Nielda Karla Gonçalves de Melo

Interação entre a sinalização luminosa, hormonal e do óxido
nítrico durante o desestiolamento e desenvolvimento plastidial em
plântulas de tomateiro

Interaction between light, hormonal and nitric oxide signaling
during greening and plastid development in tomato seedlings

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Orientador: Luciano Freschi

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e ao Frank Oliveira,
por tudo e muito mais.

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e Dedico.**

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I. INTRODUÇÃO GERAL

1.1 Fotomorfogênese e diferenciação plastidial

O desenvolvimento vegetal é profundamente influenciado pelo meio ambiente circundante; conseqüentemente, as plantas dependem sobremaneira de mecanismos eficientes para perceber, interpretar, responder e se adaptar às frequentes mudanças nas condições ambientais (NEMHAUSER, 2008). Dentre os diversos sinais ambientais percebidos e interpretados pelas plantas, a aquisição de informações acerca da intensidade, duração e qualidade luminosa impacta diretamente o estabelecimento de respostas fisiológicas diversas, tais como germinação, síntese de clorofilas, expansão foliar, ritmo circadiano e floração (FANKHAUSER & CHORY, 1997). Esse controle da luz sobre o desenvolvimento vegetal é conhecido como fotomorfogênese e torna-se especialmente conspícuo durante os primeiros estágios de desenvolvimento da planta onde ocorrem eventos cruciais para o estabelecimento desta no ambiente (SYMONS & REID, 2003).

A fotomorfogênese vegetal é controlada por pelo menos quatro classes de fotorreceptores: os fitocromos que absorvem principalmente os comprimentos de luz vermelho (V) e vermelho-extremo (VE), os criptocromos que absorvem luz azul e UV-A, as fototropinas que absorvem luz azul e a proteína UVR8, recentemente identificada em *Arabidopsis* como fotorreceptor dos comprimentos de onda na faixa do UV-B (GYULA *et al.* 2003; WU *et al.*, 2012).

As fototropinas são conhecidas classicamente pela sua participação nas respostas fototrópicas dos vegetais, bem como no deslocamento de cloroplastos. Em gimnospermas e angiospermas, existem basicamente dois tipos de resposta de deslocamento de cloroplastos, uma relacionada à alteração de sua distribuição no interior da célula para maximizar a captura de luz em ambientes pouco iluminados e

outra associada ao deslocamento dessas organelas com vista à minimizar a captura excessiva da luz e, conseqüentemente, prevenir a ocorrência de fotoinibição ou geração excessiva de radicais livres (CHEN *et al.*, 2004).

Os fitocromos, por sua vez, mediam grande parte das respostas fotomorfogênicas em plantas, apresentando papel crucial na regulação de respostas à luz desde a germinação até a transição do estado vegetativo para o reprodutivo (HENNIG *et al.*, 1999). Em conjunto com os criptocromos, diversos membros da família dos fitocromos são particularmente importantes durante o processo de desestiolamento, o qual envolve uma massiva reorganização no programa transcricional da planta e uma alteração dramática em seu padrão de desenvolvimento (CHEN *et al.*, 2004; WANG *et al.*, 2001).

Em termos gerais, logo após a germinação, dois programas distintos de desenvolvimento podem ser seguidos pela plântula. Na ausência de luz, plântulas de eudicotiledôneas seguem o programa de desenvolvimento chamado estocomorfogênese, apresentando fenótipo estiolado caracterizado pela presença do gancho apical, cotilédones pouco expandidos, hipocótilos e epicótilos alongados, ausência de pigmentos fotossintéticos e presença de plastídios com reduzido desenvolvimento de seus sistemas internos de membranas (VON-ARNIM & DENG, 1996; SYMONS *et al.*, 2008; CHEMINANT *et al.*, 2011). Em contrapartida, quando o sinal luminoso é recebido, diversas mudanças fisiológicas são desencadeadas, as quais fazem parte de outro programa de desenvolvimento, a fotomorfogênese. Tais alterações preparam a plântula para produzir seus próprios fotoassimilados através do estabelecimento do processo fotossintético, marcando, portanto, a transição do estado heterotrófico para o autotrófico. Em plântulas de eudicotilêdoneas, por exemplo, as principais mudanças morfológicas durante o desestiolamento consistem na perda do gancho apical, expansão de cotilédones e desenvolvimento de folhas primárias, redução do alongamento de

hipocótilos e epicótilos, diferenciação e desenvolvimento de cloroplastos e síntese e acúmulo de clorofilas e demais pigmentos (FANKHAUSER & CHORY, 1997; SYMONS *et al.*, 2008). Assim sendo, o processo de desestiolamento das plântulas nada mais é do que a transição entre o programa de escotomorfogênese para o de fotomorfogênese, sendo a luz o sinal ambiental desencadeador desse processo (NEMHAUSER, 2008).

Dentre estas diversas mudanças morfológicas e fisiológicas associadas ao processo de desestiolamento, a biogênese e maturação dos cloroplastos constituem uma etapa fundamental para o pleno desenvolvimento do vegetal, pois além de serem essenciais para a ocorrência do processo fotossintético, estas organelas também atuam de forma crítica em diversas outras importantes vias bioquímicas, tais como na síntese e degradação de amido, redução do nitrogênio, biossíntese de ácidos graxos e isoprenóides e, até mesmo, na biossíntese de fitormônios, tais como o ABA e as giberelinas (NEUHAUS & EMES, 2000; RODRÍGUEZ-CONCEPCIÓN & BORONAT, 2002; LÓPEZ-JUEZ & PYKE, 2005).

Diante desse papel primordial, mesmo células não fotossintetizantes possuem plastídios, os quais exercem uma grande diversidade de funções metabólicas (WATERS & PYKE, 2004). Plastídios de células embrionárias ou meristemáticas, por exemplo, são denominados proplastídios e caracterizam-se pelo seu tamanho reduzido e por apresentarem um sistema interno de membranas pouco desenvolvido, atuando principalmente como precursores de outros plastídios (cloroplastos, cromoplastos, amiloplastos) em tecidos maduros da planta (PYKE & LEECH, 1992; WATERS & PYKE, 2004). Em células de raízes e de folhas muito jovens, as quais possuirão cloroplastos em algum momento de seu desenvolvimento, são encontrados inicialmente plastídios com uma alta variabilidade morfológica, sendo maiores que os proplastídios e

apresentando membranas internas mais desenvolvidas, os quais são denominados plastídios ameboides (LÓPEZ-JUEZ & PYKE, 2005).

Um dos tipos mais importantes de plastídios são os amiloplastos, uma vez que estes armazenam grãos de amido oriundos do processo fotossintético e apresentam a via oxidativa da pentose-fosfato muito ativa, a qual é responsável pela geração de energia para a assimilação de nitrogênio e outros processos fisiológicos (NEUHAUS & EMES, 2000). Adicionalmente, os amiloplastos são constituintes fundamentais das células dos órgãos de armazenamento como tubérculos, cotilédones e endosperma de sementes (STAEHELIN & NEWCOMB, 2000; WATERS & PYKE, 2004). Existem ainda os leucoplastos, plastídios especializados na estocagem de lipídios, e os elaioplastos, envolvidos na estocagem de óleos aromáticos em órgãos de armazenamento como, por exemplo, sementes oleaginosas (LÓPEZ-JUEZ & PYKE, 2005).

Os plastídios também possuem a capacidade de acumular diversos pigmentos, como os carotenoides e xantofilas que são responsáveis pela coloração amarela, laranja e vermelha de flores e frutos. Tais plastídios são chamados de cromoplastos e podem ser originados diretamente de proplastídios ou indiretamente a partir de cloroplastos ou amiloplastos (EGEA, *et al.*, 2011).

Por outro lado, em células vegetais totalmente privadas de luz ou expostas a níveis insuficientes desse sinal ambiental, os proplastídios passam a acumular lipídios, protoclorofila (molécula precursora da clorofila), protoclorofila redutase (POR, enzima responsável pela conversão de protoclorofila em clorofila), NADPH, e alguns carotenoides como a luteína e a violaxantina (ARMSTRONG *et al.*, 1995; VINTI *et al.*, 2005). Proplastídios que passam por essa diferenciação passam a ser chamados de etioplastos, e podem ser identificados pela estrutura semicristalina denominada corpo prolamelar, a qual na presença de luz dá origem aos tilacóides (LÓPEZ-JUEZ & PYKE,

2005). Tendo em vista que para grande parte das plantas terrícolas, o início do desenvolvimento da plântula normalmente ocorre num ambiente com pouco ou nenhum acesso à luz, a conversão dos proplastídios em etioplastos parece ser de grande valor adaptativo, uma vez que a formação de cloroplastos mediante a exposição da planta ao ambiente iluminado seria facilitada justamente pelo fato dos etioplastos possuírem tais corpos prolamelares (FORREITER & APEL, 1993; HEYES & HUNTER 2005; POGSON & ALBRECHT, 2011).

Logo após a exposição ao sinal luminoso de qualidade e intensidade adequada, estruturas chamadas prototilacóides originam-se a partir dos corpos prolamelares se espalhando pelo estroma desses plastídios (POGSON & ALBRECHT, 2011). Em angiospermas, a luz estimula a atividade da enzima POR que converterá a protoclorofila previamente acumulada nos corpos prolamelares em clorofilida *a*, a qual será subsequentemente convertida em clorofilas *a* e *b*. Juntamente com a produção de clorofilas, tilacóides e fotossistemas são formados, as enzimas responsáveis pelas reações fotossintéticas são produzidas e, a partir de então, o plastídio passa a ser capaz de captar a luz e executar todas as etapas do processo fotossintético (FORREITER & APEL, 1993; POGSON & ALBRECHT, 2011). Assim sendo, o padrão temporal de síntese e acúmulo de clorofilas durante o desestiolamento (*greening*, em inglês) é rigorosamente regulado pelas condições ambientais circundantes, uma vez que esta resposta fisiológica deve ocorrer apenas quando a maquinaria fotossintética já estiver plenamente funcional (LÓPEZ-JUEZ & PYKE, 2005; CHEMINANT *et al.*, 2011), caso contrário, processos deletérios, tais como danos oxidativos decorrente da formação de espécies reativas de oxigênio, podem ocorrer, comprometendo, portanto, o estabelecimento da plântula no ambiente (HEYES & HUNTER 2005; WATERS & LANGDALE, 2009; REINBOTHE *et al.* 2010; KOBAYASHI *et al.*, 2012).

A abundância de cloroplastos em cada tipo celular também deve se ajustar à importância relativa dessa célula no que tange ao processo de fixação de carbono (PYKE, 2009). Assim sendo, células epidérmicas ou meristemáticas possuem poucos cloroplastos, ao passo que as células do parênquima clorofiliano possuem uma alta densidade dessas organelas (WATERS & LANGDALE, 2009; PYKE, 2009). Dessa forma, não apenas a diferenciação plastidial, mas também a divisão dessas organelas necessita ser precisamente regulada pela célula hospedeira. Ainda há grandes lacunas no conhecimento sobre o processo de divisão plastidial nas células vegetais e sobre os mecanismos regulatórios envolvidos na coordenação da população plastidial em diferentes tipos celulares (WATERS & LANGDALE, 2009). Conforme descrito em diversos trabalhos (PYKE, 1999, 2010; LÓPEZ-JUEZ, 2007; MAPLE & MØLLER, 2007; YANG *et al.*, 2008), a biogênese de novos cloroplastos obrigatoriamente ocorre através da fissão binária de plastídios pré-existentes, sendo necessária a formação de um anel constricional e a expansão dos sistemas de membranas. Sabe-se também que a regulação da população e volume total de plastídios dentro das células vegetais depende tanto de sinais internos, tais como fatores de transcrição e fitormônios, quanto externos, com especial destaque para a disponibilidade e qualidade luminosa (GALPAZ *et al.*, 2008; JONES *et al.*, 2002; RAYNAUD *et al.*, 2005).

Durante a última década, grandes avanços foram alcançados na elucidação das cascatas de sinalização responsáveis por integrar a percepção da luz e os processos de formação de cloroplastos a partir de etioplastos ou proplastídios (RASCIO *et al.*, 1984; TERRY *et al.*, 2001; SOLYMOSI & SCHOEFS, 2010). Como veremos a seguir, estudos com mutantes portadores de alterações na percepção ou transdução do sinal luminoso foram especialmente importantes para a identificação dos elementos chave responsáveis pela interligação entre a percepção deste sinal ambiental e o controle da

abundância e diferenciação plastidial e acúmulo de pigmentos durante o processo de desestiolamento (CHORY & PETO 1990; DENG & QUAIL, 1992; MUSTILLI *et al.*, 1999; WANG *et al.*, 2001; TERRY *et al.*, 2001).

Em *Arabidopsis*, foi demonstrado que a perda de função de proteínas PIFs (PHYTOCHROME INTERACTING FACTORS) pode influenciar negativamente o estabelecimento de processos fotomorfogênicos tanto em plântulas germinadas na presença quanto na ausência de luz (SHIN *et al.*, 2009; STEPHENSON *et al.*, 2009). Em um estudo realizado por SHIN e col. (2009), foi observado que plântulas estioladas portadoras das mutações *pif1*, *pif3* ou *pif1pif3* apresentavam hipocótilos reduzidos, maior expansão cotiledonar, altos teores de protoclorofilas e etioplastos com maior quantidade de prototilacóides, indicando, portanto, que PIF1 e PIF3 atuariam como reguladores negativos do desenvolvimento dos cloroplastos.

Complementarmente, proteínas como COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) ou DET1 (DE-ETIOLATED 1), as quais atuam inibindo respostas ao estímulo luminoso (DENG & QUAIL 1992; PEPPER *et al.*, 1994; FANKHAUSER & CHORY, 1997; WEI & DENG, 1999), parecem desempenhar um papel supressor ao desenvolvimento de cloroplastos em cotilédones de plântulas crescidas no escuro, bem como em tecidos não fotossintetizantes de plantas desenvolvidas na presença de luz, tais como os tecidos radiculares (CHORY & PETO, 1990; DENG & QUAIL 1992; LEBEDEV *et al.*, 1995; DAVULURI *et al.*, 2004). Sabe-se, por exemplo, que plastídios de raízes de plântulas do mutante *det1* de *Arabidopsis* costumam se diferenciar em cloroplastos mesmo quando germinadas no escuro (CHORY & PETO, 1990). De forma semelhante, plântulas estioladas de *Arabidopsis* carregando a mutação recessiva *cop1* também apresentam características de plântulas desenvolvidas na presença de luz, tais como acentuada expansão dos cotilédones,

acúmulo de antocianinas e diferenciação de plastídios em formas intermediárias de cloroplastos, sendo que, em tratamentos mais prolongados, tais mutantes são capazes até mesmo de emitir o primeiro par de folhas verdadeiras (DENG & QUAIL, 1992). C

Curiosamente, algumas proteínas DET e COP interagem entre si e com outras proteínas reguladas pela luz como DDB1a (UV-DAMAGED DNA BINDING PROTEIN 1a), dando origem ao chamado sinalossomo COP9 (WEI & DENG, 1999; WEI *et al.*, 2008), que marca algumas proteínas como HY5 (LONG HYPOCOTYL 5) para degradação proteossômica (WEI *et al.*, 2008). HY5 é um fator de transcrição que atua a jusante de COP1 como um regulador positivo da fotomorfogênese (BAE & CHOI, 2008), cujo papel promotor do desenvolvimento plastidial e acúmulo de pigmentos tem sido recorrentemente demonstrado (LIU *et al.*, 2004; LÓPEZ-JUEZ, 2007; KOBAYASHI *et al.*, 2012).

De forma similar ao observado para *Arabidopsis*, DET1, DDB1, COP1, HY5 e outros componentes das cascatas de sinalização desencadeadas pela luz, têm sido identificados como elementos chaves no controle da biogênese e diferenciação plastidial tanto em folhas quanto em frutos de plantas de tomateiro (*Solanum lycopersicum*) (DAVULURI *et al.* 2005; KOLOTILIN *et al.*, 2007; LIU *et al.*, 2004; WANG *et al.*, 2008).

Nesse modelo vegetal de alto interesse econômico, mutantes que carregam as mutações monogênicas *high pigment* (*hp-1*, *hp-1^w*, *hp-2*, *hp-2^j* e *hp-2^{dg}*) desenvolvem fenótipo característico de respostas exacerbadas à luz, tais como plântulas com hipocótilos curtos e com altos teores de antocianinas, folhas mais escuras devido aos teores elevados de clorofilas, pigmentação mais escura em frutos imaturos e maduros, bem como um nítido aumento no tamanho e número de cloroplastos por célula (BINO

et al., 2005; LEVIN *et al.*, 2006; LIEBERMAN *et al.*, 2004; WANG *et al.*, 2008). Mutações em *hp-1* e em *hp-2* ocorrem em genes homólogos e ortólogos ao *DDB1* e *DET1* de *A. thaliana*, respectivamente (LIEBERMAN *et al.*, 2004; LIU *et al.*, 2004; MUSTILLI *et al.*, 1999).

Em consonância ao observados no mutante *hp-1*, frutos de tomateiro transgênicos que tiveram a expressão de *HP1/DDB1* ou *HP2/DET1* reduzida, apresentaram um aumento significativo no número de plastídios e nos teores de pigmentos (DAVULURI, *et al.*, 2004; WANG *et al.*, 2008). Tais resultados indicam que as proteínas HP1/DDB1 e HP2/DET1 desempenham papel crítico na divisão e no desenvolvimento plastidial também em tecidos reprodutivos de tomateiro (YEN *et al.*, 1997; COOKSON *et al.*, 2003; KOLOTILIN *et al.*, 2007; WANG, *et al.*, 2008). Da mesma forma, a repressão de outros reguladores negativo da fotomorfogênese, como COP1, também resultou em fenótipos com respostas exageradas à luz, tais como plântulas com fotomorfogênese exacerbada, folhas com pigmentação mais escura e teores elevados de carotenoides em frutos (LIU *et al.*, 2004).

Em contrapartida, transgênicas deficientes em HY5 apresentam reduzida resposta à luz, de tal forma que plantas que carregam essa alteração são caracterizadas por apresentarem folhas com teores reduzidos de clorofilas, inibição de respostas fotomorfogênicas nas plântulas, perda da organização dos tilacóides e reduzido conteúdo de carotenoides totais em frutos maduros (LIU *et al.*, 2004).

Além disso, a influência de fitocromos e criptocromos no desenvolvimento de cloroplastos e acúmulo de pigmentos tem sido alvo de excelentes estudos (WELLER *et al.*, 2000, 2001; GILIBERTO *et al.*, 2005). Por exemplo, WELLER e col. (2000) investigaram a interação entre os fitocromos A (PHYA), B1 (PHYB1) e B2 (PHYB2)

em plantas de tomateiro. Neste estudo, quando a perda de função de PHYB2 ocorria em conjunto com a mutação *phyB1*, o fenótipo estiolado dessas plantas tornava-se bastante conspícuo. Em contrapartida, a perda de função de PHYB2 em indivíduos que conservavam *PHYB1* funcional não resultou em nenhum fenótipo aparente durante o desestiolamento, indicando uma possível ação redundante entre esses dois fitocromos. Por outro lado, a atuação de PHYA mostrou-se amplamente independente de PHYB1 e PHYB2 no que concerne ao controle de alongamento de hipocótilos e com uma clara interação antagônica em relação à PHYB1 durante o controle de síntese de antocianinas, indicando, portanto, que as interações entre tipos específicos de fitocromos em tomateiro podem variar de acordo com o evento em questão (WELLER *et al.*, 2000).

Em um estudo conduzido com os mutantes *cry1* (*cryptochrome 1*), *phyA*, *phyB1* e *phyB2* de tomateiro, WELLER e col. (2001) demonstraram que esses quatro receptores são capazes de mediar respostas à luz azul. No entanto, a importância de suas contribuições individuais parece variar de acordo com a irradiância, presença de outros fotorreceptores e resposta de desenvolvimento examinada. Dessa forma, PHYA e CRY1 foram caracterizados como os principais mediadores do desestiolamento induzido por luz azul em baixa e alta irradiância, respectivamente. É interessante ressaltar que esse estudo, foi o primeiro a realizar uma caracterização detalhada das funções de CRY1 em uma espécie vegetal que não fosse *Arabidopsis*.

Recentemente, por meio do silenciamento e super-expressão do gene *CRYPTOCHROME 2* (*CRY2*), GILIBERTO e col. (2005) obtiveram importantes informações sobre as funções desse tipo específico de criptocromo em plantas de tomateiro. Até então, CRY2 havia sido descrito em *Arabidopsis* apenas como um dos elementos envolvidos no controle do florescimento e da fotomorfogênese em condições luminosas de baixa fluência (GUO *et al.*, 1998; LIN & SHALITIN, 2003). Por outro

lado, em tomateiro, a super-expressão de *CRY2* revelou uma significativa influência desse fotorreceptor em uma série de eventos fisiológicos adicionais àqueles sob controle de seu homólogo em *Arabidopsis*. Por exemplo, verificou-se que plantas portadoras de super-expressão de *CRY2* apresentavam maior acúmulo de antocianinas e clorofilas em folhas, flavonoides e licopeno em frutos e atraso no florescimento (GILIBERTO *et al*, 2005). Em consonância, o silenciamento deste gene resultou no fenótipo oposto, desencadeando um menor acúmulo de antocianinas nas nervuras das folhas e no florescimento tardio, indicando, assim, que *CRY2* desempenha um papel importante no desenvolvimento de plantas de tomateiro, tanto em tecidos vegetativos quanto em tecidos reprodutivos (GILIBERTO *et al*, 2005).

Contudo, apesar dos avanços atingidos ao longo dos últimos anos, o nosso entendimento atual acerca das interações específicas entre os diferentes fotorreceptores e destes com os sinais endógenos responsáveis pela conversão de etioplastos em cloroplastos durante o desestiolamento vegetal ainda é um campo que carece de estudos mais aprofundados em diferentes espécies vegetais, incluindo o tomateiro. Dentre os sinalizadores endógenos que interagem com a luz, destacam-se os hormônios vegetais (CHORY *et al.*, 1994; KRAPIEL & MAGIANIC, 1997). Assim sendo, discutiremos a seguir as relações já conhecidas entre luz e fitormônios no que concerne à diferenciação plastidial e acúmulo de pigmentos fotossintéticos que tipicamente ocorrem durante o desestiolamento vegetal.

1.2 Diferenciação plastidial e fitormônios vegetais

De forma similar à outras respostas fotomorfogênicas, a diferenciação de etioplastos em cloroplastos em resposta à irradiação luminosa parece depender sobremaneira da participação de hormônios vegetais e outras moléculas sinalizadoras, as quais interagem

direta ou indiretamente com as cascatas de sinalização desencadeadas pelos sinais luminosos (EGEA *et al.* 2010). Auxinas, citocininas, giberelinas (GAs), etileno, ácido abscísico (ABA) e brassinosteróides desempenham papel importante em diversos eventos fotomorfogênicos (SYMONS & REID, 2003), sendo que cada hormônio pode atuar tanto de forma sinérgica quanto antagônica em relação ao estímulo luminoso, dependendo do evento fisiológico, espécie vegetal ou condições experimentais sob análise (HALLIDAY & FANKHAUSER, 2002; KRAEPIEL & MAGINIAC, 1997).

Em conjunto com a luz, citocininas e ABA têm sido caracterizados como importantes sinais hormonais controladores do desenvolvimento do aparato fotossintético em angiospermas (LÓPEZ-JUEZ, 2007; WATERS & LANGDALE, 2009). Desta forma, essas duas classes hormonais têm sido foco de diversos estudos relacionados à sinalização e interação hormonal durante a diferenciação de cloroplastos a partir de etioplastos (KUSNETSOV *et al.*, 1998; YARONSKAYA *et al.*, 2006; KRAVTSOV *et al.*, 2011).

Estudos conduzidos em diversas espécies indicam um papel estimulatório das citocininas sobre a conversão de etioplastos em cloroplastos, ativação de enzimas plastidiais, regulação do acúmulo de pigmentos fotossintéticos e das taxas fotossintéticas (LERBS *et al.*, 1984; CHORY *et al.*, 1994; KUSNETSOV *et al.*, 1994, 1998; YARONSKAYA *et al.*, 2006). Sabe-se, por exemplo, que tratamentos com citocininas podem mimetizar diversos processos classicamente regulados pela luz (BRACALE *et al.*, 1988; COHEN *et al.*, 1988; CHORY *et al.*, 1994; HALLIDAY & FANKHAUSER, 2002; SYMONS & REID, 2003). De forma condizente, mutantes de *Arabidopsis* que possuem níveis elevados de citocininas, apresentam fenótipo parcialmente desestiolado mesmo quando continuamente mantidos no escuro (CHIN-ATKINS *et al.*, 1996; HALLIDAY & FANKHAUSER, 2002).

A aplicação exógena de citocininas pode induzir a expansão de cotilédones, acúmulo de clorofilas, biogênese plastidial e acúmulo de proteínas e pigmentos fotossintéticos (LERBS *et al.*, 1984; KRAVTSOV *et al.*, 2011). Tratamentos com citocininas ou alterações na percepção dessa classe hormonal podem, inclusive, mimetizar o fenótipo de mutantes fotomorfogênicos com hipersensibilidade ao sinal luminoso, tais como *det1* de *Arabidopsis* e *hp-2* de tomateiro (MARTINEAU *et al.*, 1994; MUSTILLI *et al.*, 1999; KUBO & KAKIMOTO, 2000). Em tomateiro, por exemplo, plântulas tratadas com citocininas exógenas e submetidas à luz branca, desenvolvem hipocótilos mais curtos e acumulam altos teores de antocianinas em comparação àquelas não tratadas com esse hormônio (MUSTILLI *et al.*, 1999). De forma condizente, mutantes de *Arabidopsis* que apresentam hipersensibilidade às citocininas apresentam maior desenvolvimento de cloroplastos (KUBO & KAKIMOTO, 2000). Além disso, frutos de tomateiro onde a expressão ectópica do gene *ipt* do plasmídeo Ti de *Agrobacterium tumefaciens* foi induzida e, portanto, portadores de teores elevados de citocininas, apresentaram fenótipo bastante diferenciado, incluindo uma pigmentação verde não uniforme e alterações marcantes na dinâmica de diferenciação de cloroplastos em cromoplastos, resultando em manchas verdes remanescentes em meio a um *continuum* intensamente vermelho (MARTINEAU *et al.*, 1994).

Diversos trabalhos indicam relações antagônicas entre citocininas e ABA durante a diferenciação plastidial e transcrição gênica tanto em plântulas quanto em tecidos maduros (KUSNETSOV *et al.*, 1998; KRAVTSOV *et al.*, 2011). De modo geral, o ABA tem sido caracterizado como um fitormônio que, entre outras funções, é capaz de suprimir a biogênese plastidial (KHOKHLOVA *et al.*, 1978). Relatos indicam que tratamentos com ABA usualmente resultam na repressão do acúmulo de clorofilas e da

transcrição de genes plastidiais (KRAVTSOV *et al.*, 2011). Além disso, de modo interessante, o mutante *high pigment 3 (hp3)* de tomateiro, deficiente na biossíntese de ABA, apresenta frutos contendo um maior número de plastídios em suas células e teores de carotenoides cerca de 30% superiores àqueles detectados no genótipo selvagem, além de apresentarem uma nítida alteração no padrão de organização tilacoidal em seus cloroplastos (GALPAZ *et al.*, 2008). Adicionalmente, experimentos realizados com outros dois mutantes de tomateiro deficientes na biossíntese de ABA, *flacca* e *sittiens*, apresentaram resultados semelhantes, uma vez que foi possível observar maior abundância plastidial e acúmulo de pigmentos nos tecidos desses mutantes, sugerindo, portanto, o envolvimento de ABA no controle da divisão plastidial (GALPAZ *et al.*, 2008).

De forma interessante, a exposição de tecidos vegetais a tratamentos luminosos comumente resulta na redução dos níveis endógenos de ABA (TOYOMASU *et al.*, 1994), e, de modo condizente, mutantes de tabaco (*Nicotiana tabacum*) deficientes na síntese do cromóforo dos fitocromos, apresentam elevados teores de ABA em sementes e folhas, indicando, portanto, a participação de fitocromos como reguladores dos teores endógenos desse fitormônio (KRAEPIEL *et al.*, 1994). De forma equivalente, mutantes de *Arabidopsis* com perda de função no gene *ABA*, o qual codifica uma enzima chave na rota biossintética de ABA (a zeaxantina epoxidase), apresentam alteração na capacidade de reprimir a fotomorfogênese mesmo quando mantidos no escuro, resultando num fenótipo parcialmente desestiolado caracterizado pela redução tanto no alongamento do hipocótilo quanto na expansão dos cotilédones e no desenvolvimento de folhas verdadeiras (BARRERO *et al.*, 2008).

Curiosamente, GAs e luz também parecem atuar de modo sinérgico em alguns processos fotomorfogênicos e antagônico em outros (BETHKE *et al.*, 2007; SYMONS

et al., 2008). Um exemplo de ação sinérgica acontece durante a germinação, onde o tratamento de sementes com luz vermelha induz a germinação através de um aumento tanto na biossíntese quanto na sensibilidade às GAs (TOYOMASU *et al.*, 1994; BETHKE *et al.*, 2007).

Em contrapartida, GAs e luz parecem atuar de forma antagônica em processos que envolvem alongamento celular. Por exemplo, a redução do alongamento de hipocótilos ou entrenós estimulada pela luz depende de uma redução nos teores endógenos de GAs, bem como de uma diminuição na percepção desse hormônio pelos tecidos iluminados (KRAEPIEL & MIGINIAC, 1997, O'NEILL *et al.*, 2000, SYMONS *et al.*, 2008). Em consonância, plântulas de mutantes com menor biossíntese ou sensibilidade às GAs costumam apresentar fenótipo desestiolado, tais como hipocótilos curtos, acúmulo da enzima POR e de carotenoides bem como maior formação de corpos prolamelares, mesmo quando mantidos no escuro (ALABADI *et al.*, 2008; CHEMINANT *et al.*, 2011). Interessantemente, tais respostas parecem estar relacionadas à alterações na biossíntese e acúmulo de uma família de reguladores de transcrição que reprimem respostas mediadas por GAs, as proteínas DELLAs (ALABADI *et al.*, 2008). Tais proteínas têm sido caracterizadas como elementos que interagem fortemente com a sinalização luminosa em diversos outros eventos de desenvolvimento (ACHARD *et al.*, 2006). No caso do desestiolamento, por exemplo, elas podem atuar regulando tanto a ativação de genes que codificam proteínas envolvidas no processo fotossintético, tais como a enzima POR, quanto o acúmulo de pigmentos associados como as protoclorofilas e os carotenoides, contribuindo, assim, para a prevenção de danos foto-oxidativos (CHEMINANT *et al.*, 2011). Além disso, tais proteínas também parecem estimular a formação de corpos prolamelares, indicando, portanto, uma grande

importância dessa família de reguladores durante o desestiolamento vegetal (CHEMINANT *et al.*, 2011).

Com relação aos brassinoesteróides, mutantes deficientes ou insensíveis à essa classe hormonal apresentam características de desestiolamento mesmo quando mantidos no escuro, tais como hipocótilos reduzidos e desrepressão de genes regulados pela luz, como *CAB2* (*CHLOROPHYLL A/B- BINDING PROTEIN2*) e *RbcS* (*RUBISCO SMALL-SUBUNIT*) (CLOUSE & SASSE, 1998; SCHUMACHER & CHORY, 2000).

Com relação às auxinas, a luz representa um fator de grande influência tanto nos teores e transporte quanto na responsividade dos tecidos à essa classe hormonal (HALLIDAY *et al.*, 2009). Sabe-se que auxinas atuam de forma estimulatória ao alongamento celular de hipocótilos e caules em condições de escuro, ao passo que a luz atua inibindo esse processo (SYMONS & REID, 2003). Relatos indicam que plântulas de mutantes deficientes na biossíntese de auxinas apresentam hipocótilos curtos e maior expansão de cotilédones (TAO *et al.*, 2008), enquanto mutantes com abundância dessa classe hormonal apresentam fenótipo estiolado, tais como hipocótilo alongado e reduzida expansão de cotilédones (ZHAO *et al.*, 2001; HOECKER *et al.*, 2004; KIM *et al.*, 2007), denotando, dessa forma, a existência de uma relação antagônica entre luz e auxinas durante os eventos em questão (HALLIDAY *et al.*, 2009).

Adicionalmente, relatos onde o tratamento de plântulas com luz vermelha reduz os teores de auxinas sugerem a participação dos fitocromos como reguladores dos níveis endógenos desse hormônio, regulando tanto sua biossíntese quanto o seu catabolismo (HALLIDAY & FANKHAUSER, 2002). Em concordância com essas observações, estudos têm demonstrado um acréscimo considerável nos teores de auxinas tanto em mutantes de tabaco deficientes na síntese do cromóforo do fitocromo (KRAEPIEL *et al.*, 1994) quanto em mutantes de tomateiro deficientes em tipos específicos de

fitocromos (KERCKHOFFS *et al.*, 1997). Além disso, a luz é capaz de modular genes envolvidos no transporte polar das auxinas, tais como *PGP19*, *PGP1*, e *PIN3*, regulando, assim, a distribuição celular e tecidual das auxinas e, conseqüentemente, a expansão celular e outras respostas fisiológicas controladas por esse hormônio (HALLIDAY *et al.*, 2009).

Com relação aos estudos envolvendo auxinas e diferenciação plastidial, JONES e col. (2002) demonstraram que o gene *DR12/ARF4*, membro da família multigênica de fatores de resposta a auxinas ARF, participa da regulação tanto da divisão celular quanto da diferenciação plastidial em frutos de tomateiro (JONES *et al.*, 2002). Observou-se que a repressão constitutiva do fator de resposta DR12/ARF4 resultou em frutos com fenótipo verde-escuro, com amadurecimento irregular, tecido externo do pericarpo do fruto com maior número de cloroplastos por célula e um aumento dramático na formação de grana (JONES *et al.*, 2002). Curiosamente, esse fenótipo manteve-se restrito aos tecidos do fruto.

Por outro lado, raízes destacadas de *Arabidopsis* tratadas com auxinas apresentaram menor acúmulo de clorofilas e menor diferenciação plastidial quando comparadas com raízes destacadas que não foram submetidas ao tratamento com esse fitormônio, sugerindo, portanto, que as auxinas atuam reprimindo a diferenciação plastidial e acúmulo de pigmentos nesse órgão (KOBAYASHI *et al.*, 2012).

No que tange às interações entre luz e etileno, a aplicação exógena desse fitormônio pode anular diversas respostas fotomorfogênicas, tais como a expansão de cotilédones e abertura do gancho apical (HALLIDAY & FANKHAUSER, 2002). Além disso, esse fitormônio também tem sido relacionado ao desenvolvimento de cotilédones em situações de estresse bem como em mutantes deficientes no processo de desenvolvimento desencadeado pela luz (ZHOU *et al.*, 1998; ZHONG *et al.*, 2009).

De modo interessante, estudos têm demonstrado que a luz pode inibir o acúmulo de etileno diretamente ou através da regulação fotossintética (FINLAYSON *et al.*, 1999; TEPPERMAN *et al.*, 2001). A emissão de etileno em plântulas estioladas de feijão, por exemplo, é inibida quando essas plântulas são transferidas para tratamentos com luz vermelha, indicando o envolvimento dos fitocromos na regulação desse hormônio (YANG & HOFFMAN, 1984; VANGRONSVELD *et al.*, 1988). Além disso, a luz parece inibir a percepção de etileno durante o desestiolamento de plântulas de ervilha (*Pisum sativum*), permitindo a abertura do gancho apical, expansão de cotilédones e acúmulo de clorofilas (GOESCHL *et al.*, 1967; FOO *et al.*, 2006).

Em um estudo realizado por FOO e col. (2006) utilizando o duplo mutante *phyAphyB* de ervilha, o fenótipo pálido, encurtado e espessado dos caules foi relacionado à elevada produção de etileno observada nesses mutantes. De modo interessante, demonstrou-se que o fenótipo alterado dessas plantas mutantes podia ser parcialmente ou completamente recuperado quando estas eram tratadas com um inibidor da síntese de etileno. Assim sendo, em plantas de ervilha, o etileno parece participar em um amplo conjunto de respostas fotomorfogênicas, incluindo a supressão de alongamento caular, regulação da expressão do gene regulado pela luz *CAB9*, acúmulo de clorofilas e desenvolvimento foliar.

Estudos têm caracterizado também quais seriam as etapas específicas da via de biossíntese de etileno afetadas pela luz (YANG & HOFFMAN, 1984; JIAO *et al.*, 1987; VANGRONSVELD *et al.*, 1988; HALLIDAY & FANKHAUSER, 2002). Em plantas, o etileno é gerado a partir da conversão da metionina, a qual passa basicamente por três reações enzimáticas até formar o hormônio. Primeiramente a metionina é convertida à S-adenosil-metionina (S-AdoMet) através da atividade da S-AdoMet sintase e, em seguida, a S-AdoMet é convertida no ácido 1-amminociclopropano-1-carboxílico

(ACC), por meio da atividade da enzima ACC sintase (ACS). O ACC produzido é então degradado pela enzima ACC oxidase (ACO) dando origem ao etileno (LIN *et al.*, 2009). Dessas três reações-chaves, a formação de ACC tem sido considerada um fator limitante da via de biossíntese de etileno (GARAY-ARROYO *et al.*, 2012). De modo interessante, a influência da luz sobre essas três reações-chaves tem sido frequentemente demonstrada na literatura, especialmente durante a indução de processos relacionados ao desestiolamento vegetal, onde alterações nos teores endógenos dessas enzimas são frequentemente observados em mutantes fotomorfogênicos (VANGRONSVELD *et al.*, 1988; VRIEZEN *et al.*, 2004; FOO *et al.*, 2006; VANDENBUSSCHE *et al.*, 2007).

Com relação à influência do etileno sobre a biogênese e diferenciação plastidial, a maior parte dos estudos realizados até o momento focaram no processo de conversão de cloroplastos em cromoplastos em tecidos reprodutivos ou, então, na degradação de clorofilas e desestruturação dos cloroplastos durante a senescência foliar (EGEA *et al.*, 2010; SARWAT *et al.*, 2013). Por exemplo, alterações na pigmentação dos frutos resultante de distúrbios na dinâmica de formação de cromoplastos a partir de cloroplastos têm sido frequentemente observadas em mutantes e transgênicos de tomateiro defectivos na biossíntese ou em elementos da cascata de transdução de sinal de etileno (EGEA *et al.*, 2010).

No que tange a interação entre fitormônios, a relação entre etileno e auxinas tem sido amplamente estudada e relacionada a diversos eventos importantes durante o desestiolamento, tais como a regulação de alongamento do hipocótilo e a formação, manutenção e abertura do gancho apical em eudicotiledôneas (SYMONS & REID, 2003). Entretanto, a influência desses hormônios sobre a biogênese plastidial e diferenciação de etioplastos em cloroplastos durante o desestiolamento de plântulas ainda é muito pouco conhecida. Dessa forma, estudos dedicados à caracterização da

atuação e interação entre essas duas classes hormonais com a luz e demais fatores endógenos é de suma importância para o maior entendimento dos mecanismos envolvidos na biogênese e diferenciação de cloroplastos e acúmulo de pigmentos durante o início do desenvolvimento vegetativo.

Além dos hormônios vegetais, outras moléculas sinalizadoras endógenas parecem participar do processo de diferenciação plastidial e acúmulo de pigmentos durante a transição do desenvolvimento escotomorfogênico para o fotomorfogênico (BELIGNI & LAMATTINA, 2000; ZHANG *et al.*, 2006; LIU *et al.*, 2013). Dentre essas substâncias, o radical livre óxido nítrico (NO), tem sido proposto como um composto sinalizador potencialmente envolvido na indução de respostas fotomorfogênicas em diferentes espécies, interagindo com a luz na regulação de processos que vão desde a germinação de sementes, alongamento de hipocótilo até expansão de cotilédones e acúmulo de pigmentos (BELIGNI & LAMATTINA, 2000; ZHANG *et al.*, 2006; LOZANO-JUSTE & LEON, 2011). Assim sendo, discutiremos a seguir as principais evidências acerca da interação entre NO, fitormônios e luz durante a indução de respostas fotomorfogênicas, com especial ênfase na formação de cloroplastos e acúmulo de pigmentos fotossintéticos em tecidos vegetais em processo de desestiolamento.

I.3. Relações entre NO, fitormônios e luz durante a fotomorfogênese vegetal.

O NO é um composto gasoso, inorgânico, lipofílico, de pequeno tamanho molecular e moderada solubilidade em água (DURNER *et al.*, 1999), o qual é produzido por vários seres vivos tais como bactérias, fungos, plantas e animais (MOILANEN & VAPAATALO, 1995; LESHEN & HARAMATY, 1996; GUPTA *et al.*, 2011). Esse radical livre tem ganhado crescente destaque ao longo das últimas décadas, tornando-se alvo de diversos estudos na área das ciências biológicas (LESHEN & HARAMATY,

1996; BELIGNI & LAMATTINA, 2000; FRESCHI *et al.*, 2010; LIU *et al.*, 2013). Na biologia vegetal, o estudo da participação do NO como um composto sinalizador no crescimento e desenvolvimento vegetal é tópico ainda relativamente recente e com diversas lacunas e controvérsias (MAGALHÃES *et al.*, 2006; NEILL *et al.*, 2008).

Atualmente, sabe-se que a relação das plantas com esse radical livre é muito mais complexa do que o inicialmente proposto. O NO parece ser um sinal ubíquo em plantas, atuando no controle de diversos eventos do desenvolvimento vegetal (LAMATTINA *et al.*, 2003; BESSON-BARD *et al.*, 2008) e inúmeras respostas das plantas à estresses ambientais (LAXALT *et al.*, 1997; FRESCHI *et al.*, 2010; OLIVEIRA *et al.*, 2013).

Por ser um tópico relativamente recente na biologia vegetal e, em partes, devido à sua reduzida estabilidade química, questões sobre o real papel desempenhado pelo NO em plantas ainda permanecem em aberto (NEILL *et al.*, 2008). Alguns autores o descrevem como um mensageiro secundário (PAGNUSSAT *et al.*, 2004), ao passo que outros sugerem que este possa ser um novo hormônio vegetal, por ser produzido em baixas concentrações, atuar de maneira dose-dependente e possuir fácil difusão nos tecidos da planta (BELIGNI & LAMATTINA, 2001).

O NO também parece participar de processos controlados pela luz, estimulando respostas tão diversas quanto a germinação de sementes fotoblásticas positivas, alongamento de hipocótilos, expansão de cotilédones e acúmulo de clorofilas (BELIGNI & LAMATTINA 2000; ZHANG *et al.*, 2006; LOZANO-JUSTE & LEON, 2011;). Em um estudo seminal, BELIGNI e LAMATTINA, (2000) constataram que tratamentos com nitroprussiato de sódio (SNP), um doador de NO, eram capazes de mimetizar diferentes respostas normalmente desencadeadas pela luz, tais como acúmulo de clorofilas em plântulas de trigo (*Triticum aestivum*), indução da germinação em

alface (*Lactuca sativa*), redução do alongamento de hipocótilos em plântulas de *Arabidopsis* e redução dos entrenós em plantas de batata (*Solanum tuberosum*) mantidas no escuro ou cultivadas em condições de baixa luminosidade. Com relação à expansão foliar e acúmulo de clorofilas, sabe-se que discos foliares de ervilha apresentam incrementos na expansão quando tratados com baixas concentrações de NO (LESHEM & HARAMATY, 1996) e que tratamentos com SNP induzem, de forma dose dependente, o acúmulo de clorofilas em plântulas de trigo mantidas no escuro (BELIGNI & LAMATTINA, 2000) ou intensificam o efeito da luz no acúmulo desses pigmentos e na produção de proteínas das membranas dos tilacóides em plântulas de cevada (*Hordeum vulgare*) (ZHANG *et al.*, 2006).

A inibição da produção de NO também pode resultar em alterações no programa de desenvolvimento vegetal (CORREA-ARAGUNDE *et al.*, 2004; LOZANO- JUSTE & LEON, 2010, 2011; LIU *et al.*, 2013). Em um estudo realizado com plântulas do triplo mutante *nial1,2noal-2* de *Arabidopsis*, o qual possui a biossíntese de NO severamente reduzida, demonstrou-se que tal mutante apresenta severas alterações fenotípicas, tais como reduzido desenvolvimento de raízes e caule, atraso no desenvolvimento de folhas e hipocótilos mais alongados mesmo em plântulas crescidas na presença de luz (LOZANO- JUSTE & LEON, 2010). De forma condizente, plântulas de trigo estioladas apresentaram aumento na produção de NO quando submetidas à luz, indicando, assim, uma possível relação positiva desse sinalizador durante o desestiolamento induzido pela luz (LIU *et al.*, 2013).

Adicionalmente, estudos realizados com mutantes de *Arabidopsis* deficientes na produção de fitocromos A e B demonstraram a influência do NO particularmente sobre a germinação de sementes controladas pelos fitocromos A, ao passo que sua influência sobre a germinação de sementes reguladas pelos fitocromos B foi quase nula (BATAK

et al., 2002). Entretanto, a interação entre NO e demais fotorreceptores tanto durante a germinação de sementes quanto em outras respostas fotomorfogênicas permanece ainda pouco elucidada.

No que tange às interações entre NO e fitormônios, sabe-se que esse sinalizador interage com praticamente todas as classes hormonais durante a regulação das mais diversas respostas do desenvolvimento vegetal, seja através da participação em cascatas de sinalização ou pela modulação direta do metabolismo, percepção ou transdução de sinais hormonais (PAGNUSSAT *et al.*, 2004; LOZANO-JUSTE & LEON, 2010; FENG *et al.*, 2012; FRESCHI, 2013). Ao mesmo tempo, as evidências indicam que praticamente todas as classes hormonais podem influenciar a produção de NO sob diferentes circunstâncias, etapas do desenvolvimento e modelos vegetais (BELIGNI & LAMATTINA, 2001; CORREA-ARAGUNDE *et al.*, 2004; XIAO-PING & XI-GUI, 2006; LOZANO-JUSTE & LEON, 2011).

Por exemplo, múltiplos níveis de interação entre citocininas e NO têm sido relatados na literatura durante os últimos anos. Tais interações podem ser tanto sinérgicas quanto antagônicas dependendo do evento em questão, modelo vegetal e abordagem experimental (CARIMI *et al.*, 2005; WILHELMOVÁ *et al.*, 2006; MISHINA *et al.*, 2007; FRESCHI, 2013, LIU *et al.*, 2013). São exemplos de interações sinérgicas entre essas duas moléculas sinalizadoras o controle de senescência foliar (MISHINA *et al.*, 2007), morte celular programada (CARIMI *et al.*, 2005), ajuste fotossintético ao estresse hídrico (SHAO *et al.*, 2010) e diferenciação e divisão celular (SHEN *et al.*, 2012).

Em relação às interações antagônicas, existem relatos de que o tratamento exógeno com citocininas resulte na redução da emissão de NO em células guardas de *Vicia faba*

tratadas com SNP (XIAO-PING & XI-GUI, 2006). Em concordância com esses resultados, evidências de correlação negativa entre citocininas e NO foram estabelecidas em plantas transgênicas de tabaco, onde plantas com teores reduzidos de citocininas apresentavam maior emissão de NO enquanto plantas com maior produção endógena de citocininas apresentavam menor emissão desse sinalizador (WILHELMOVÁ *et al.*, 2006). Além disso, relatos indicam que o tratamento com a citocinina zeatina alivia o fenótipo atribuído ao excesso de NO dos mutantes *nox1* (do inglês: *nitric oxide overexpression 1*) de *Arabidopsis* (LIU *et al.*, 2013).

A influência do NO em eventos do desenvolvimento onde GAs desempenha papel importante tais como germinação, alongamento de hipocótilos, fotomorfogênese e o crescimento da raiz primária, entre outros, também tem sido frequentemente descrita na literatura (BELIGNI & LAMATTINA, 2000; TONÓN *et al.*, 2010; LOZANO-JUSTE & LEON, 2011). No entanto, a maior parte do conhecimento atual dos mecanismos relativos à interação entre GAs e NO é restrita à regulação da germinação de sementes (BELIGNI *et al.*, 2002; BETHKE *et al.*, 2007) e controle do alongamento de hipocótilos durante o desestiolamento (LOZANO-JUSTE & LEON, 2011). Sabe-se, por exemplo, que hipocótilos estiolados de plântulas *gal-3* de *Arabidopsis*, as quais apresentam deficiência na síntese de GAs, apresentam maior emissão de NO do que hipocótilos de plântulas do tipo selvagem. De forma condizente, plântulas do triplo mutante de *Arabidopsis nial,2noal-2*, apresentam alterações no acúmulo de proteínas DELLA, resultando, assim, num aumento da sensibilidade à GAs e deficiência do desestiolamento mesmo em presença de luz vermelha (LOZANO-JUSTE & LEON, 2011). Tais resultados sugerem que as GAs exercem controle negativo sobre a produção de NO nessas plantas.

Consideradas duas moléculas importantes em respostas relacionadas ao estresse (SAVOURÉ *et al.*, 1997; HAO *et al.*, 2008; FRESCHI *et al.*, 2010; FRESCHI, 2013), NO e ABA interagem intensamente em diversas cascatas de sinalização desencadeadas por alterações ambientais, tais como o estresse hídrico e a radiação UV-B, as quais, em última instância, levam à indução de respostas adaptativas, tais como fechamento estomático e acúmulo de antioxidantes (NEILL *et al.*, 2002; TOSSI *et al.*, 2009; HANCOCK *et al.*, 2011). O NO também tem sido relacionado à regulação da dormência e germinação de sementes, dois eventos também regulados por ABA (LOZANO-JUSTE & LEON, 2011). Com relação à germinação, sabe-se que sementes tratadas com doadores de NO apresentam redução nos teores de ABA, o qual atua promovendo a dormência (BETHKE *et al.*, 2006, 2007; GNIAZDOWSKA *et al.*, 2007; LIU *et al.*, 2009). De forma condizente, reduções nos teores endógenos de NO têm sido associados à hipersensibilidade ao ABA, resultando em maior dormência de sementes e menores taxas de germinação (LOZANO-JUSTE & LEON, 2011).

Interações sinérgicas entre auxinas e NO têm sido frequentemente observadas durante organogênese radicular (PAGNUSSAT *et al.*, 2002, 2003, 2004; LANTERI *et al.*, 2006), respostas gravitrópicas (HU *et al.*, 2005), ativação da divisão celular (ÖTVÖS *et al.*, 2005), estimulação da enzima nitrato redutase (NR) (DU *et al.*, 2008), entre outros processos. Na maior parte dos casos, a atuação do NO parece estar posicionada a jusante das auxinas (HU *et al.*, 2005; LOMBARDO *et al.*, 2006; FRESCHI, 2013), uma vez que tratamentos com auxinas ou mutações que levam à uma produção exacerbada desse hormônio frequentemente desencadeiam incrementos nos teores endógenos de óxido nítrico (PAGNUSSAT *et al.*, 2002; CORREA-ARAGUNDE *et al.*, 2004; HU *et al.*, 2005; LOMBARDO *et al.*, 2006; CHEN *et al.*, 2010). Entretanto, tendo em vista que em algumas condições experimentais a produção de NO

parece não estar associada às auxinas (TUN *et al.*, 2001; GUO *et al.*, 2003), a ação promotora dessa classe hormonal na produção de NO não pode ser generalizada para todos os eventos de desenvolvimento vegetal (HU *et al.*, 2005).

Por fim, estudos indicam que os sinalizadores gasosos NO e etileno podem apresentar tanto interações antagônicas quanto sinérgicas de acordo com o evento estudado (MAGALHÃES *et al.*, 2000; MANJUNATHA *et al.*, 2010; FRESCHI, 2013). Dos eventos onde esses dois sinalizadores atuam de forma antagônica, os que têm recebido maior atenção são a senescência foliar e floral e o amadurecimento de frutos (LESHEM *et al.*, 1998; MANJUNATHA *et al.*, 2010). A aplicação exógena de NO têm sido descrita como sendo capaz de atrasar a senescência tanto de tecidos vegetativos quanto reprodutivos através da regulação de diversos elementos envolvidos na produção de etileno (WILLS *et al.*, 2000; PARANI *et al.*, 2004; LIU *et al.*, 2007; MANJUNATHA *et al.*, 2010, 2012). O NO possui a capacidade de modular a ativação transcricional de enzimas como ACS e ACO, impactando diretamente o acúmulo de ACC e a emissão de etileno (MANJUNATHA *et al.*, 2010).

No que tange às relações positivas entre esses dois sinalizadores, sabe-se que embriões de maçã (*Malus domestica*) submetidos a tratamentos com SNP apresentaram emissão de etileno cerca de duas vezes maior do que observado em plântulas controle (GNIAZDOWSKA *et al.*, 2007). Por outro lado, embriões tratados com cPTIO, um sequestrador de NO, apresentavam redução na emissão desse hormônio (GNIAZDOWSKA *et al.*, 2007). Além disso, relatos indicando uma influência estimulatória de tratamentos com doadores de NO sobre as taxas de emissão de etileno também têm sido descritos em diferentes modelos vegetais, incluindo *Arabidopsis*, tabaco e milho (*Zea mays*) (MAGALHÃES *et al.*, 2000; WANG *et al.*, 2006; EDERLI *et al.*, 2009).

Tendo visto essa pronunciada interação do NO com tão amplo leque de moléculas sinalizadoras durante os diversos eventos de desenvolvimento supracitados, questiona-se na literatura quais seriam os mecanismos responsáveis por conferir ao NO a especificidade necessária para desencadear/participar de tantos eventos fisiológicos em plantas. Entre outros aspectos, o controle da biossíntese e degradação de NO parece desempenhar papel fundamental para que esta molécula esteja ou não disponível em momento e local oportuno (NEILL *et al.*, 2008; FRESCHI, 2013).

Em animais, o NO é sintetizado a partir de reações de oxidação catalisadas por enzimas do tipo sintase do óxido nítrico (NOS) (IGNARRO, 1996). Com o avanço dos conhecimentos acerca do funcionamento dessa via em animais, estudos desenvolvidos em plantas esperavam por similaridades nos mecanismos moleculares de produção deste composto (CRAWFORD, 2006). Embora alguns estudos tenham revelado atividade enzimática do tipo NOS em tecidos vegetais (MODOLO *et al.*, 2002, 2005, 2006; WILSON *et al.*, 2008), nenhuma enzima NOS de planta foi purificada ou clonada até o momento.

Nesse contexto, GUO e col. (2003) isolaram e clonaram um gene em *Arabidopsis thaliana* que codifica uma proteína potencialmente envolvida com a regulação dos níveis de NO nessa espécie. Inicialmente denominada AtNOS1 (CRAWFORD, 2006), essa proteína mostrou-se capaz de influenciar a regulação de vários processos fisiológicos da planta claramente mediados pelo NO, tais como crescimento, transição floral e abertura estomática (GUO *et al.*, 2003). Porém, após a purificação das proteínas recombinantes, não foi confirmada a atividade NOS para a AtNOS1 (ZEMOJTEL *et al.*, 2006). Com a constatação de que o mutante *atnos1* apresentava deficiência de NO, mas não codificava uma enzima do tipo NOS, CRAWFORD (2006) propôs a mudança do nome do gene para *AtNOA1* (*NITRIC OXIDE ASSOCIATED 1*). Atualmente, sabe-se

que *AtNOA1* codifica uma enzima GTPase localizada nos cloroplastos, a qual provavelmente está envolvida na produção de ribossomos e subsequente tradução de proteínas nessa organela (FLORES-PEREZ *et al.*, 2008; MOREAU *et al.*, 2008).

Contudo, as plantas apresentam diferentes vias alternativas para a produção enzimática de NO além daquelas descritas em sistemas animais. Tais alternativas vão desde a produção de NO via hemeproteínas (NEILL *et al.*, 2008; WILSON *et al.*, 2008) e mecanismos não-enzimáticos (COONEY *et al.*, 1994; BETHKE *et al.*, 2004), até a síntese de NO através da atividade da enzima NR e outras enzimas envolvidas no metabolismo do nitrogênio (STHÖR *et al.*, 2001). O NO pode ainda ser produzido em cloroplastos como resultado de reações enzimáticas envolvendo nitrito e arginina (JASID *et al.*, 2006) ou a partir da redução não-enzimática do nitrito nas condições de baixo pH do apoplasto (BETHKE *et al.*, 2004).

Dentre essas possíveis rotas biossintéticas de NO em plantas, a NR tem sido considerada a via mais provável para a produção de NO em condições fisiologicamente relevantes (KAISER & PLANCHET, 2006; GUPTA *et al.*, 2011). Estudos em modelos vegetais, órgãos, tecidos e condições experimentais variadas têm relacionado a inibição da atividade da enzima NR com a queda da produção de NO (PLANCHET & KAISER, 2006; OLIVEIRA *et al.*, 2009; FRESCHI *et al.*, 2010). Além disso, o duplo mutante de *Arabidopsis nia1,nia2*, deficiente nas duas isoformas da NR dessa espécie, sabidamente apresenta reduzida capacidade de síntese de NO, evidenciando, portanto, a importância dessa via biossintética na determinação dos teores endógenos desse sinalizador em plantas (DESIKAN *et al.*, 2002; NEILL *et al.*, 2003).

A NR é uma enzima bifuncional que participa de uma cadeia de transporte de elétrons localizada no citossol (CAMPBELL, 1999). Sua principal função é catalisar a

transferência de dois elétrons do NAD(P)H para o nitrato (+5), que é reduzido a nitrito (+3), e posteriormente a amônio (-3) nos plastídios (KAISER & HUBER, 2001). Em outra situação, ela também pode catalisar a transferência de um elétron do NAD(P)H para o nitrito e, dessa forma, levar à produção de NO (DEAN & HARPER, 1988; YAMASAKI *et al.*, 1999).

De forma interessante, a NR pode ser fosforilada em um resíduo de serina, na região hinge 1, criando um sítio de inativação para proteínas 14-3-3. Na presença de Mg^{2+} livre, as proteínas 14-3-3 ligam-se ao complexo P-NR (NR fosforilada), inativando-o. Na ausência de cátions divalentes, todas as formas da NR permanecem completamente ativas (KAISER & HUBER, 2001).

Diversos estímulos ambientais e endógenos podem regular a NR tanto em nível transcricional quanto pós-traducional (KAISER & HUBER, 2001). Nesse aspecto, não é surpreendente o fato de que a luz atua como um importante sinal para a regulação de sua atividade, controlando tanto a sua transcrição gênica (controle transcricional) quanto o seu estado de fosforilação (controle pós-traducional) (LILLO & APPENROTH, 2001). Plântulas estioladas de tomateiro, por exemplo, apresentam aumento dramático nos teores de transcritos e de proteínas da NR horas após exposição à luz vermelha (BECKER *et al.*, 1992). Tal resposta parece estar relacionada à percepção da luz através dos fitocromos, uma vez que plantas do mutante de tomateiro deficiente em fitocromos (*aurea*), submetidos às mesmas condições experimentais não apresentaram alterações significativas nos teores de atividade da NR (BECKER *et al.*, 1992). Em contrapartida, a atividade da NR em plântulas do mutante de tomateiro com resposta exacerbada à luz *hp-1*, manteve-se em níveis superiores aos observados em plantas selvagens (GOUD & SHARMA, 1994).

A produção de NR também está relacionada aos fitormônios vegetais. Tratamentos com citocininas, por exemplo, reconhecidamente estimulam o acúmulo de transcritos da NR em cevada (LU *et al.*, 1990, 1992), bem como a sua atividade (KENDE *et al.*, 1971). Por outro lado, ABA parece reprimir NR nessas mesmas condições (LU *et al.*, 1992).

Tendo em vista a diversidade de relatos indicativos de interações entre o óxido nítrico e fitormônios (BELIGNI & LAMATTINA, 2001; CORREA-ARAGUNDE *et al.*, 2004; PAGNUSSAT *et al.*, 2004; XIAO-PING & XI-GUI, 2006; LOZANO-JUSTE & LEON, 2010, 2011; FRESCHI, 2013) e o fato dessas substâncias interagirem de forma bastante intensa com as cascatas de sinalização desencadeadas pela luz, parece-nos plausível hipotetizar que esses sinalizadores possam interagir no controle da conversão de etioplastos em cloroplastos e o acúmulo de pigmentos fotossintéticos durante o desestiolamento de plântulas. No entanto, essa relação ainda não foi demonstrada.

I.4. O tomateiro como um modelo para estudos sobre fotomorfogênese vegetal

Muito do que se sabe sobre a fotomorfogênese vegetal deve-se à estudos que fazem uso de plantas mutantes ou transgênicas (KENDRICK *et al.*, 1997; TERRY *et al.*, 2001; CASPI *et al.*, 2008; HARRISON *et al.*, 2011; SAITO *et al.*, 2011). Importantes descobertas acerca da relação entre as sinalizações luminosa e hormonal, por exemplo, têm sido obtidas através do uso de mutantes defectivos em fotorreceptores ou em elementos da transdução do sinal luminoso, bem como, na sensibilidade/metabolismo de fitormônios (CHORY & PETO, 1990; HALLIDAY & FANKHAUSER, 2002). Grande parte desses estudos tem sido realizada na planta modelo *Arabidopsis thaliana*

(CHORY & PETO, 1990, 1994; PYKE & LEECH, 1992; WANG *et al.*, 2001; ALURU *et al.*, 2006; VANDENBUSSCHE *et al.*, 2007; KOBAYASHI *et al.*, 2012). Devido ao seu pequeno tamanho, ciclo de vida curto, genoma sequenciado, facilidade de crescer em ambientes controlados e, principalmente, pela identificação e caracterização de diversos mutantes, essa planta tornou-se a primeira opção para o desenvolvimento de pesquisas em diversos campos da fisiologia vegetal (CHORY & PETO, 1990; DENG & QUAIL, 1992; CHORY *et al.*, 1994; PYKE & LEECH, 1994; FANKHAUSER & CHORY, 1997; GYULA *et al.*, 2003; STEPHENSON *et al.*, 2008; WATERS & LANGDALE, 2009; KOBAYASHI *et al.*, 2012; SARWAT *et al.*, 2013). Entretanto, algumas plantas de interesse agrônomo direto também têm se revelado excelentes modelos genéticos para estudos sobre a fotomorfogênese vegetal (KENDRICK *et al.*, 1997; PRATT *et al.*, 1997).

Nesse aspecto, o tomateiro (*Lycopersicon esculentum* Mill. *syn.* *Solanum lycopersicum* L.) ganha destaque, pois é uma espécie de grande importância econômica, para a qual se conhece grande número de mutações e variações genéticas naturais (KENDRICK *et al.*, 1997; LIU *et al.*, 2003; LIEBERMAN *et al.*, 2004; CAMPOS *et al.*, 2010). Além disso, essa espécie apresenta diversas características difíceis ou até mesmo impossíveis de se estudar em *Arabidopsis*, tais como ausência de crescimento em roseta, folhas compostas, floração simpodial independente de fotoperíodo, tricomas multicelulares, frutos carnosos e climatéricos, biossíntese de metabólitos secundários, e associação com fungos micorrízicos (CAMPOS *et al.*, 2010, CARVALHO *et al.*, 2011). O tomate é atualmente a principal fonte de licopeno na dieta humana, destacando-se ainda pela alta concentração de elementos de alto valor nutricional, tais como β -caroteno, luteína e fitoeno, flavonóides, fenilpropanóides, ácido ascórbico (vitamina C)

e tocoferol (vitamina E), considerados altamente benéficos para a saúde humana (ROSS & KASUM, 2002; GIUNTINI *et al.*, 2008; AZARI *et al.*, 2010).

Atualmente, encontram-se descritos na literatura mutantes de tomateiro com alteração na produção de fitocromos (TERRY & KENDRICK, 1996), criptocromos e outros elementos-chaves das vias de transdução do sinal luminoso (KENDRICK *et al.*, 1997), bem como mutantes com alterações nas vias metabólicas e/ou de sensibilidade à diversas classes hormonais, incluindo auxinas, citocininas, ABA, GAs, etileno, brassinoesteróides e ácido jasmônico (HICKS *et al.*, 1989; BENSEN & ZEEVAART, 1990; BURBIDGE *et al.*, 1999; OH *et al.*, 2006; WILKINSON *et al.*, 1995; CAMPOS *et al.* 2010; CARVALHO *et al.* 2011).

Com relação à diferenciação plastidial, a manipulação de componentes envolvidos na percepção e transdução do sinal luminoso, tais como HP1/DDB1, HP2/DET1, COP1 e HY5, e a utilização de mutantes hormonais tem se mostrado uma estratégia eficaz para o entendimento dos mecanismos envolvidos nas cascatas de sinalização desencadeadas durante o desenvolvimento, biogênese plastidial e acúmulo de pigmentos (DAVULURI *et al.* 2005; LIU *et al.* 2004; WANG *et al.*, 2008).

Além dos mutantes fotomorfogênicos e hormonais, o uso de transgênicas portadoras de promotores responsivos a classes hormonais específicas ou que, alternativamente, possuem alterações em genes correlacionados com a cascata de transdução dos mesmos, possibilitam análises ainda mais detalhadas dos processos estudados (WANG *et al.*, 2008; EGEA *et al.*, 2010).

Em suma, para desenvolver estudos que avaliem a participação das diversas classes hormonais, fotorreceptores e outros compostos sinalizadores no controle da fotomorfogênese vegetal, torna-se fundamental o uso de modelos vegetais que

possibilitem diferentes abordagens experimentais e cujo cultivo e obtenção de novos indivíduos sejam relativamente simples. Nesse aspecto, a cultivar de tomateiro Micro-Tom (MT) ganha destaque.

Inicialmente produzida com propósitos ornamentais, plantas de MT apresentam um fenótipo “anão” tanto na parte vegetativa quanto reprodutiva (SCOTT & HARBAUGH, 1989). Seu tamanho reduzido, ciclo de vida curto e fácil transformação genética fez com que este se tornasse um modelo conveniente para a investigação sobre a regulação do desenvolvimento de frutos (MEISSNER *et al.*, 1997; EYAL & LEVY, 2002). Apesar das mutações que lhe conferem esse fenótipo “anão”, estudos recentes demonstraram que MT é um modelo apto para pesquisas em tomateiro, inclusive em pesquisas sobre interações hormonais (CAMPOS *et al.*, 2010).

Nos últimos anos uma ampla gama de mutantes fotomorfogênicos e hormonais (CARVALHO *et al.*, 2011), bem como transgênicas foram introgrididos em MT (D’AGOSTINO *et al.*, 2000; MARTÍ *et al.*, 2010; PINO *et al.*, 2010). A introgressão de diversas mutações e transgênicas dentro de uma mesma cultivar facilita a comparação entre resultados obtidos em diferentes estudos, bem como possibilita o cruzamento entre mutantes e entre mutantes e transgênicas para melhor investigar os mecanismos envolvidos em determinados eventos fisiológicos, como, por exemplo, a diferenciação plastidial e acúmulo de pigmentos fotossintéticos durante o processo de desestiolamento vegetal, o qual é o foco do presente trabalho.

Apesar dos diversos estudos desenvolvidos na área, grande parte das informações disponíveis acerca do controle da biogênese e diferenciação plastidial em tomateiro tem sido obtida por meio de pesquisas conduzidas exclusivamente em tecidos reprodutivos (FORTH & PYKE, 2006; WANG *et al.*, 2008; EGEA *et al.*, 2010, 2011), tornando essa

espécie um dos principais modelos para estudos voltados à caracterização estrutural, bioquímica e regulatória dos processos de biogênese e diferenciação de cloroplastos em cromoplastos (FORTH & PYKE, 2006; BARSAN *et al.*, 2010; EGEA *et al.*, 2010, 2011).

Muito desse interesse se deve ao fato de que grande parte dos compostos nutracêuticos do fruto carnoso do tomateiro são sintetizados e/ou acumulados em seus plastídios (WANG *et al.*, 2008; EGEA *et al.*, 2010) e, portanto, dependem da dinâmica de formação e diferenciação dessas organelas. Entretanto, ainda existem enormes lacunas no que concerne à participação conjunta de sinais luminosos, hormonais e do NO durante a regulação da biogênese e diferenciação dos cloroplastos e do acúmulo de pigmentos tanto em tecidos reprodutivos quanto vegetativos de tomateiro.

Desconhece-se, por exemplo, os mecanismos regulatórios implicados no controle da abundância e da diferenciação plastidial durante o processo de desestiolamento de plântulas de tomateiro, e sabe-se muito pouco acerca dos processos de sinalização implicados no controle da biossíntese e acúmulo de pigmentos nessas plântulas. Estudar tais eventos durante o desestiolamento de plântulas torna-se especialmente interessante uma vez que é justamente nesse período que ocorre a diferenciação dos cloroplastos e o acúmulo de pigmentos fotossintéticos (FANKHAUSER & CHORY, 1997).

Nesse contexto, o presente estudo traz evidências da atuação do NO como sinal estimulatório ao desenvolvimento de cloroplastos e ao acúmulo de pigmentos durante o desestiolamento de plântulas de tomateiro, bem como relata uma forte interação entre o NO e os hormônios etileno e auxinas, onde o NO atuaria reprimindo a produção de etileno enquanto intensificaria os efeitos promotores das auxinas sobre essas respostas fotomorfogênicas. Adicionalmente, o presente trabalho também demonstra que a

enzima NR é provavelmente a principal fonte responsável pela biossíntese de NO nos tecidos cotiledonares de tomateiro em processo de desestiolamento e que tratamentos com NO exógeno são capazes de recuperar o fenótipo estiolado de plântulas de tomateiro mutantes para fitocromos quando essas são crescidas em condições luminosas não indutoras do desestiolamento.

II. OBJETIVOS

O presente estudo visou investigar, de forma integrada, o envolvimento e interações existentes entre o óxido nítrico (NO), etileno e auxinas, bem como o envolvimento da enzima nitrato redutase (NR) como possível rota biossintética de NO, durante os processos de diferenciação plastidial e acúmulo de pigmentos fotossintéticos em cotilédones de plântulas de tomateiro induzidos em respostas a estímulos luminosos.

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III CAPÍTULO ÚNICO

Cross talk between nitric oxide, ethylene and auxins during light-mediated greening and plastid development in de-etiolating tomato seedlings

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Short running title

NO and hormonal control of plastid development during deetiolation

ABSTRACT

The transition from etiolated to green seedlings involves the conversion of etioplasts into mature, functional chloroplasts via a multifaceted light-driven process comprising multiple and tightly coordinated endogenous signaling networks. Plant hormones and other signaling molecules, such as the free radical nitric oxide (NO), are believed to play important roles in controlling the acquisition of these photomorphogenic traits. In the present study, we investigated, in an integrated way, the influence of NO, ethylene and auxins on the light-evoked greening and chloroplast development in tomato (*Solanum lycopersicum*) seedlings. By determining the time course of photosynthetic pigments accumulation, etioplast-to-chloroplast differentiation, fluctuations in endogenous NO content and in nitrate reductase (NR) activity in wild type (Micro-Tom cultivar, MT) and in photomorphogenic mutants (*aurea* and *high pigment-1*) seedlings maintained under continuous darkness or exposed to monochromatic red (RL) or blue light (BL), we evidenced a clearly positive correlation between the NO production via NR and the light-induced cotyledon greening and chloroplast maturation. Supporting a role for NR as an important biosynthetic source of NO in de-etiolating tomato seedlings, different strategies employed to inhibit the light-evoked increment in the activity of this enzyme have successfully reduced the endogenous NO production. Interestingly, exogenous NO stimulated greening and chloroplast differentiation in cotyledon cells of *aurea* seedlings maintained under RL, thereby indicating that this signaling molecule might complement the partial deficiency in RL perception characteristic of this phytochrome-deficient mutant. In parallel, a mutual antagonism between NO and ethylene was evidenced by a number of findings. (i) RL- or BL-induced greening and chloroplast differentiation in tomato seedlings temporally coincided with increases and decreases in NO and ethylene emission, respectively. (ii) Whereas NO stimulated cotyledon greening, treatments with gaseous ethylene or its precursor (1-aminocyclopropane-1-carboxylic acid, ACC) severally impaired either RL- or BL-induced greening in MT. (iii) Ethylene- or ACC-treated de-etiolating seedlings presented significantly lower NO levels whereas the ethylene-insensitive *Never ripe* (*Nr*) mutant exhibited increased endogenous NO content. (iv) De-etiolating *Nr* seedlings exhibited increased total activity and activation state of the NO-generating enzyme NR. (v) Exogenous NO drastically reduced ethylene emission in *au* seedlings maintained under RL. On the other hand, a series of evidence indicated a mutual synergism between

auxins and NO in de-etiolating tomato seedlings. (i) The light-induced NO accumulation coincided with an increased activation of the synthetic auxin-responsive promoter *DR5* in both RL- and BL-exposed MT seedlings. (ii) Exogenous NO completely rescued the reduced activation of the *DR5* promoter observed in *au* seedlings under RL. (iii) Endogenous NO was drastically decreased and increased in de-etiolating seedlings of auxin-insensitive (*diageotropica*) and auxin-hypersensitive (*entire*) tomato mutants, respectively. Taken together, these data reveal that negative and positive feedback regulatory loops orchestrate ethylene-NO and auxin-NO interactions during the light-triggered cotyledon greening and chloroplast differentiation in de-etiolating tomato seedlings, reinforcing the importance of these signaling molecules during the coordination of seedling transition from the etiolated state to photomorphogenic growth.

Keywords: Tomato; De-etiolation; Nitric oxide; Nitrate reductase; Ethylene, Auxins, Phytochrome

III.1 INTRODUCTION

Chloroplast biogenesis and maturation are critical steps during plant growth and development since this organelle not only serves as the site of photosynthesis but also functions as the center of many other essential metabolic pathways. During seedling development, chloroplasts may differentiate directly from the plastid progenitor known as proplastid or from the dark-grown transitory form, the etioplast (POGSON & ALBRECHT, 2011). Considering that under most natural conditions, light is frequently unavailable or insufficient during the initial development of germinating seedlings, etioplasts formation and its subsequent differentiation into chloroplasts is of great adaptive value for most, if not all, terrestrial seed plants. Facilitating the prompt differentiation of etioplast into functional chloroplast, etioplasts typically accumulate thylakoid lipids and large amounts of the chlorophyll precursor protochlorophyllide (Pchlde) bound to Pchlde reductase, forming a semicrystalline membranous structure known as prolamellar body (PLB).

Unarguably, light perception and signaling represent the central master switcher for the etioplast-to-chloroplast conversion, controlling the synthesis of chlorophylls and the transcription of a large number of genes encoding proteins that comprise the photosynthetic apparatus, ultimately leading to the final structural configuration and biochemical composition of mature chloroplasts (WATERS & LANGDALE, 2009). Light is also an essential element for the activation of angiosperm Pchlde reductase, which is a light-dependent enzyme responsible for the conversion of the Pchlde pools accumulated in etioplasts into chlorophyll molecules (HEYES & HUNTER, 2005; REINBOTHE *et al.*, 2010). As in other photomorphogenic responses, the initiation of these processes by light primarily relies on the combined action of phytochromes and

cryptochromes, which are the two photoreceptor families mainly responsible for the gene expression reprogramming typical of de-etiolating seedlings (JIAO *et al.*, 2007; LÓPEZ-JUEZ *et al.*, 2008).

During the last decade, impressive advances have been achieved in clarifying the signaling networks interconnecting light perception and etioplast-to-chloroplast transition, revealing key roles for proteins involved in the transduction of light perception via both phytochromes and cryptochromes. In *Arabidopsis*, losses of specific PHYTOCHROME INTERACTING FACTORS (PIFs) have been shown to perturb chloroplast biogenesis and development in both dark- and light-grown seedlings (SHIN *et al.*, 2009; STEPHENSON *et al.*, 2009) whereas mutations in light-inactivable proteins such as CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) or DE-ETIOLATED 1 (DET1) have indicated a clear suppressor role for these light-regulated proteins on chloroplast development in cotyledons of dark-grown seedlings as well as in non-photosynthetic tissues of light-grown plants (CHORY *et al.*, 1989; CHORY & PETO, 1990; DENG & QUAIL, 1992). Interestingly, some COP and DET proteins interact among themselves and with other light-regulated proteins, such as UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1), giving rise to the so-called COP9 signalosome, which targets specific proteins such as LONG HYPOCOTYL 5 (HY5) for proteosomal degradation (WEI *et al.*, 2008). Not surprisingly, HY5 has also been identified as a key promoter signal for chloroplast development (KOBAYASHI *et al.*, 2012).

As in *Arabidopsis*, DET1, DDB1, COP1 and HY5 and some other light signaling components have also been demonstrated to control plastid biogenesis and development in tomato (*Solanum lycopersicum*) leaf and fruit tissues (LIU *et al.*, 2004; DAVULURI *et al.*, 2005; KOLOTILIN *et al.*, 2007; WANG *et al.*, 2008). In this economically and

experimentally important crop, mutations in *HIGH PIGMENT 1* (*HP1*) and *HP2*, which respectively encodes a homolog and an ortholog of *Arabidopsis* DDB1 and DET proteins, have long been characterized to increase chlorophyll and carotenoid accumulation as well as chloroplast number and size in fully expanded leaf and fruit pericarp cells (MUSTILLI *et al.*, 1999; COOKSON *et al.*, 2003; KOLOTILIN *et al.*, 2007). More recently, manipulation of light signaling components (*e.g.*, HP1/DDB1, HP2/DET1, COP1, HY5) has emerged as an efficient strategy to improve plastidial biogenesis and development and, consequently, the nutritional quality in tomato fruits (LIU *et al.*, 2004; DAVULURI *et al.*, 2005; WANG *et al.*, 2008). Some excellent studies have also characterized the influence of specific phytochromes and cryptochromes on chloroplast development and pigment accumulation in tomato leaf and fruit tissues (WELLER *et al.*, 2000, 2001; GILIBERTO *et al.*, 2005; HUSAINI *et al.*, 2007), but, in contrast, relatively rare studies have focused on the interplay among photoreceptors and their interaction with other endogenous signals during the conversion of etioplasts into chloroplasts in de-etiolating tomato seedlings.

As in many other plant photomorphogenic responses, plant hormones and other signaling molecules are believed to interact with the light signaling cascades to precisely coordinate plastid biogenesis and differentiation with changes in the environment context (EGEA *et al.*, 2010). An antagonistic relationship has frequently been described between cytokinins and abscisic acid (ABA) during greening, plastid gene transcription and chloroplast differentiation in de-etiolating seedlings (KUSNETSOV *et al.*, 1998; KRAVTSOV *et al.*, 2011).

Exogenous application of cytokinins has long been demonstrated to activate cotyledon and leaf greening in numerous species, stimulating plastid biogenesis and the accumulation of plastid transcripts, proteins and pigments (KRAVTSOV *et al.*, 2011).

Moreover, treatment with exogenous cytokinins or mutations that increases the production or sensitivity to this plant hormone usually stimulate partial differentiation of the membranous system in plastids of dark-grown seedlings, particularly by triggering the conversion of PLBs into prothylakoid membranes (CHORY *et al.*, 1994). In this and some other aspects, cytokinin seems to mimic the *cop* and *det* mutant phenotypes (CHORY *et al.*, 1994; MUSTILLI *et al.*, 1999).

ABA, on the other hand, has been frequently shown to repress chloroplast biogenesis. For instance, ABA-deficient mutants have been described to exhibit increased plastid abundance and abnormal chloroplast structure either in *Arabidopsis* leaf tissues (ROCK *et al.*, 1992) or tomato fruit pericarp (GALPAZ *et al.*, 2008). Moreover, during barley seedling de-etiolation, ABA represses both chlorophyll accumulation and plastid gene transcription (KRAVTSOV *et al.*, 2011).

In contrast to cytokinins and ABA, the influence of auxins and ethylene on plastid biogenesis and etioplast-to-chloroplast differentiation has received considerably less attention. Curiously, an intimate connection between these hormones has been widely reported during other important seedling de-etiolation processes, such as the regulation of hypocotyl elongation and the formation, maintenance and light-induced opening of the apical hook in eudicotyledons (SYMONS & REID, 2003). Moreover, the biosynthesis and signaling of these plant hormones in de-etiolating seedlings have also been shown to significantly change upon illumination (SYMONS & REID, 2003; LÓPEZ-JUEZ *et al.*, 2008).

Besides plant hormones, other endogenous signal molecules also seem to be implicated in controlling seedling greening and chloroplast development. The gaseous free radical nitric oxide (NO), for instance, has recently emerged as a stimulator signal for the acquisition of photomorphogenic traits in different plant species, closely

interacting with light during the regulation of processes such as seed germination, hypocotyl elongation and cotyledon and leaf greening (BELIGNI & LAMATTINA, 2000; ZHANG *et al.*, 2006; LOZANO-JUSTE & LEON, 2011). During seedling greening, NO seems to intensify the stimulatory effect of the light stimulus, promoting the accumulation of both chlorophylls and chloroplast-related proteins (BELIGNI & LAMATTINA, 2000; ZHANG *et al.*, 2006). In agreement, increased NO production has also been recently reported in de-etiolating *Arabidopsis* and wheat seedlings (LOZANO-JUSTE & LEON, 2011; LIU *et al.*, 2013).

Although still not demonstrated, interactions between NO and plant hormones during seedling greening might be expected since intertwined NO-hormonal signaling cascades have increasingly been reported during recent years (FRESCCHI, 2013; SIMONTACCHI *et al.*, 2013; LEÓN *et al.*, 2014). These NO-hormonal cross-talks involve not only the influence of NO on plant hormone metabolism, perception or signal transduction, but also the modulation of endogenous NO levels by plant hormones. In fact, although NO biosynthetic pathways in plants are still not fully characterized, accumulating evidence indicates that key enzymes involved in NO production in plant tissues might be regulated by plant hormones.

Among the potential biosynthetic sources of NO in plants, nitrate reductase (NR) has been considered as one of the most likely candidates responsible for NO production under physiologically relevant conditions (KAISER & PLANCHET, 2006; GUPTA *et al.*, 2011). Under strict control by multiple environmental and endogenous cues, NR is regulated at both transcription and post-translational levels (KAISER & HUBER, 2001). Not surprisingly, light is a critical environmental signal regulating plant NR levels and activity, controlling both the transcription of genes encoding NR (transcriptional control) as well as the phosphorylation state (post-translational control)

of the enzyme (LILLO & APPENROTH, 2001). In presence of nitrate, NR transcript and protein levels dramatically increase in de-etiolating seedlings soon after the exposure to illumination, a response apparently depending mostly on the light perception via phytochromes (BECKER *et al.*, 1992; GOUD & SHARMA, 1994), which, at least in some species, can be mimicked or intensified by the exogenous application of cytokinins and/or auxins (LU *et al.*, 1992; YU *et al.*, 1998). ABA, on the other hand, seems to repress light-driven increases in NR transcripts, protein and activity (LU *et al.*, 1992). As a result, light and plant hormones intensively interact to determine the induction of NR concomitantly to the de-etiolation process.

Here, we show that NR-derived NO stimulates chloroplast development in de-etiolating tomato seedlings by counteracting the repressor influence of ethylene and intensifying the positive effect of auxins.

III.2 MATERIAL AND METHODS

III.2.1 Plant material and growth conditions

Tomato (*S. lycopersicum* L.) cv. Micro-Tom (MT) and the near-isogenic lines (NILs) harbouring the mutations *aurea* (*au*), *high pigment-1* (*hp-1*), *entire* (*e*), *diageotropica* (*dgt*) and *Never ripe* (*Nr*) were obtained as previously described (CARVALHO *et al.*, 2011). All these genotypes, as well as transgenic MT carrying the synthetic auxin-responsive (*DR5*) and ethylene-responsive (*EBS*) promoters fused to the reporter gene *uid* (encoding a β -glucuronidase, GUS) were obtained from the tomato mutant collection maintained at ESALQ, Universidade de São Paulo (USP), Brazil (<http://www.esalq.usp.br/tomato/>). Additionally, crosses and phenotypical screening were performed to generate the double mutants *au,DR5::GUS* and *dgt,DR5::GUS*.

III.2.2 Growth conditions and treatments

Seeds were surface sterilized as described in LOMBARDI-CRESTANA *et al.* (2012) and directly sown in magenta vessels containing sterile medium composed of half-strength Murashige and Skoog (MS) salts and 2% (w/v) Phytigel®. After 5 days pre-germination in absolute darkness, seedlings were transferred to continuous red light (RL), blue light (BL) or maintained under absolute darkness (D). RL and BL were supplied by an array of SMD5050 Samsung LEDs mounted in a temperature-controlled growth chamber maintained at 25±1°C and installed inside a dark room. Both BL and RL were delivered at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with peak output at 470 and 625 nm respectively, as defined by the manufacturer. In all cases, tissue was harvested either

under the specific light conditions used for seedling growth or under dim green light, as appropriate.

For treatment of seedlings with inhibitors of NR activity, seeds were grown in the same manner described above except that the MS media was modified, as appropriated. In the nitrogen-free MS medium, NH_4NO_3 was omitted and KNO_3 was replaced by 0.35 g/L KCl and 0.4 g/L K_2SO_4 . Modified MS media containing ammonium or glutamine as the exclusive sources of nitrogen were essentially the same formulation of the nitrogen-free MS medium added of 1.61 g/L NH_4Cl or 2.19 g/L glutamine, respectively. Therefore, when present in the medium, nitrogen was available at a final concentration of 30 mM. The medium containing glutamine was sterilized by ultra-filtration whereas the others were autoclaved.

Hormonal and NO treatments were initiated two days before starting the light treatments and were maintained thereafter. Treatments with gaseous substances, such as ethylene (100 ppm) and NO (50, 100, 150 ppm), were conducted in sealed magenta boxes (total airspace volume of 360mL). Ethylene application was renewed in a daily basis, whereas NO concentrations were either renewed in daily basis (50 and 100ppm final concentration) or continuously fluxed inside the sealed magenta boxes containing the plants at a concentration of 150 ppm and a rate of 100mL/min. Ethylene and NO concentrations were obtained by mixing commercial standard mixtures of these gases with ethylene- and NO-free breathing air at appropriate flow rate ratios. NO gas mixtures were bubbled through a flask containing 500 mM KOH to eliminate any nitrous acid traces and humidify the gas mixture. Other plant growth regulators were dissolved in water, sterilized by ultra-filtration and directly applied to the growth medium containing the seedlings. In all cases, at least 100 seedlings were harvested at each time point and each experimental treatment was performed at least twice.

III.2.3 Chlorophylls and carotenoids quantification

For chlorophylls and carotenoids extraction, samples of cotyledons were weight (typically 20mg), immersed in a 100 times excess volume of N, N-dimethylformamide and incubated for 48 h at 25°C in absolute darkness. The supernatant absorbance was recorded at 480, 647 and 664 nm and the total chlorophyll and carotenoid contents were estimated using the equations published by PORRA *et al.* (1989) and WELLBURN (1994).

III.2.4 Protochlorophyllide determination

Protochlorophyllide content of etiolated cotyledons was determined by room-temperature fluorescence spectroscopy of 80% (v/v) acetone extracts (OUGHAM *et al.*, 2001). Five cotyledon pairs were dissected in complete darkness, weighted, grounded in liquid nitrogen and extracted in 1 ml of cold 80% acetone for 1 h at 4°C in absolute darkness. After centrifugation (13 000 g, 10 min, 4°C), fluorescence emission spectra of the supernatants were obtained with a Perkin Elmer LS55 fluorescence spectrophotometer set at an excitation wavelength of 435 nm, and the excitation and emission slit widths set at 10 and 5 nm, respectively. Protochlorophyllide levels were determined using the formula: $Pchlde = (F_{628} - 0.09 \times F_{668} + 0.006 \times F_{650}) \times 2.2$. Measurements were repeated three times using independent sets of cotyledons.

III.2.5 NO measurements

For fluorometric NO determination, the cell-impermeant fluorophore diaminorhodamine-4M (DAR-4M) was used. Cotyledons were gently fragmented into small pieces (typically 5 x 5 mm) and immediately weighted (~100mg) and incubated with 1mL of 50 mM phosphate buffer (pH 7.2) containing 37.5 μ M DAR-4M for 30 min at 25°C in absolute darkness on a rotary shaker (200 rpm). The supernatant fluorescence was measured using a spectrofluorometer (LS55, Perkin Elmer) with 560 nm excitation and 575 nm emission wavelength (5 nm band width). Fluorescence was measured at the same instrument settings in all experiments and was expressed as arbitrary fluorescence units (AU) per gram dry weight per hour (AU/g DW/h).

III.2.6 NR activity assay and activation state

NR maximum activity and activation state were assayed according to TUCKER *et al.* (2004) with some modifications. Briefly, samples were ground in liquid nitrogen and subsequently homogenized in extraction buffer (~300 mg tissue/mL) composed of 100 mM Hepes-KOH (pH 7.6), 10 μ M FAD, 10 μ M Na₂MoO₄, 1 mM EDTA, 0.5% (w/v) polyvinylpyrrolidone (PVP), 5 mM dithiotreitol (DTT) and 10 μ M leupeptine. After centrifugation (13 000 g, 10 min, 4°C), 100 μ L of the supernatant was added to 900 μ L of reaction medium composed of 100 mM Hepes-KOH (pH 7.6), 10 μ M FAD, 5 mM DTT, 20 mM KNO₃ and 200 μ M NADH, and 10 mM EDTA (for NR maximum activity), or 15 mM MgCl₂ (for actual NR activity). The reactions were incubated for 5 min at 30°C and, then, stopped by adding 100 μ L 500 mM zinc acetate. The unreacted NADH was oxidized by incubation with 100 μ M phenazine methosulfate for 20 min and the nitrite produced by the reactions quantified by standard colorimetric reaction

(HAGEMAN & REED, 1980). For the determination of NR maximum activity, the same extract was pre-incubated in ice for 12 min in the presence of 20 mM AMP, 15 mM EDTA and 10 mM KH_2PO_4 . In all cases, blanks were made by adding zinc acetate to the reaction medium prior the addition of the protein extract. NR activation state is given as the ratio of maximum NR activity (measured after preincubation of extracts with excess EDTA plus AMP) and the values of NR active (measured in the presence of excess Mg^{2+}) (MAN *et al.*, 1999). NR activities obtained in the presence of excess Mg^{2+} are believed to reflect NR activity *in situ* whereas maximum NR activity indicates the total pool of NR protein available in the tissues (MAN *et al.*, 1999).

III 2.7 Ethylene measurements

Ethylene emission was analyzed as described in FRESCHI *et al.* (2010). Briefly, intact tomato seedlings (typically 100 individuals) growing inside sealed magenta boxes, were flushed with ethylene-free air (1 L/min) for 5 min, and incubated for 24 h under specific experimental conditions, as appropriate. After incubation, 1-mL gas samples were withdrawn with a gas-tight syringe and injected into a Trace Ultra gas chromatograph (Thermo Electron) fitted with a flame-ionization detector (GC-FID) and a RT-alumina Plot column (Restek Corporation). Nitrogen was used as the carrier gas at a flow rate of 3 mL/min, and commercial standard mixtures of ethylene were used for the calibration curves. Column, injector and detector temperatures were 40°C, 250°C and 250°C, respectively.

III.2.8 Quantitative GUS activity assay

GUS activity was assayed according to JEFFERSON *et al.* (1987) with some modifications. Briefly, cotyledon samples were ground in liquid nitrogen and subsequently homogenized in MUG extraction buffer (~150 mg tissue/mL) composed of 50 mM Hepes-KOH (pH 7.0), 5 mM DTT and 0.5% (w/v) PVP. After centrifugation (13 000 g, 20 min, 4°C), 200 µL aliquots of the supernatant were mixed with 200 µL GUS assay buffer composed of 50 mM HEPES-KOH (pH 7.0), 5 mM DTT, 10 mM EDTA and 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) and incubated at 37°C for 30 minutes. Subsequently, aliquots of 100 µL were taken from each tube and the reactions were stopped with 2.9 mL of 0.2 M Na₂CO₃ (pH 9.5) and fluorescence was determined using a spectrofluorometer (LS55, Perkin Elmer) with 365 nm excitation and 460 nm emission wavelength (5 nm band width). Fluorescence was measured at the same instrument settings in all experiments.

III.2.9 Histochemical analysis of GUS activity

GUS staining was carried out according to JEFFERSON *et al.* (1987) with some modifications. Whole seedlings were fixed using pre-chilled 90% acetone at -20°C for 1 h, rinsed three times with 50 mM phosphate buffer (pH 7.0) and subsequently soaked in 50 mM phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM 2-mercaptoethanol, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 2 mM 5-bromo-4-chloro-3-indolyl β-d-glucuronide, vacuum infiltrated, and incubated at 37°C. The reaction was stopped by 70% (v/v) ethanol after 16h and 4h in *DR5::GUS* and *EBS::GUS*, respectively.

III.2.10 Measurement of ACO activity

The extraction and activity assay of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), a key enzyme in the ethylene biosynthetic pathway, was carried according to VRIEZEN *et al.* (1999), with some modifications. Briefly, samples were ground in liquid nitrogen and subsequently homogenized in extraction buffer (~500 mg tissue/mL) composed of 300 mM Tris-HCl (pH 8.0), 50 mg/mL insoluble polyvinylpyrrolidone, 10% (v/v) glycerol and 30 mM sodium ascorbate. After centrifugation (20 000 g, 20 min, 4°C), 200 µL of the supernatant was added to 1.7 mL of reaction medium composed of 100 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 30 mM sodium ascorbate, 100 µM FeSO₄, 50 mM NaHCO₃, 5 mM DTT and 2 mM 1-aminocyclopropane-1-carboxylic acid (ACC). ACO activity was determined by measuring the ability of the extract to convert exogenous ACC to ethylene after incubation at 30°C for 20 min. The ethylene levels were measured by GC-FID as described above.

III.2.11 Transmission Electron Microscopy

Cotyledons were cut into small pieces (1 x 1 mm) and fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2), dehydrated in a graded acetone series and embedded in resin Spurr. Semi-thin sections were stained with 1% toluidine blue and the ultrathin sections were counterstain with the uranyl acetate (WATSON, 1958) and lead citrate (REYNOLDS, 1963), being analyzed with a Zeiss EM 900 transmission electron microscope. The electron micrographs illustrate the relevant results obtained.

III.3 RESULTS

III.3.1 Etioplast-to-chloroplast transition and greening in tomato photomorphogenic mutants

As expected, marked differences in the accumulation of photosynthetic pigments (chlorophylls and carotenoids) were observed in tomato photomorphogenic mutant seedlings exposed to distinct light conditions (Fig. 1). In cotyledons of Micro-Tom (MT) seedlings, maximum levels of chlorophylls and carotenoids were achieved as soon as 48h after the start of either RL or BL treatments, remaining relatively stable thereafter.

The time course of pigment accumulation was virtually undistinguishable in MT and *aurea* (*au*) seedlings exposed to BL (Fig. 1c,f) whereas RL failed to induce the accumulation of either chlorophylls or carotenoids in this phytochrome-deficient mutant (Fig. 1b,e). Consistent with its light-hypersensitive phenotype, *high pigment 1* (*hp-1*) seedlings exhibited chlorophyll and carotenoid levels considerably higher than MT under RL, and even more conspicuously under BL. Significant differences in Chl *a*/Chl *b* ratio were not observed among these mutants and light treatments (Fig. S1).

Virtually no photosynthetic pigment accumulation was observed in darkness in all genotypes, with the exception of some carotenoid accumulation in the cotyledons of dark-grown *hp-1* seedlings (Fig. 1d). Besides accumulating more carotenoids, dark-grown *hp-1* seedlings also presented Pchl_{ide} levels slightly higher than MT (Fig. S1). Dark-grown *au* seedlings, on the other hand, presented about 60% of Pchl_{ide} content found in cotyledons of MT seedlings grown under similar conditions (Fig. S1).

Fig. 1. Time course of photosynthetic pigment accumulation in seedlings of tomato photomorphogenic mutants exposed to distinct light conditions. Seedlings were maintained under complete darkness (a,d) or transferred to continuous RL (b,e) or BL (c,f) treatments. MT, Micro-Tom; *high pigment 1*, *hp1*; *aurea*, *au*. Means $\pm$ SD.

In terms of plastid ultrastructure, these mutants also presented some significant differences both under dark and light conditions (Fig. 2). As expected, in dark-grown MT and *au* seedlings, plastids presented an internal structure typical of etioplasts (Fig. 2a,g), exhibiting the characteristic lattice-like membranous structure known as the prolamellar body (PLB). On the other hand, cotyledon cells of dark-grown *hp-1* seedlings presented semi-developed chloroplasts instead of etioplasts, exhibiting PLBs converted into prothylakoid membranes (Fig. 2d).

The development of the internal membranous structure of the chloroplast in MT and *hp-1* under RL or BL (Fig. 2b,c,e,f) as well as in the *au* mutant under BL (Fig. 2i) was virtually indistinguishable, with the formation of granal thylakoids, disappearance of the PLBs and sometimes with the presence of starch grains. On the other hand, although PLBs were no longer present in chloroplasts of *au* under RL, these plastid did

not show the formation of granal thylakoids (Fig. 2h). Ultrastructural plastid features observed in cotyledon cells of MT, *hp-1* and *au* seedlings exposed to white light (Fig. S2) resembled those observed under BL; therefore, D, RL and BL treatments were selected for the subsequent steps of this study. As expected, the highest and lowest plastidial abundances were observed in cotyledons of *hp-1* and *au*, respectively (data not shown).

Fig. 2. Plastid structure in cotyledon cells of tomato photomorphogenic mutants exposed to distinct light conditions. Seedlings of MT (a-c), *high pigment-1* (d-f) and *aurea* (g-i) were analyzed upon 72h continuous darkness (D), red light (RL) or blue light (BL) treatment. PLB, prolamellar body; S, starch; G, granal thylakoid. Bars: 0.5 μ m.

III.3.2 NR-dependent NO production temporally coincides with light-driven greening and etioplast-to-chloroplast conversion

To gain information on the possible role of NO in the regulation of tomato seedling greening and etioplast-to-chloroplast differentiation, NO release in cotyledon tissues of MT, *hp-1* and *au* was compared by using the fluorometric quantification method based on the cell-impermeant NO probe DAR-4M. Simultaneously, the maximum activity and activation state of NR were also measured in the cotyledon tissues of these seedlings since this enzyme has been identified as one of the main sources of NO in plant systems (KAISER & PLANCHET, 2006; GUPTA *et al.*, 2011).

Under continuous darkness, all genotypes exhibited reduced endogenous NO release (Fig. 3a) associated with extremely low levels of maximum NR activity (Fig. 3d). On the other hand, striking differences in the dynamics and intensity of NO release and NR activity were observed among MT, *au* and *hp-1* under either RL (Fig. 3b,e) or BL (Fig. 3c,f).

The endogenous NO release in MT dramatically increased during the first 24h of RL or BL treatment, progressively decreasing thereafter (Fig. 3b,c). In contrast, the phytochrome-deficient mutant *au*, which remains partially etiolated under RL and completely de-etiolated under BL, failed to exhibit RL-dependent increases in NO generation (Fig. 3b) but presented a BL-dependent up-regulation of NO release very similar to that observed in the MT seedlings (Fig. 3c). These differences in NO release in *au* seedlings under RL or BL were mirrored by similar patterns in maximum NR activity (Fig. 3e,f), which were also only up-regulated under BL conditions.

Among the three genotypes, the light-hypersensitive mutant *hp-1* exhibited the highest values of both NO release and maximum NR activity under either RL or BL, which frequently exceed in several times the levels observed in MT or *au* seedlings.

hp-1 seedlings also exhibited distinctly higher values of NR activation state, which ranged between 40 and 70% regardless the light treatment, whereas MT and *au* never presented more than 40% of their NR protein in the active state (Fig. 3 g-i).

Fig. 3. Increases in NO levels during light-driven tomato seedling greening temporally coincide with the rise in NR activity. Fluorometric quantification of endogenous NO release (a-c), NR maximum activity (d-f) and NR activation state (g-h) in seedlings of wild type (MT) and photomorphogenic mutants (*high pigment-1*, *hp1* and *aurea*, *au*) maintained under continuous darkness (a,d,g) or transferred from darkness to continuous RL (b,e,h) or BL (c,f,i) treatments. Means $\pm$ SD.

III.3.3 Exogenous NO promotes greening in phytochrome-deficient tomato mutants

Treating *au* seedlings under RL with 50, 100 or 150 ppm gaseous NO caused significant increases in both chlorophyll and carotenoid levels (Fig. 4 a,c). Significant increases in pigment accumulation were observed as fast as 24h after simultaneous treatment with RL and 150 ppm NO. On the other hand, 50 and 100ppm led to increases in chlorophylls and carotenoids only after 48h of simultaneous treatment with RL and NO. Treating *au* seedlings with NO under complete darkness failed to induce photosynthetic pigments accumulation in this mutant (data not shown).

Under RL, the final chlorophyll concentration was similar in *au* seedlings treated with 100 or 150ppm NO whereas 50 ppm resulted in significantly lower chlorophyll and carotenoid accumulation (Fig. 4 a,c). The levels of pigments in *au* seedlings under RL and 150 ppm NO was similar to those observed when this mutant was exposed to BL in the absence of exogenous NO (Fig. 1 c,f) and was also not markedly different from that observed in MT seedlings exposed to RL or BL (Fig. 1 b,c,e,f).

Interestingly, even the highest concentration of NO used in the experiments (150 ppm) promoted only limited development of the internal membranous structure of chloroplasts in *au* seedlings under RL (Fig. 4b,d). However, although the formation of granal thylakoids in NO-treated *au* seedlings was relatively modest, it was clearly more intense than in seedlings of this phytochrome-deficient mutant exposed to RL with no NO supplementation (Fig. 2h).

Treating etiolated MT seedlings with 100 ppm NO under continuous darkness, RL or BL did not result in significant increments in chlorophyll or carotenoid contents (Fig. S3). Similarly, etiolated *au* seedlings exposed to NO did not show any differences in

terms of chlorophyll and carotenoid pigments (data not shown) or even in terms of Pchl_a content (Fig. S1). Moreover, MT seedlings treated with 2 mM of the NO scavenger 2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (PTIO) exhibited no significant changes in pigment accumulation under either RL or BL (Fig. S3).

Fig. 4. Exogenous NO promotes cotyledon greening and plastid differentiation in the phytochrome-deficient *aurea* (*au*) mutant. (a,c) Photosynthetic pigments accumulation in the photomorphogenic mutant *au* exposed to continuous RL in the presence or absence of different concentrations of exogenous NO. Means $\pm$ SD. (b,d) Plastid structure in *au* cotyledons upon 72h of continuous RL and 150 ppm NO treatment. Bars: 2 μ m (b); 0.5 μ m (d).

In an attempt to consistently reduce endogenous NO and, at the same time, investigate the relevance of NR as a source of NO in tomato cotyledons, different treatments were conducted to inhibit the activity of this enzyme, including the use of a nitrogen-free medium and two modified MS media containing ammonium or glutamine as the sole source of nitrogen.

As indicated in Table 1, all these approaches significantly inhibit both NR activity and NO release from cotyledon cells of MT either in the presence of RL or BL treatment. Regardless of the light conditions, MT seedlings cultivated in nitrogen-free medium or in modified MS medium containing ammonium as the sole source of nitrogen exhibited a reduction between 85 and 98% in NR activity, which was associated with a decrease between 72 and 81% in endogenous NO release compared to nitrate-sufficient, control seedlings.

MT seedlings grown in modified MS medium containing glutamine as the only source of nitrogen displayed undetectable NR activity and a reduction of about 83% in NO release under either RL or BL treatment (Table 1). Interestingly, regardless of the strategy used to inhibit NR activity, the reduction in NO release from the cotyledon tissues was relatively homogenous, representing a reduction of about 80% when compared with untreated, control seedlings.

Table 1. Maximum and actual NR activity and endogenous NO release in MT seedlings cultivated in different sources of nitrogen under red light (RL) or blue light (BL).

Treatments	Maximum NR activity ($\mu\text{mol NO}_2^-/\text{h/g DW}$)		Actual NR activity ($\mu\text{mol NO}_2^-/\text{h/g DW}$)		NO Fluorescence (AU/g DW/h)	
	RL	BL	RL	BL	RL	BL
Control	$0.601 \pm 0.039\text{a}$	$0.874 \pm 0.070\text{a}$	$0.275 \pm 0.030\text{a}$	$0.343 \pm 0.034\text{a}$	$76.9 \pm 15.4\text{a}$	$65.5 \pm 2.9\text{a}$
Nitrogen-free	$0.045 \pm 0.002\text{b}$	$0.030 \pm 0.007\text{c}$	$0.020 \pm 0.003\text{b}$	$0.008 \pm 0.003\text{c}$	$21.0 \pm 2.4\text{b}$	$16.5 \pm 0.6\text{b}$
Ammonium	$0.046 \pm 0.004\text{b}$	$0.133 \pm 0.020\text{b}$	$0.023 \pm 0.008\text{b}$	$0.042 \pm 0.012\text{b}$	$14.4 \pm 1.2\text{c}$	$14.8 \pm 1.6\text{b}$
Glutamine	n.d.	n.d.	n.d.	n.d.	$11.6 \pm 1.1\text{c}$	$11.4 \pm 0.9\text{b}$

Values are means $\pm$ SD of two different experiments with at least three replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. n.d., not detectable.

No significant nitrite accumulation was detected in the cotyledons of de-etiolating tomato seedlings (data not shown); therefore, a physiologically relevant levels of non-enzymatic NO production from nitrite (BETHKE *et al.*, 2004) seems not very probable.

Finally, treatments with the mammalian nitric oxide synthase (NOS) inhibitor N^G -nitro-L-arginine methyl ester (L-NAME) had no effect on the greening process of RL- or BL-exposed tomato seedlings (Fig. S3).

III.3.4 NO and ethylene antagonistically interact during light-driven cotyledon greening

Next, the interconnection between NO and ethylene during tomato seedling greening and chloroplast differentiation was also investigated. Firstly, the emission of ethylene by MT and *au* seedlings was quantified, revealing increased ethylene production in the phytochrome-deficient mutant during most sampling times, especially under dark and RL conditions and less conspicuously under BL treatment (Fig. 5 a-c). In agreement with these observations, ACO activity under all light conditions was also higher in *au* than in MT seedlings (Fig. 5d,e). Whereas RL treatment decreased ethylene emission and ACO activity in MT seedlings (Fig. 5b,d), this light treatment failed to produce the same effects in *au* seedlings (Fig. 5b,e). During the first 48h of treatment, ethylene activity in tomato cotyledons, estimated by fluorimetric quantifications of GUS activity in transgenic seedlings carrying the *EBS::GUS*, was slightly higher under dark conditions than under RL and BL (Figs 5f and S3).

Considering the clearly opposite trends in NO and ethylene emissions during the greening process in tomato seedling (Figs. 3b and 5b), we next analyzed whether these signaling substances interact at biosynthetic or action levels as a mechanism to regulate chloroplast development and accumulation of photosynthetic pigments. As indicated in Fig. 5b, treating *au* seedlings with exogenous NO drastically decreased the ethylene emission under RL conditions, reducing the emission rates of this hormone in the

mutant to levels as low as those observed for the MT seedlings under the same light conditions.

On the other hand, no indication of NO action on the tissue sensitivity to ethylene was observed when etiolated *EBS::GUS* seedlings were simultaneously treated with ethylene and NO since the GUS activity observed in seedlings exposed to this combined treatment was virtually identical to that observed in plants only exposed to ethylene (Fig. 5g).

Curiously, though, compared with dark-grown *EBS::GUS* cotyledons not exposed to exogenous NO supplementation (Fig. 5f), dark-grown seedlings exposed to continuous fumigation with gaseous NO in the absence of exogenous ethylene exhibited a clear reduction in the activation of the ethylene-responsive promoter *EBS* (Fig. 5g). Also curiously, a gradual increase in the tissue responsiveness to exogenous ethylene was observed between 0 and 72h of the dark treatment since progressively higher GUS activity was observed following the exposure of the *EBS::GUS* seedlings to the same concentration and duration of ethylene fumigation (Fig. 5g).

Besides these indications that NO might down-regulate ethylene production in tomato seedlings, evidence obtained also suggests a negative influence of ethylene on the endogenous NO levels. As shown in Fig. 6, treating MT seedlings with gaseous ethylene or its precursor (ACC) resulted in a drastic reduction in endogenous NO release, almost completely abolishing the RL- or BL-driven up-regulation in endogenous NO production. Interestingly, both these hormonal treatments also significantly reduced the accumulation of chlorophylls and carotenoids in cotyledons of de-etiolating MT seedlings (Fig. 7).

Similar analysis conducted in seedlings of the ethylene-insensitive *Nr* mutant, revealed a clearly opposite pattern. Chlorophyll and carotenoid levels in *Nr* cotyledons

were higher than in MT at all sampling times either under either RL or BL treatment (Fig. 7). This increased pigment accumulation in the *Nr* mutant was associated with significantly increased NO release (Fig. 6 a,b), total NR activity (Fig. 6 c,d) and NR activation state (Fig. 6 e,f). The highest differences in these parameters were observed 48h after the start of the RL treatment, where the values of NO release, total NR activity and NR activation state in the *Nr* mutant under RL were respectively 2.3-, 3.9- and 2.7-fold higher than those detected in the cotyledons of MT seedlings under the same light conditions (Fig. 6). Under BL, an equivalent temporal pattern was observed; however, even higher values of total NR activity were observed in the *Nr* mutant, exceeding in more than 7-fold those detected in the wild type. When compared to MT seedlings growing under the same circumstances (Fig. 2), no striking differences in plastid structure were observed either in dark-grown or BL-treated *Nr* seedlings (Fig. 7c,f).

Fig. 5. Ethylene metabolism and signaling during tomato seedling greening and its modulation by exogenous NO. (a-c) Ethylene emission by seedling of wild type (MT) and the phytochrome-deficient mutant *aurea* (*au*) maintained under continuous darkness (a) or exposed to RL (b) or BL (c) treatment. (d) ACO activity in MT seedlings exposed to D, RL or BL treatment. (e) ACO activity in *au* seedlings exposed to BL, RL or RL plus 150 ppm NO. (f) *In vitro* GUS activity in *EBS::GUS* seedlings exposed to D, RL or BL treatment. (g) *In vitro* GUS activity in *EBS::GUS* tomato seedlings maintained under complete darkness in the presence of 150 ppm NO or 100 ppm ethylene (ET), or both. *, not sampled. Means $\pm$ SD.

Fig. 6. Ethylene negatively influences endogenous NO production in de-etiolating tomato seedlings. Fluorometric quantification of endogenous NO release in cotyledons of MT seedlings treated with 100 ppm gaseous ethylene (ET) or 100 μM ACC as well as in cotyledons of the ethylene-insensitive *Never ripe* (*Nr*) mutant exposed to either RL (a) or BL (b) treatment. Both ethylene and ACC treatments were initiated 2 days before starting the light exposure. Ethylene concentration was daily renewed, and ACC was only supplemented once to the growth medium. NR maximum activity (c,d) and activation state (e,f) in MT and *Nr* seedlings under BL (c,e) and RL (d,f). Means ± SD.

Fig. 7. Ethylene inhibits chlorophyll and carotenoid accumulation in de-etiolating tomato seedlings. Chlorophyll and carotenoid content in cotyledons of MT seedlings treated with 100 ppm gaseous ethylene (ET) or 100 μ M ACC as well as in cotyledons of the ethylene-insensitive *Never ripe* (*Nr*) mutant exposed to either RL (a,d) or BL (b,e) treatment. Treatment details as described in Fig. 6. Means $\pm$ SD. Plastid structure in cotyledon cells of the *Nr* mutant upon 72h of continuous darkness (c) or BL (f). Bars: 0,5 μ m.

III.3.5 NO positively interacts with auxins during light-driven cotyledon greening

Evidencing a positive interaction between NO and auxins, the auxin-insensitive tomato mutant *diageotropica* (*dgt*) exhibited clearly reduced NO levels in cotyledon tissues during most sampling times (Fig. 8a,b) whereas, in contrast, the mutant *entire* (*e*), which presents exaggerated responsiveness to auxins due to a single-base deletion in the coding region of the auxin signaling transcriptional repressor AUX/IAA9 (ZHANG *et al.*, 2007), exhibited increased endogenous NO levels (Fig. 8a,b) and increased chlorophyll and carotenoid contents under either RL or BL (data not shown).

Similar to the pattern of endogenous NO release (Fig. 3a-c), the activation of the auxin-responsive promoter *DR5* remained relatively more stable and at lower levels in tomato seedlings maintained under continuous darkness than in those exposed to RL or BL (Fig. 8c). The highest activation of the *DR5* promoter was observed after 24h of BL and 48h of RL treatments, decreasing thereafter.

Seedlings of the double mutant *au,DR5::GUS* exposed to RL exhibited reduced GUS activity values (Fig. 8d). Surprisingly, though, *au,DR5::GUS* seedlings simultaneously exposed to RL and exogenous NO (150 ppm) exhibited conspicuously higher GUS activity (Fig. 8d), resembling or even exceeding the GUS activity levels observed in light-treated *DR5::GUS* cotyledons (Fig. 8c). Confirming the specificity of the *DR5* promoter towards auxin signaling, seedlings of the double mutant *dgt,DR5::GUS* exhibited extremely low levels of GUS activity when compared with those observed in *DR5::GUS* seedlings exposed to BL or RL or maintained under continuous darkness (data not shown).

In all cases, the sensitivity of the histochemical GUS staining method was not adequate to detected changes in spatial pattern of auxin action along the seedlings of *DR5::GUS*, *au,DR5::GUS* and *dgt,DR5::GUS* since only the apical root and shoot meristems presented visible GUS staining (Fig. S4).

Fig. 8. NO and auxin interactions during tomato greening. Fluorometric quantification of endogenous NO release in the seedlings of *diageotropica* (*dgt*) and *entire* (*e*) mutants under RL (a) or BL (b) treatment. Time course of GUS activity (c) in cotyledons of *DR5::GUS* seedlings exposed to different light treatments. Time course of GUS activity (d) in cotyledons of *au,DR5::GUS* seedlings exposed to RL in the presence or absence of exogenous NO (150 ppm). Means $\pm$ SD.

III.4 DISCUSSION

The rapid acquisition of mature, functional chloroplast is of obvious importance for a seedling exposed to a light environment compatible with the occurrence of photosynthetic activity, marking the transition of a purely heterotrophic metabolism to the essentially autotrophic behavior (WATERS & LANGDALE, 2009). Not surprisingly, light is the central environmental cue eliciting and orchestrating the endogenous signaling events responsible for controlling chloroplast biogenesis and differentiation in de-etiolating seedlings (LÓPEZ-JUEZ *et al.*, 2008), however, the exact role played by some endogenous signals in the light-triggered cascades leading to chloroplast development and seedling greening still remains elusive.

The free radical nitric oxide (NO), for instance, has been demonstrated to influence cotyledon and leaf greening when exogenously applied (BELIGNI & LAMATTINA, 2000; ZHANG *et al.*, 2006; GNIAZDOWSKA *et al.*, 2010); however, the proposed role of NO on such a process still requires physiological support to be considered functionally significant during natural seedling de-etiolation. Here, we provide a series of evidence for the involvement of endogenous NO as an important element interconnecting light perception and the acquisition of photomorphogenic traits in de-etiolating tomato seedlings, revealing that the production of this free radical via NR might be closely associated with the induction of cotyledon greening and chloroplast maturation.

As a first line of evidence, treating tomato photomorphogenic mutant seedlings with specific monochromatic lights revealed a clear temporal correlation between the time courses of NR activity, NO production, photosynthetic pigment accumulation and plastid development (Figs. 1 to 3). Confirming the phytochrome-dependent regulation

of NR in tomato (BECKER *et al.*, 1992; GOUD & SHARMA, 1994), red light completely failed to induce NR activity in *au* seedlings and, very interestingly, the RL-induced increase in NO production observed in the control genotype (MT) was totally abolished in this mutant (Fig. 3b). Moreover, in agreement with studies conducted in other species (APPENROTH *et al.*, 2000), we found no effect of phytochromes on NR activation state, thus confirming that this class of photoreceptor apparently do not influence the post-translational regulation of this enzyme (LILLO & APPENROTH, 2001). On the other hand, the light-hypersensitive mutant *hp-1* exhibited exacerbated accumulation of photosynthetic pigments (Fig. 1) and intensified plastidial differentiation (Fig. 2), which were associated with high levels of NR activity and NO content (Fig. 3), thereby indicating that HP1/DDB1 acts as a repressor of these light-dependent physiological responses. Similar to the *Arabidopsis cop* and *det* mutants (CHORY *et al.*, 1989; DENG & QUAIL, 1992) as well as the *hp-2* tomato mutant (MUSTILLI *et al.*, 1999), here we demonstrate that dark-grown *hp-1* tomato seedlings partially formed thylakoid network instead of normal etioplasts with typical PLBs (Fig. 2d). As expected, only partial chloroplast development was observed in the dark since chlorophyll synthesis requires light, and photosystems cannot assemble without chlorophyll (HEYES & HUNTER, 2005; REINBOTHE *et al.*, 2010). In addition, it was also interesting to notice that the *hp-1* mutation led to increased NR activation state under RL, BL and particularly under continuous darkness (Fig. 3), suggesting that HP1/DDB1 not only represses total NR activity levels but also negatively influences NR post-translational regulation. To the best of our knowledge, no other light-signaling molecule has been identified as a potential regulator of NR at the post-translational levels in plants.

Besides these correlations between endogenous NO content and NR activity, we also observed that all distinct strategies employed to inhibit the light-evoked accumulation of this enzyme successively reduced both NR activity and NO production (Table 1), which further suggests nitrate reductase as possible source of NO in de-etiolating tomato seedlings. In agreement, NR has also been identified as one of the main sources of NO in several other NO-mediated physiological events such as stomatal closure, floral transition, and plant defense responses against pathogens, drought, cold and UV stress (reviewed by MUR *et al.* 2012). NR has also been demonstrated to be a critical source of NO in intact plants and cell suspensions of the other solanaceas species, such as *Nicotiana tabacum* and *N. plumbaginifolia* (LEA *et al.*, 2004; PLANCHET & KAISER, 2006; PLANCHET *et al.*, 2006).

Besides the reduction of nitrite into NO via NR, an oxidative pathway based on the production of NO from arginine via a nitric oxide synthase-like (NOS-like) activity has also been frequently linked to NO production during diverse plant developmental and stress responses (GUPTA *et al.*, 2011; MUR *et al.*, 2012). However, in spite of a great deal of efforts, the actual molecular identity of such NOS-like enzymes in plants still remains elusive (GUPTA *et al.*, 2011). In one study conducted with etiolated barley seedlings, ZHANG *et al.* (2006) reported a concomitant increase in NO emission and NOS-like activity during the light-induced greening process and observed delayed chlorophyll accumulation when seedlings were treated with a mammalian NOS inhibitor or a NO scavenger. More recently, LIU *et al.* (2013) also described that either the NOS inhibitor L-NAME (0.2mM) nor the NO scavenger cPTIO (0.1mM) blocked the restoration of chlorophyll content in etiolated wheat seedlings. Here, we have observed that treatments with even higher concentrations of either L-NAME (1mM) or cPTIO (2mM) completely failed to show any clear influence on RL- or BL-induced

tomato seedling greening (Fig. S3). The lack of response to cPTIO seems to indicate that NO-independent routes controlling light-induced tomato seedling greening might exist or, alternatively, the concentration of cPTIO used might have been insufficient for removing all NO generated following light exposure. As demonstrated by ARITA *et al.* (2006), VITECEK *et al.* (2008) and some other studies, fluorescent detection of NO is not adequate to determine the endogenous NO levels following cPTIO application; therefore, the effectiveness of this treatment cannot be easily determined.

Contrary to previous studies in grasses such as wheat (BELIGNI & LAMATTINA, 2000) and barley (ZHANG *et al.*, 2006), which reported dramatic increases in chlorophyll content in de-etiolating seedlings sprayed with the NO donor sodium nitroprusside (SNP), we observed that exogenous NO completely failed to induce further increases in photosynthetic pigment accumulation in de-etiolating tomato seedlings (Fig. S3). A critical difference, however, is that in the present study we have direct gassed the seedlings with a NO-enriched atmosphere instead of using SNP and we have to consider that SNP decomposition not only generates nitric oxide but also evolves other physiologically relevant compounds such as cyanide gas (BETHKE *et al.*, 2006). In a similar way, treating etiolated MT seedlings with gaseous NO under complete darkness also failed to induce any greening (Fig. S3), which seems to be in agreement with the fact that light irradiation is an indispensable requisite for the activation of Pchlide reductase in angiosperm (HEYES & HUNTER, 2005; REINBOTHE *et al.*, 2010). In addition, exogenous NO did not influence the Pchlide content of etiolated *au* seedlings (Fig. S1), maintaining this chlorophyll precursor at levels as low as that found in etiolated *au* seedlings of MT (Fig. S1) and other tomato cultivars (~ 60% of wild type) (TERRY & KENDRICK, 1999; RYBERG & TERRY, 2002).

In contrast, very clear dose-response stimulation in the accumulation of photosynthetic pigments was observed when phytochrome-deficient tomato mutant seedlings were simultaneously exposed to RL and gaseous NO (Fig. 4), which seems to indicate that NO might complement in some way the partial deficiency in RL perception characteristic of such mutant. As reported elsewhere, etiolated *au* seedlings present a small amount of functional phytochromes, which is estimated to be between 3 and 5% of wild-type levels (PARKS *et al.*, 1987; ADAMSE *et al.*, 1988; SHARMA *et al.*, 1993; GOUD & SHARMA, 1994). Therefore, some RL perception still remains in such mutant, thus allowing some partial responses even to monochromatic RL conditions, such as the conversion of etioplasts into semi-developed chloroplasts (Fig. 2h) and a very modest accumulation of photosynthetic pigments (Figs. 1b). Since NR activity and endogenous NO levels under these circumstances are extremely low (Fig. 3), it seems plausible to hypothesize that gassing these mutant seedlings with NO might have facilitated the achievement of a certain threshold level of this signaling molecule inside the etiolated tissues, which in association with some residual RL perception due to the remaining functional phytochromes, might have promoted a more extensive development of the chloroplast internal membranous system (Fig. 4d) and incipient synthesis and accumulation of photosynthetic pigments (Fig. 4a,c).

Therefore, the stimulator effect of NO on seedling greening and chloroplast differentiation apparently depends on the activation of at least some phytochrome molecules, which might also explain previous observations about the importance of concomitant light pluses for the NO-induced partial greening in etiolated wheat seedlings (BELIGNI & LAMATTINA, 2000). Besides seedling and leaf greening, NO has also been implicated in other phytochrome-dependent responses, such as photoblastic seed germination (BELIGNI & LAMATTINA, 2000; JOVANOVIĆ *et al.*,

2005; GUO *et al.*, 2008), hypocotyl elongation and anthocyanin accumulation (LOZANO-JUSTE & LEON, 2011). Moreover, BATAK *et al.* (2002) have demonstrated that NO donors particularly promoted PHYA-specific *Arabidopsis* seed germination, having a much more limited effect on PHYB-specific germination.

In *Arabidopsis* seedlings, NO-deficiency has been shown to reduce PHYB protein levels under RL (LOZANO-JUSTE & LEON, 2011). Therefore, considering that PHYB is the main photoreceptor mediating seedling de-etiolation in RL (DE LUCAS *et al.*, 2008), LOZANO-JUSTE and LEON (2011) have suggested that this phytochrome type probably is an important target of NO during RL-induced seedling photomorphogenesis. More recently, though, LIU *et al.* (2013) have demonstrated that SNP promoted the accumulation of the phytochromes photointerconvertible active form (Pfr) as well as promoted the expression of *PHYA* gene in etiolated wheat seedling leaves maintained under continuous darkness, thereby suggesting NO as an upstream regulator of phytochromes in this species. In the case of the tomato *au* seedlings, which are deficient in the biosynthesis of the phytochrome chromophore (MURAMOTO *et al.*, 2005), it seems not very likely that some enrichment in the internal pool of specific phytochrome apoproteins might be responsible for the NO-induced greening of this mutant under RL conditions, but obviously such possibility cannot be ruled out.

Besides potentially acting upstream of phytochromes, NO might alternatively play a role downstream of these photoreceptors, participating at some point in the phytochrome-triggered signaling network. For example, single cell assays carried out in tomato *au* hypocotyls by microinjection have consistently demonstrated that the NO second messengers cyclic guanosine 3',5'-cyclic monophosphate (cGMP) and cytosolic calcium play significant roles in mediating the phytochrome-dependent signaling cascades leading to chloroplast development (BOWLER *et al.*, 1994a; WU *et al.*, 1996).

Moreover, these same authors have also demonstrated that the NO donor SNP stimulates gene expression of chalcone synthase, an anthocyanin biosynthetic enzyme, either in the dark- or light-adapted soybean cell cultures, resembling in many ways the responses obtained with cGMP analog supplementation, and consequently implying that NO might act as a possible activator of guanylate cyclase (GC) activity during light-triggered plant responses (BOWLER *et al.*, 1994b). More recently, additional examples of close interactions between NO and cGMP during the induction of plant responses have been reported (PAGNUSSAT *et al.*, 2003; SALMI *et al.*, 2007; PASQUALINI *et al.*, 2009; KEYSTER *et al.*, 2010; WANG *et al.*, 2010). Based on these reports, the modulation of cGMP endogenous levels might also represent a possible target of NO during the light-evoked cotyledon greening in tomato seedlings.

On the other hand, accumulating evidence also indicates profuse and intricate NO-hormonal cross-talks in plants (FRESCHI, 2013; SIMONTACCHI *et al.*, 2013; LEÓN *et al.*, 2014) as well as pronounced involvement of phytohormones in plant photomorphogenic responses (KRAEPIEL & MIGINIAC, 1997; HALLIDAY & FANKHAUSER, 2003; SYMONS & REID, 2003; LAU & DENG, 2010). In agreement with this scenario, in the present study we discovered a mutual antagonism between NO and ethylene in de-etiolating tomato seedlings, which seems to significantly impact the light-mediated greening and chloroplast development processes. Several lines of evidence support this finding. (i) RL or BL-induced greening and chloroplast differentiation in tomato seedlings temporally coincide with increases and decreases in NO (Fig. 3) and ethylene emission (Fig. 5), respectively. (ii) Whereas NO stimulated cotyledon greening (Fig. 4), treatments with ethylene or its precursor ACC severally impaired both RL- and BL-induced greening in MT (Fig. 7). (iii) Ethylene- or ACC-treated de-etiolating seedlings present significantly lower NO levels whereas the

ethylene-insensitive *Nr* mutant exhibited increased endogenous NO content (Fig. 6). (iv) De-etiolating *Nr* seedlings exhibited increased total activity and activation state of the NO-generating enzyme NR. (v) Exogenous NO drastically reduced ethylene emission in *au* seedlings maintained under RL. Taken together, these data seem to indicate that the mutual antagonism between NO and ethylene in de-etiolating tomato seedlings might mainly occur at the biosynthetic level.

The negative influence of NO on the production of ethylene has already been reported for physiological events such as fruit ripening and the regulation of leaf and flower senescence (LESHEM *et al.*, 1998; WILLS *et al.*, 2000; MANJUNATHA *et al.*, 2010; MANJUNATHA *et al.*, 2012), which, in many cases, essentially relies on a restriction in the transcript abundance and/or activity of key ethylene biosynthetic enzymes (reviewed by MANJUNATHA *et al.*, 2012; FRESCHI, 2013). On the other hand, some studies have indicated that ethylene might either positively or negatively impact NO production (LESHEM & HARAMATY, 1996; GARCIA *et al.*, 2011; PARRA-LOBATO & GOMEZ-JIMENEZ, 2011). Scarcer reports have also demonstrated that NO might not only impact ethylene biosynthesis but also its perception and signaling (YANG *et al.*, 2010; NIU & GUO, 2012). In the present study, NO treatment apparently had no effect on the tissue sensitivity to ethylene since the activation of the ethylene-responsive promoter EBS was virtually indistinguishable in seedlings exclusively treated with ethylene or with a combination of gaseous ethylene and NO (Fig. 5g).

The negative influence of ethylene on tomato greening contrasts with recent findings in *Arabidopsis* seedlings, in which this hormone seems to promote cotyledon greening by minimizing photobleaching due to an excessive Pchl_a accumulation under stressful conditions or in mutant or transgenic seedlings with disturbances in the light-

mediated greening process (ZHOU *et al.*, 1998; ZHONG *et al.*, 2009, 2010). In the present study, however, no signals of photobleaching were observed even in cotyledons of the light-hypersensitive tomato mutant *hp-1*, which accumulates about 15% more Pchl_a than the control genotype (Fig. S1) (TERRY & KENDRICK, 1999); therefore, potential ethylene-induced reductions in Pchl_a levels seem more prone to impair than facilitate the establishment of a green tomato seedling during the transition from the etiolated state to photomorphogenic growth.

In agreement with our findings in tomato, phytochrome-deficient pea (*Pisum sativum*) mutants have also presented increased ethylene production (FOO *et al.*, 2006). In a very interesting manner, the application of an ethylene biosynthesis inhibitor completely rescued many of the phenotype alterations observed in this pea mutant (FOO *et al.*, 2006), thereby suggesting that light perception via phytochromes plays a critical role in maintaining ethylene synthesis at adequate levels. In most cases, the phytochrome-mediated repression in ethylene production at least partially relies on a down-regulation in key ethylene biosynthetic enzymes (VANGRONSVELD *et al.*, 1988; FOO *et al.*, 2006), which is in agreement with the increased levels of ACO activity observed in tomato *au* mutant (Fig. 5e). Besides reducing ethylene biosynthesis, light perception might also decreased the sensitivity of the seedling tissues to ethylene (HALLIDAY & FANKHAUSER, 2003), and further experiments are currently being carried out to clarify whether this would also be the case in de-etiolating tomato seedlings.

In the present study, data indicating a possible interaction between NO and auxins during the greening of de-etiolating tomato seedlings was also obtained. However, in contrast with the above described antagonistic NO-ethylene crosstalk, the NO-auxin interactions seemed to occur in a particularly synergist manner. Firstly, a positive

influence of auxin signaling on endogenous NO content was evidenced by the significant decrease and increase in light-induction of NO production in seedlings of *dgt*, an auxin-insensitive mutant, and *entire*, a mutant hypersensitive to auxin, respectively (Fig. 8). In agreement with these data, increases in endogenous NO have frequently been observed in diverse plant models soon after exogenous auxin application (PAGNUSSAT *et al.*, 2002; CORREA-ARAGUNDE *et al.*, 2004; LOMBARDO *et al.*, 2006), being particularly conspicuous in plant tissues undergoing auxin-mediated developmental processes.

According to the auxin-responsive promoter *DR5* activation pattern in de-etiolation tomato seedlings (Fig. 8c), either RL or BL exposure stimulates auxin activity in MT cotyledon tissues. Accordingly, the *au* mutation completely eliminated the RL-induced stimulation of auxin-dependent reporter expression in these tissues (Fig. 8d). Interestingly, the same NO concentration that triggered greening in *au* seedlings under RL also activated the auxin-responsive promoter to similar levels found in MT seedlings at an equivalent light treatment (Fig. 8d). Based on these data, it seems likely that NO might have facilitated cotyledon greening in RL-exposed *au* seedlings by increasing the abundance of active auxins and/or by stimulating the sensitivity of this organ to the endogenous pool of auxins. Quantifications of endogenous auxins as well as investigations on the sensitivity of the NO-treated tissues are currently being performed to test these possibilities. If proven, such NO-auxin interaction might represent a positive feedback regulatory loop triggered by light, in which auxin perception induces NO production (as indicated in Fig. 8a,b) and endogenous NO promotes auxin accumulation, perception and/or signaling.

In accordance with this potential NO-auxin positive crosstalk in de-etiolating tomato seedlings, accumulating evidence indicates that NO might modulate auxin

metabolism, transport, and signaling (reviewed by FRESCHI, 2013). For instance, NO has been demonstrated to modulate auxin levels in *Medicago truncatula* seedlings (XU *et al.*, 2010) and auxin transport in *Arabidopsis* roots (FERNÁNDEZ-MARCOS *et al.*, 2011). Moreover, NO is also believed to promote auxin-dependent gene expression via post-translationally modifying the *Arabidopsis* auxin receptor protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1) (TERRILE *et al.*, 2012).

Besides this more direct interplay between NO and auxins, it is also possible to suggest an indirect interaction between these signaling molecules, in which NO might influence the cotyledon tissue auxin activity specifically by targeting elements of the light perception and signaling cascades as discussed above (*e.g.*, positive influence of NO on active phytochrome abundance or on downstream steps of the light signaling cascade). Given the wide range of crosstalk possibilities between light and auxin transduction networks described in the literature, such as the effects of light on auxin biosynthesis, catabolism, transport, and tissue responsiveness (reviewed by HALLIDAY *et al.*, 2009), a relatively complex light-NO-auxin crosstalk network might be expected to coordinate the critically important conversion of colorless, etiolated seedlings in a green, photosynthetic competent young plants.

In conclusion, the present study brings evidence of a close interaction between NO, ethylene and auxins during the light-induced transition of etioplast in chloroplasts and consequent accumulation of photosynthetic pigments in de-etiolating tomato seedlings. During this physiological response, light-induced production of NO via NR activity apparently antagonizes ethylene production and intensifies auxin activity at the cotyledon tissues. Negative and positive feedback regulatory loops apparently orchestrate ethylene-NO and auxin-NO interactions, respectively. Finally, the capacity of rescuing the etiolated phenotype of phytochrome-deficient tomato seedlings grown

under monochromatic RL indicates that NO might either facilitate red light perception or, alternatively, mimic its effects by acting downstream in the phytochrome signaling cascades. Therefore, the exact nature of the NO-phytochromes-phytohormones crosstalk during acquisition of photomorphogenic traits, such as greening and chloroplast development, still deserves further investigation not only in tomato but also in other plant species.

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SUPPLEMENTARY FIGURES

Fig. S1. Pigment accumulation in tomato photomorphogenic mutants under diverse light conditions. (a-b) Chlorophyll *a/b* ratio in cotyledons of Micro-Tom (MT), *high pigment 1* (*hp-1*) and *aurea* (*au*) seedlings maintained transferred from darkness to RL (a) or BL (b) treatments. (c) Protochlorophyllide levels in 9-day-old etiolated cotyledons of MT, *hp-1* and *au* and in *au* cotyledons after continuous NO (150 ppm) treatment. (d) Room-temperature fluorescence emission spectra from acetone extracts obtained from cotyledons of 9-day-old etiolated seedlings of MT (red line), *hp-1* (green line) and *au* (blue line). Protochlorophyllide levels are given on a per-cotyledon pair basis. Error bars represent SD.

Fig. S2. General view of plastid structure in cotyledon cells of MT (a-d), *high pigment 1* (e-h) and *aurea* (i-l) and seedlings upon 72h of dark, red, blue or white light ($100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of white light) treatment. PLB, prolamellar body; S, starch; G, granal thylakoid. Bars: 1 μm (f, g, i, j); 2 μm (a-e, h, k, l).

Fig. S3. Time course of photosynthetic pigment accumulation in tomato seedlings exposed to distinct light conditions in the presence of exogenous NO, NO scavenger or NOS-like inhibitor. Micro-Tom (MT) seedlings were treated with 100 ppm gaseous NO, 2 mM of the NO scavenger 2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (PTIO) or 1 mM N^G -nitro-L-arginine methyl ester (L-NAME) under continuous darkness (a,d), RL (b,e) or BL (c,f). Treatments with L-NAME were exclusively conducted during the first 24h of light treatment (RL or BL). Means $\pm$ SD.

Fig. S4. Histochemical localization of GUS activity in *EBS::GUS* tomato seedlings exposed to D, RL or BL treatment after 0 (a), 24 (b-d), 48 (e-g) and 72 h (h-j). Bars: 0,8 mm (c); 1 mm (e,h,i) 2 mm (a,b,d,f,g,j).

Fig. S5. Histochemical localization of GUS activity in *DR5::GUS* tomato seedlings after 72h of D, RL or BL treatment. Bars: 1 mm (c,h,i) 2 mm (a,b,d).

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IV. CONCLUSÕES

Em conclusão, o conjunto de resultados obtidos ao longo desta pesquisa indica um importante papel sinalizador para o NO durante o desencadeamento dos eventos fotomorfogênicos de diferenciação plastidial e acúmulo de pigmentos fotossintéticos em plântulas de tomateiro em processo de desestiolamento. Durante a indução dos eventos em questão, a enzima NR parece ser a principal rota biossintética responsável pela produção de NO, uma vez que houve uma drástica diminuição na emissão desse radical livre em todos os tratamentos inibitórios à atividade dessa enzima.

Além de confirmar a função crítica dos fitocromos na indução da NR em resposta à luz vermelha, os dados obtidos também sugerem que a proteína HP1/DDB1 regularia negativamente a abundância e regulação pós-transcricional dessa enzima em cotilédones de plântulas de tomateiro.

Verificou-se, ainda, que o fenótipo estiolado observado em plântulas do mutante *aurea* crescidas sob luz vermelha foi parcialmente recuperado através do tratamento com NO, sugerindo uma possível atuação desse radical livre como facilitador da percepção da luz vermelha ou, alternativamente, como um elemento capaz de mimetizar as respostas de desestiolamento tipicamente desencadeadas pelos fitocromos.

Além do envolvimento com fotorreceptores, os resultados obtidos também sugerem estreitas interações entre o NO, o etileno e as auxinas durante a conversão de etioplastos em cloroplastos e consequente acúmulo de pigmentos fotossintéticos. Conforme indicado por uma série de observações, o etileno parece atuar reprimindo os eventos fotomorfogênicos em questão muito provavelmente devido à existência de uma relação antagônica mútua com o NO. Em contrapartida, uma clara interação sinérgica mútua

foi observada entre as auxinas e o NO durante o desestiolamento em plântulas de tomateiro.

Em conjunto, os resultados obtidos sugerem que a indução da síntese do NO via NR em resposta aos estímulos luminosos antagonizaria a produção de etileno e intensificaria a atividade das auxinas nas células cotiledonares de tomateiro, provavelmente através de um controle regulatório de *feedbacks* negativos e positivos, respectivamente. Dessa forma, o NO atuaria estimulando o desenvolvimento de cloroplastos e o acúmulo de pigmentos durante o desestiolamento de plântulas de tomateiro, possivelmente desempenhando um papel integrador entre as redes de sinalização controladas pela luz e por fitormônios.

RESUMO

O desestiolamento vegetal envolve a conversão de etioplastos em cloroplastos maduros e plenamente funcionais, sendo desencadeado pela luz através de um processo multifacetado que se baseia em redes de sinalização endógenas diversificadas e altamente coordenadas. Acredita-se que hormônios vegetais ou outras moléculas sinalizadoras, tais como o radical livre óxido nítrico (NO), desempenham papel importante na regulação desse conjunto de respostas fotomorfogênicas. No presente estudo, buscamos investigar, de forma integrada, a influência do NO, do etileno e das auxinas na indução do acúmulo de pigmentos fotossintéticos e desenvolvimentos dos cloroplastos desencadeados pela luz em plântulas de tomateiro (*Solanum lycopersicum*). Por meio da determinação do padrão temporal de acúmulo de pigmentos fotossintéticos, diferenciação de etioplastos em cloroplastos, flutuações nos teores endógenos de NO e na atividade e estado de ativação da nitrato redutase (NR) em plântulas do tipo selvagem (cultivar Micro-Tom, MT) e de mutantes fotomorfogênicos (*aurea* and *high pigment 1*) mantidas sob escuro contínuo ou expostas às luzes monocromáticas vermelha e azul, pudemos constatar uma clara correlação positiva entre a produção de NO via NR e a indução do acúmulo de pigmentos e desenvolvimento dos cloroplastos em resposta à luz. Dando suporte à importância da NR como fonte biossintética de NO nas plântulas de tomateiro em processo de desestiolamento, constatou-se que as diferentes estratégias empregadas com o intuito de inibir a indução da atividade dessa enzima em resposta à luz resultaram em reduções consideráveis na produção endógena de NO. De modo interessante, tratamentos com NO estimularam o acúmulo de pigmentos e a diferenciação plastidial nas células cotiledonares do mutante *aurea* sob luz vermelha, indicando, portanto, que essa molécula sinalizadora seria capaz de complementar a deficiência parcial na percepção da luz vermelha característica desse mutante deficiente em fitocromos. Em paralelo, um antagonismo mútuo entre o NO e o etileno foi evidenciado por meio de uma série de constatações. (i) O acúmulo de pigmentos e diferenciação de cloroplastos induzidos nas plântulas de tomateiro em resposta às luzes vermelha e azul coincidiram temporalmente com um aumento e diminuição nas emissões de NO e etileno, respectivamente. (ii) Enquanto o NO se mostrou estimulatório ao acúmulo de pigmentos, tratamentos com etileno gasoso ou com o seu precursor (o ácido 1-aminociclopropano-1-carboxílico, ACC) drasticamente inibiram o acúmulo de pigmentos em resposta às luzes vermelha ou azul. (iii) Plântulas

em processo de desestiolamento tratadas com etileno ou ACC apresentaram níveis reduzidos de NO, ao passo que plântulas do mutante com baixa sensibilidade ao etileno *Never ripe* (*Nr*) exibiram teores de NO endógeno significativamente aumentados. (iv) Plântulas de *Nr* em processo de desestiolamento apresentaram incrementos consideráveis tanto na atividade total quanto no estado de ativação da NR, uma enzima produtora de NO. (v) NO exógeno reduziu drasticamente a emissão de etileno em plântulas do mutante *aurea* mantidas sob luz vermelha. Em contrapartida, diversas evidências revelaram um sinergismo mútuo entre auxinas e NO durante o processo de destiolamento em plântulas de tomateiro. (i) O acúmulo de NO em resposta à luz coincidiu com um aumento na ativação do promotor sintético responsivo à auxinas *DR5* em plantas de MT expostas às luzes vermelha ou azul. (ii) A suplementação com NO gasoso reestabeleceu a reduzida ativação do promotor *DR5* observada em plântulas de *aurea* sob luz vermelha. (iii) Os teores endógenos de NO foram drasticamente aumentados e diminuídos em plântulas do mutante com baixa sensibilidade à auxinas (*diageotropica*) e no mutante hipersensível à auxinas (*entire*), respectivamente. Em conjunto, os dados obtidos parecem indicar que durante a indução do acúmulo de pigmentos fotossintéticos e diferenciação de cloroplastos em plântulas estioladas de tomateiro as interações NO-etileno e NO-auxinas seriam controladas via mecanismos regulatórios de retroalimentação positiva e negativa, respectivamente; e, assim, tais relações hormonais desempenhariam papel importante na coordenação da transição dessas plântulas do estado estiolado para o desenvolvimento fotomorfogênico.

ABSTRACT

The transition from etiolated to green seedlings involves the conversion of etioplasts into mature, functional chloroplasts via a multifaceted light-driven process comprising multiple and tightly coordinated endogenous signaling networks. Plant hormones and other signaling molecules, such as the free radical nitric oxide (NO), are believed to play important roles in controlling the acquisition of these photomorphogenic traits. In the present study, we investigated, in an integrated way, the influence of NO, ethylene and auxins on the light-evoked greening and chloroplast development in tomato (*Solanum lycopersicum*) seedlings. By determining the time course of photosynthetic pigments accumulation, etioplast-to-chloroplast differentiation, fluctuations in endogenous NO content and in nitrate reductase (NR) total activity and activation state in wild type (Micro-Tom cultivar, MT) and in photomorphogenic mutants (*aurea* and *high pigment 1*) seedlings maintained under continuous darkness or exposed to monochromatic red (RL) or blue light (BL), we evidenced a clearly positive correlation between the NO production via NR and the light-induced cotyledon greening and chloroplast maturation. Supporting a role for NR as an important biosynthetic source of NO in de-etiolating tomato seedlings, different strategies employed to inhibit the light-evoked increment in the activity of this enzyme successfully reduced the endogenous NO production. Interestingly, exogenous NO stimulated greening and chloroplast differentiation in cotyledon cells of *aurea* seedlings maintained under RL, thereby indicating that this signaling molecule might complement the partial deficiency in RL perception characteristic of this phytochrome-deficient mutant. In parallel, a mutual antagonism between NO and ethylene was evidenced by a number of findings. (i) RL- or BL-induced greening and chloroplast differentiation in tomato seedlings temporally coincided with increases and decreases in NO and ethylene emission, respectively. (ii) Whereas NO stimulated cotyledon greening, treatments with gaseous ethylene or its precursor (1-aminocyclopropane-1-carboxylic acid, ACC) severally impaired either RL- or BL-induced greening in MT. (iii) Ethylene- or ACC-treated de-etiolating seedlings presented significantly lower NO levels whereas the ethylene-insensitive *Never ripe* (*Nr*) mutant exhibited increased endogenous NO content. (iv) De-etiolating *Nr* seedlings exhibited increased total activity and activation state of the NO-generating enzyme NR. (v) Exogenous NO drastically reduced ethylene emission in *au* seedlings maintained under RL. On the other hand, a series of evidence indicated a mutual synergism between

auxins and NO in de-etiolating tomato seedlings. (i) The light-induced NO accumulation coincided with an increased activation of the synthetic auxin-responsive promoter *DR5* in both RL- and BL-exposed MT seedlings. (ii) Exogenous NO completely rescued the reduced activation of the *DR5* promoter observed in *au* seedlings under RL. (iii) Endogenous NO was drastically decreased and increased in de-etiolating seedlings of auxin-insensitive (*diageotropica*) and auxin-hypersensitive (*entire*) tomato mutants, respectively. Taken together, these data reveal that negative and positive feedback regulatory loops orchestrate ethylene-NO and auxin-NO interactions during the light-triggered cotyledon greening and chloroplast differentiation in de-etiolating tomato seedlings, reinforcing the importance of these signaling molecules during the coordination of seedling transition from the etiolated state to photomorphogenic growth.