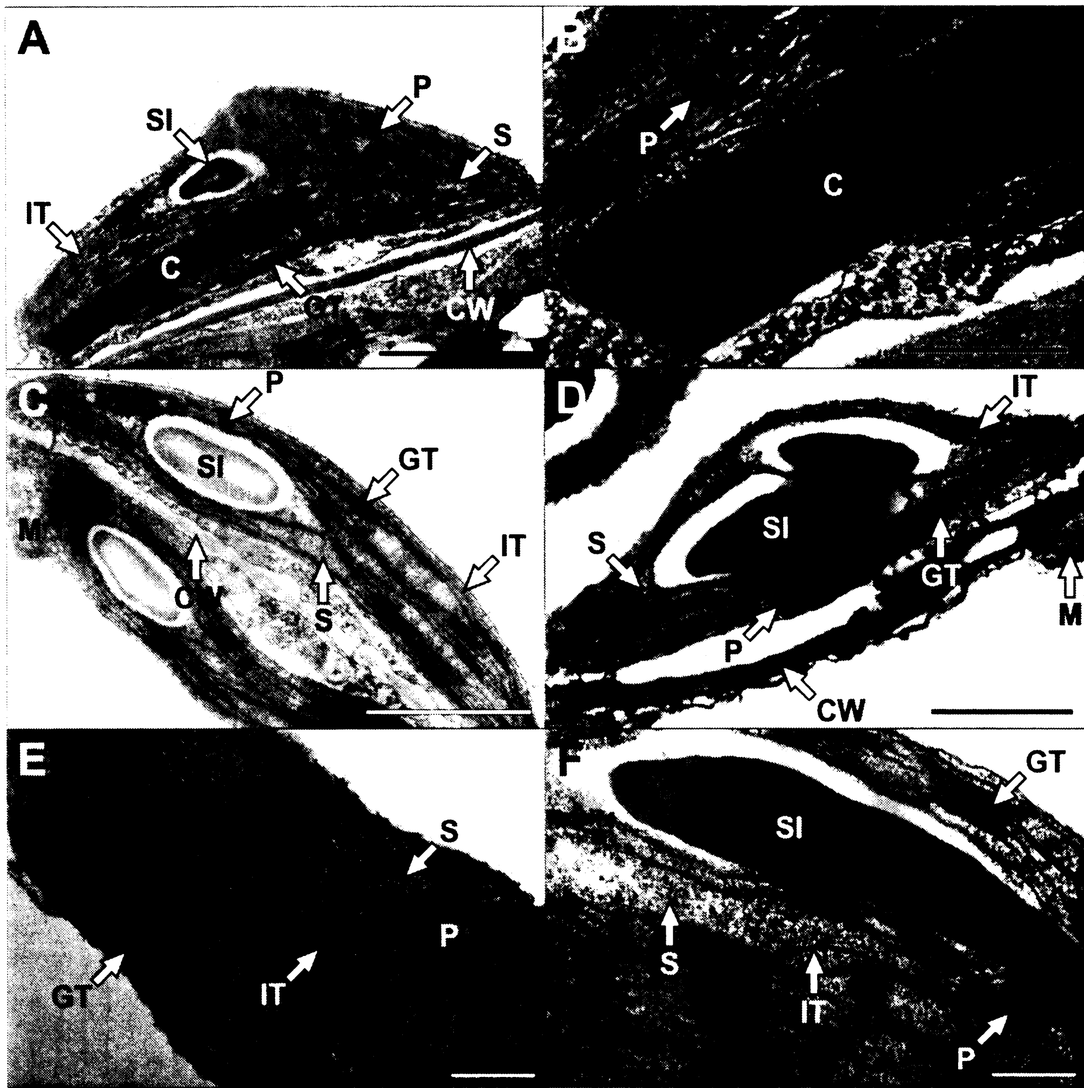


Figure 6