Rafael Zuccarelli

Influência da luz sobre o metabolismo de
óxido nítrico em tecidos vegetativos e
reprodutivos de tomateiro

Light influence on nitric oxide metabolism in
tomato vegetative and reproductive tissues

São Paulo

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Dedicatória

Dedico este trabalho a todas as
pessoas que não puderam,
por dificuldades da vida,
estudar e alcançar seus sonhos.

Epígrafe

EU SOU TREZENTOS

Eu sou trezentos, sou trezentos-e-cincoenta,
As sensações renascem de si mesmas sem repouso,
Ôh espelhos, ôh Pireneus! Ôh caiçaras!
Si um deus morrer, irei no Piauí buscar outro!

Abraço no meu leito as melhores palavras,
E os suspiros que dou são violinos alheios;
Eu piso a terra como quem descobre a furto
Nas esquinas, nos táxis, nas camarinhas seus próprios beijos!

Eu sou trezentos, sou trezentos-e-cincoenta,
Mas um dia afinal eu toparei comigo...
Tenhamos paciência, andorinhas curtas,
Só o esquecimento é que condensa,
E então minha alma servirá de abrigo

(Mário de Andrade, 7/6/1929. Publicada no livro *Remate de Males*, 1930)

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Introdução Geral

1. Óxido nítrico como sinalizador em sistemas biológicos

O óxido nítrico (NO) é um composto gasoso que pode ser produzido industrialmente a partir da oxidação da amônia e aparece como poluente atmosférico, sendo precursor da chuva ácida e do “*smog*” fotoquímico, subproduto do funcionamento de motores de combustão interna. Assim como seus análogos químicos monóxido de carbono (CO) e cianeto (CN⁻), o NO se liga irreversivelmente aos centros metálicos de proteínas e por esse motivo, em determinadas concentrações, possui efeito tóxico, particularmente no que diz respeito aos processos fisiológicos de respiração celular e trocas gasosas, como por exemplo na cadeia de transporte de elétrons mitocondrial, na ligação do oxigênio com a hemoglobina sanguínea ou no funcionamento dos pulmões (Cassina & Radi, 1996; Weinberger *et al.*, 2001). No entanto, o NO e algumas outras espécies reativas de oxigênio e nitrogênio vêm sendo caracterizadas recentemente como importantes compostos sinalizadores em sistemas biológicos, atuando em organismos filogeneticamente distantes como bactérias, fungos, plantas e animais (Lamattina *et al.*, 2003; Baidya *et al.*, 2011; Barnes *et al.*, 2014). Devido ao histórico de sua descoberta e interesse médico, a maior parte do conhecimento atual e investimento em pesquisas acerca da atuação e metabolismo do NO encontra-se centrada em modelos animais, particularmente em mamíferos (Palmer *et al.*, 1987; Radomski *et al.*, 1990; Nguyen *et al.*, 1992; Thomsen *et al.*, 1995; Hirata & Yokoyama, 1996; Förstermann & Sessa, 2012). Um dos primeiros trabalhos desenvolvidos nessa temática demonstrou a participação do NO no relaxamento da musculatura lisa do endotélio dos vasos sanguíneos por meio da ativação da guanil-ciclase, enzima responsável pela produção do mensageiro secundário monofosfato

cíclico de guanosina cíclico (GMPc) (Arnold *et al.*, 1977). Trabalhos posteriores, também em animais, indicaram a participação do NO em diversos processos, tais como na regulação da neurotransmissão no cérebro (Jaffrey & Snyder, 1995), agregação plaquetária (Radomski *et al.*, 1987), aprendizado e memória (Zoubovsky *et al.*, 2011), função sexual masculina (Melis & Argiolas, 1997), citotoxicidade e citoproteção (Kröncke *et al.*, 1997), desenvolvimento de aterosclerose (Lloyd-jones & Bloch, 1996), ativação das defesas do sistema imune (Wei *et al.*, 1995), entre muitas outras.

Apesar das evidências acumuladas até o momento, os mecanismos exatos de atuação em cada um desses processos ainda é objeto de enorme debate, envolvendo diferentes rotas de transformação química, assim como múltiplas vias de produção, degradação e sinalização (Stamler, 1994; Lundberg *et al.*, 2008; Sato *et al.*, 2012). A partir de 1998, um número crescente de trabalhos tem indicado um importante papel sinalizador para o NO em um grande leque de respostas fisiológicas em plantas (Baudouin, 2011). Além das limitações técnicas em transpor resultados *in vitro* para as condições *in vivo*, as evidências atualmente disponíveis sugerem que em comparação com sistemas animais, as plantas metabolizam o NO através de vias consideravelmente mais complexas e diversificadas (Yu *et al.*, 2014).

Um papel de destaque tem sido atribuído ao NO como molécula sinalizadora em inúmeras respostas à estresses bióticos e abióticos em plantas, tais como alterações bioquímicas em resposta à deficiência nutricional (Lamattina *et al.*, 2003; Graziano & Lamattina, 2007), ativação de mecanismos de defesa a patógenos (Klessig *et al.*, 2000; Romero-Puertas *et al.*, 2004), aumento da tolerância à salinidade (Zhang *et al.*, 2006a) e ao estresse oxidativo (Velikova *et al.*, 2008), resistência a metais pesados (Kopyra & Gwózdź, 2003), dentre outras.

Em processos ligados ao desenvolvimento, o NO tem sido descrito como sinalizador da germinação e desestiolamento (Beligni & Lamattina, 2000), formação de raízes laterais e adventícias (Lanteri *et al.*, 2006), senescência (Leshem & Haramaty, 1996), amadurecimento de frutos (Lamattina *et al.*, 2003), entre muitos outros.

Até o momento, a maioria dos mecanismos de interação do NO com diversas classes de hormônios vegetais sugere sua atuação como um possível mensageiro secundário (Neill *et al.*, 2003). Em contrapartida, tendo em vista sua produção em baixas concentrações, capacidade de desencadear respostas de forma dose-dependente e fácil difusão pelos tecidos, alguns autores consideram que o próprio NO poderia ser classificado como um novo hormônio vegetal (Beligni & Lamattina, 2001).

2. Propriedades químicas e vias de sinalização do óxido nítrico em plantas

O óxido nítrico, ou monóxido de nitrogênio, de fórmula NO, é um composto inorgânico, lipofílico que se destaca por ser um dos poucos compostos gasosos que atuam como sinalizadores em sistemas biológicos (Bleecker & Kende, 2000; Lefer, 2007; Song *et al.*, 2008; Wang *et al.*, 2011; Mur *et al.*, 2013). É também notável por consistir, em sua forma neutra (NO[•]), de um radical livre, possuindo um elétron em seu orbital anti-ligante 2p- π (Stamler *et al.*, 1992), característica essa que confere alta reatividade e instabilidade química, permitindo sua participação em uma grande variedade de reações químicas com compostos presentes em seres vivos. No entanto, nas baixas concentrações em que o NO é encontrado no meio aquoso das células vegetais (pM a nM), este é relativamente estável, apresentando uma meia-vida na faixa de minutos (Planchet & Kaiser, 2006).

O NO prontamente forma complexos com íons de metais de transição, incluindo aqueles encontrados em metaloproteínas (McCleverty, 2004). Reações com proteínas contendo o grupo heme têm sido extensivamente estudadas, particularmente as reações envolvendo hemoglobinas (Wei *et al.*, 1995; Igamberdiev & Hill, 2004). Sua participação em processos bioquímicos envolve uma cadeia de formas redox intercambiáveis: o cátion nitrosênio (NO^+), o óxido nítrico ($\text{NO}^\bullet$) e o ânion nitroxil (NO^-). Tanto o NO^+ quanto o $\text{NO}^\bullet$ podem nitrosilar grupos tióis de cisteínas em proteínas e peptídios (R-S-NO), potenciamente regulando as atividades de proteínas e fatores de transcrição (Lamattina *et al.*, 2003). Além dessas formas redox, diversos produtos resultantes de sua reação com outras substâncias são também candidatos prováveis na cadeia de reações envolvendo a sinalização por NO. Por exemplo, em presença de superóxido ($\text{O}_2^{\cdot-}$) o NO é rapidamente convertido em peroxinitrito (ONOO^-) que por sua vez pode levar a formação de nitrito (NO_2) e o potente radical livre hidroxila $\text{OH}^\bullet$. O peroxinitrito pode, também, promover a nitração de tirosinas (Tyr-NO_2) ou a oxidação dos resíduos tióis em ácidos sulfênicos e sulfônicos, modificando a estrutura terciária e funções de proteínas e fatores de transcrição (Lamattina *et al.*, 2003).

Também chama a atenção o fato do NO não poder ser facilmente armazenado na célula, e possuir uma difusibilidade maior do que outros solutos de natureza sólida ou líquida. Além de possuir características lipofílicas e carga neutra, permeando com facilidade as membranas celulares, possui também moderada solubilidade em meio aquoso (McCleverty, 2004). Essas características únicas permitem ao NO ser facilmente transportado através dos meios intra e extracelular, difundindo-se livremente. A especificidade de sua ação como molécula sinalizadora em plantas, parece ser, portanto, altamente dependente do fino controle de sua rápida produção, e

em especial, do controle temporal e espacial de sua degradação ou conjugação com outros compostos.

3. Vias de produção de NO em plantas

A produção de NO em plantas pode ser dividida em duas vias principais: vias dependentes de nitrito (vias redutivas) e vias dependentes de L-arginina (vias oxidativas) (Gupta *et al.*, 2011).

3.1. Vias de produção de NO dependentes de nitrito

Sistemas vegetais parecem apresentar diferentes sistemas de produção de NO a partir de nitrito, os quais seguem a reação química geral:

A nitrato redutase (NR), enzima que normalmente reduz nitrato a nitrito, às custas do consumo de NAD(P)H, é também capaz de transferir um elétron do NAD(P)H para o nitrito resultando na formação de NO (número de oxidação do nitrogênio +5, +3 e +2, respectivamente). Essa enzima, que ocupa uma posição central no metabolismo de nitrogênio das plantas, por realizar uma vez que realiza o primeiro passo na conversão do nitrato em amônia (forma assimilável de N), tem se mostrado uma importante fonte de óxido nítrico, sendo a produção desse radical livre dependente da regulação da atividade da enzima por fosforilação (Rockel *et al.*, 2002).

Além da NR citossólica, estudos demonstram que uma NR associada com uma nitrito redutase, ambas ligadas à membrana plasmática, também seriam capazes de produzir NO, estando esse complexo está presente exclusivamente apenas nas raízes e levando à produção de NO no espaço apoplástico (Stöhr & Stremlau, 2006). Essa via

de produção de NO é altamente dependente de condições ambientais, tais como disponibilidade de nitrato ou oxigênio, e evidências recentes têm demonstrado sua importância no controle da formação de micorrizas (Gupta *et al.*, 2011).

A cadeia de transporte de elétrons mitocondrial também tem sido indicada como uma fonte potencial de NO, especialmente em situações de baixas tensões de oxigênio (Igamberdiev & Hill, 2004). Na impossibilidade de utilizar o oxigênio comoceptor final de elétrons, a citocromo c oxidase exibe a capacidade de reduzir nitrito a NO, condição inversa a que ocorre em aerobiose, onde esta enzima é capaz de consumir NO e produzir nitrito, mantendo, ao menos parcialmente, o *status* energético da célula (Gupta & Igamberdiev, 2011).

Nos cloroplastos, as membranas dos tilacóides também parecem estar envolvidas no processo de produção de NO a partir de nitrito. Contudo, cabe ressaltar que o cloroplasto é uma importante fonte de radical O_2^- , e a formação de peroxinitrito a partir do NO e O_2^- estaria associada à ocorrência de peroxidação de lipídios e proteínas nessa organela, ambos importantes na modulação de seu funcionamento (Jasid *et al.*, 2006). Ademais, estudos *in vitro* indicam a possibilidade da participação dos peroxissomos na produção de NO a partir da redução do nitrito, sendo esta reação catalizada pela enzima xantina oxidoreductase (XOR). Os produtos predominantes da ação desta enzima em condições aeróbicas são o ácido úrico e o superóxido, entretanto, em condições de anaerobiose, essa enzima reduz o nitrito em NO, utilizando NADH e xantina como agente redutor (Gupta *et al.*, 2011).

Finalmente, existe a produção de NO no espaço apoplástico através da redução não enzimática de nitrito, em pH ácido, segundo a reação reversível:

Agentes redutores como ácido ascórbico e compostos fenólicos podem deslocar o equilíbrio da reação no sentido da formação do NO, aumentando sua taxa de produção (Bethke *et al.*, 2006).

3.2. Vias de produção de NO dependentes de L-arginina

Uma diversificada família de enzimas conhecidas como NO sintases (NOS) estão amplamente presentes em animais, sendo responsáveis pela reação de oxidação de L-arginina em L-citrulina com a liberação de NO. A reação de oxidação ocorre por meio de um grupo heme, em presença de oxigênio, com o consumo de NADPH. Seu funcionamento é dependente da presença de calmodulina e Ca^{2+} (Alderton *et al.*, 2001). Até o presente momento, a existência de NO sintases, sejam elas homologas ou não-homologas às encontradas em animais, não foi demonstrada de maneira definitiva em plantas. Apesar disso, a atividade do tipo NOS, com produção de óxido nítrico dependente de L-arginina já foi extensivamente demonstrada em plantas (Zhao *et al.*, 2007; Issue, 2009; Fröhlich & Durner, 2011).

Outras vias de produção de NO com participação de L-arginina estão sendo investigadas atualmente, com resultados ainda inconclusivos. Dentre elas, destacam-se a liberação de NO durante a síntese de poliaminas e a produção de NO a partir de hidroxilamina (Gupta *et al.*, 2011).

4. Mecanismos de degradação de NO

A diversidade de origens e processos em que o NO participa sugere a existência de mecanismos de degradação ou inativação que sejam capazes de controlar seus níveis, bem como sua reatividade e função sinalizadora. Por exemplo, as hemoglobinas são reconhecidas como capazes de modular a homeostase do NO,

tanto por oxidação quanto por reações de S-nitrosilação em plantas (Perazzolli *et al.*, 2006). Por outro lado, a enzima S-nitrosoglutationa redutase (GSNOR) também tem sido indicada como uma das principais rotas pelas quais o metabolismo do NO e de moléculas derivadas do NO pode ser regulada (Salgado *et al.*, 2013). Outras vias são indicadas na literatura, com resultados ainda incertos em relação a sua importância e com poucas evidências *in vivo* de suas atuações na degradação do NO em plantas.

4.1. Hemoglobinas

As hemoglobinas são encontradas de forma ubíqua em eucariotos e estão também presentes em muitas bactérias (Watts *et al.*, 2001). Ao menos três tipos foram categorizados em plantas: simbiótica, não-simbiótica e truncada.

A hemoglobina simbiótica é certamente a mais conhecida, sendo encontrada principalmente nas células infectadas por bactérias simbióticas fixadoras de nitrogênio de nódulos de raízes de plantas leguminosas (Arredondo-Peter *et al.*, 1998). Estas se acumulam no citosol em concentrações relativamente altas, mantendo a concentração de oxigênio estável e em escala nanomolar. As baixas tensões de oxigênio evitam a inativação da enzima nitrogenase presente nas bactérias, e ao mesmo tempo a hemoglobina armazena oxigênio, permitindo a continuidade da respiração (Ott *et al.*, 2005).

Em contrapartida, as hemoglobinas não-simbióticas, como o próprio nome sugere, não estão ligadas à fixação simbiótica de nitrogênio. Ocorrem em sementes, raízes, folhas e outros órgãos de praticamente todas as espécies vegetais (Igamberdiev & Hill, 2004). São classificadas em dois tipos principais no que diz respeito à intensidade da ligação química com o oxigênio. As pertencentes ao primeiro tipo (classe 1), possuem uma altíssima afinidade com o oxigênio e como resultado

apresentam-se majoritariamente no estado oxigenado na maior parte das condições fisiológicas. São induzidas em condições de estresse, especialmente durante anóxia e hipóxia (Igamberdiev & Hill, 2004). As pertencentes ao segundo tipo (classe 2), possuem afinidade ao oxigênio semelhante a das hemoglobinas simbióticas e sua função ainda não está bem estabelecida (Dordas, 2003).

A importância das hemoglobinas de classe 1 em plantas têm sido evidenciada pela descoberta de uma possível via fermentativa em condições de hipóxia envolvendo o nitrito como aceptor final alternativo, na cadeia de transporte de elétrons mitocondrial, gerando NO como produto (Gupta & Igamberdiev, 2011). A hemoglobina atuaria convertendo o NO em nitrato (NO_3^-). O nitrato por sua vez é convertido em nitrito (NO_2^-) pela ação da enzima nitrato redutase as custas de consumo de NADH, reiniciando o ciclo (Igamberdiev & Hill, 2004).

A via sugerida nesse, e em outros trabalhos (Perazzolli *et al.*, 2004), estabelece que a oxihemoglobina [$\text{Hb}(\text{Fe}^{2+})\text{O}_2$] liga-se ao NO, formando S-nitrosohemoglobina. O NO é oxidado em nitrato (NO_3^-) tendo como produto a metHb [$\text{Hb}(\text{Fe}^{3+})$]. A metHb é então reduzida a [$\text{Hb}(\text{Fe}^{2+})$] por uma metHb redutase, as custas do consumo de NAD(P)H. A [$\text{Hb}(\text{Fe}^{2+})$] é prontamente oxigenada [$\text{Hb}(\text{Fe}^{2+})\text{O}_2$], dada a altíssima afinidade pelo oxigênio, ocorrendo mesmo em concentrações muito baixas (nanomolar), e, desse modo, a oxihemoglobina é regenerada (Figura 1). Esse ciclo mantém o funcionamento da cadeia de transporte de elétrons mitocondrial, resultando na regeneração do NAD(P)H e do NADH no processo, mantendo os níveis de NO sob controle. Essa via contribui com a produção de ATP sob hipóxia, mantendo baixos os níveis de fermentação láctica e alcoólica (Igamberdiev & Hill, 2004).

As hemoglobinas truncadas foram as últimas a serem descobertas e também possuem ampla distribuição, porém suas propriedades bioquímicas e estrutura

terciária distinta sugerem diferentes funções celulares, ainda desconhecidas (Dordas, 2003).

Figura 1. Representação esquemática do ciclo da hemoglobina/NO em plantas sob hipóxia. A enzima nitrato redutase (NR) converte nitrato em nitrito às custas do consumo de NADH. A cadeia de transporte de elétrons mitocondrial (M) utiliza o nitrito como aceptor final de elétrons alternativo. A oxihemoglobina $[\text{Hb}(\text{Fe}^{2+})\text{O}_2]$ converte o NO em NO_3^- convertendo-se em MetHb $[\text{Hb}(\text{Fe}^{3+})]$. A oxihemoglobina é regenerada pela enzima MetHb redutase seguida da ligação com o oxigênio (modificado de Igamberdiev & Hill, 2004).

4.2. S-nitrosoglutationa redutase

A glutatona é um tripeptídeo (γ -glutamilcisteinilglicina) que contém uma ligação peptídica não usual entre o grupo amino de uma cisteína ligado ao grupo carboxil da cadeia lateral de um glutamato. A cisteína está ligada também a uma glicina por uma ligação peptídica normal. É conhecido por atuar direta ou indiretamente em quase todos os processos celulares, tais como síntese protéica e de DNA, atividade enzimática, transporte de metabólitos, proteção celular, entre outros

(Meister & Anderson, 1983). É um potente antioxidante e participa de importantes mecanismos de prevenção de danos celulares causados por formas radicais livres como espécies reativas de oxigênio e nitrogênio (Szalai *et al.*, 2009; Zechmann, 2014).

Está presente em grandes concentrações celulares em tecidos de plantas, animais e micro-organismos (0,1-10 mM) (Meister, 1988). Existe sob duas formas como sulfidril (reduzido, GSH) e como dissulfeto (GSSG). O grupo tiol da cisteína, em sua forma reduzida, é capaz de doar um elétron ($H^+ + e^-$) para outras moléculas instáveis, processo pelo qual ela própria se torna instável, ligando-se a outra glutathione no mesmo estado oxidado, formando o dímero glutathione dissulfeto (GSSG). A enzima glutathione redutase (GR) converte-a novamente para a forma GSH às custas do consumo de NAD(P)H. Por esse motivo a maior parte das células possuem 90% de sua glutathione em sua forma GSH (Meister, 1988).

O óxido nítrico reage com o GSH por meio de uma reação de S-nitrosilação, formando a S-nitrosoglutathione (GSNO) (Figura 2). Essa é atualmente considerada uma das reações mais importantes no metabolismo do NO em plantas, uma vez que a GSNO consistiria num possível reservatório móvel de NO, aumentando, portanto, a possibilidade de estocagem e transporte desse composto sinalizador (Barroso *et al.*, 2006; Corpas *et al.*, 2013). O mais notável sobre essa rota metabólica é a presença de uma GSNO redutase (GSNOR) conservada, de bactérias a humanos, sendo responsável pela conversão de GSNO em GSSG e NH_3 (Figura 2) (Liu *et al.*, 2001). Assim sendo, a molécula GSNO parece atuar não apenas como um estoque móvel de NO, mas também como um composto intermediário na rota de degradação de NO via GSNOR, e, dessa forma, representaria um importante mecanismo de regulação da disponibilidade do NO em tecidos vegetais.

Figura 2. Representação esquemática do metabolismo da GSNO em plantas e sua regulação pela GSNO redutase. O NO reage com a glutathiona reduzida (GSH) para formar S-nitrosoglutationa (GSNO), que pode ser convertida em glutathiona oxidada (GSSG) e amônia (NH₃). Alternativamente, a GSNO, em presença de GSH, ascorbato e Cu⁺ pode produzir NO e GSSG. A GSNO pode transferir NO para resíduos de cisteína de outros peptídeos ou proteínas (reações de S-nitrosilação). Essa modificação pós-traducional pode modificar a função de uma ampla variedade de proteínas (modificado de Leterrier *et al.*, 2014).

4.3. Interação com espécies reativas de oxigênio.

As espécies reativas de oxigênio ROS (do inglês, *Reactive Oxygen Species*) são substâncias com grande reatividade e podem interagir com a imensa maioria das moléculas biológicas. São as principais causadoras de estresse oxidativo celular e, por esse motivo, podem apresentar efeitos tóxicos, dependendo da concentração em que se encontram (Saed-Moucheshi *et al.*, 2014). Em células vegetais, as ROS são geradas por diferentes mecanismos e ocorrem em diferentes compartimentos celulares como apoplasto, mitocôndria, peroxissomos, cloroplastos e retículo endoplasmático (Saed-Moucheshi *et al.*, 2014).

O NO é capaz de reagir com tais moléculas atuando, dependendo das concentrações e dos radicais envolvidos, ora como antioxidante, ora como fontes de outras espécies reativas com poder oxidante ainda maior (Chaki *et al.*, 2009). Tal fato pode ser ilustrado no caso da reação do NO com o radical superóxido (O₂⁻) dando

origem ao potente oxidante peroxinitrito ($\text{ONOO}^-/\text{ONOOH}$). Em baixas concentrações, ($\text{NO}/\text{O}_2^- < 1$), o incremento das taxas de NO é capaz de aumentar as taxas de peroxidação de lípidios pelo aumento na formação de peroxinitrito. Quando a concentração de NO ultrapassa a de superóxido ($\text{NO}/\text{O}_2^- > 1$) este passa a reagir com o peroxinitrito, removendo-o e inibindo a peroxidação (O'Donnell *et al.*, 1997).

Em plantas, as ROS são importantes substâncias sinalizadores em condições de estresse, tanto bióticos como abióticos, sendo responsáveis por diversas respostas de aclimação e resistência (Dat *et al.*, 2000; Baxter *et al.*, 2014). Trabalhos recentes indicam também a participação de compostos derivados da interação do NO com ROS em algumas dessas respostas à estresses (Wrzaczek *et al.*, 2010; Vandelle & Delledonne, 2011).

5. Interações entre NO e fitohormônios no desenvolvimento vegetal

É bem estabelecido que o desenvolvimento vegetal é controlado pela ação coordenada de diversas classes hormonais e outros sinalizadores endógenos, dentre os quais o NO (Freschi, 2013). Por exemplo, respostas controladas pelo hormônio ácido abscísico (ABA), tais como a abertura e o fechamento estomático e o crescimento de raízes, são induzidas concomitantemente à aumentos na produção de NO e de outras espécies reativas de oxigênio como o peróxido de hidrogênio (H_2O_2) (Hancock *et al.*, 2011).

A quebra da dormência de sementes, outro evento tipicamente controlado por ABA e giberelinas, também envolveria a participação do etileno e NO, os quais agiriam em conjunto na neutralização dos efeitos inibidores do ABA sobre a germinação (Arc *et al.*, 2013b). Em outras respostas vegetais, no entanto, a relação entre a ação do etileno e do NO ocorre de forma antagônica, tais como durante o

amadurecimento de frutos, onde o NO se mantém elevado durante os estágios imaturo do desenvolvimento e diminui sua concentração ao longo do amadurecimento, situação inversa a do etileno (Manjunatha *et al.*, 2012). Aliás, a aplicação de NO tem sido proposta por diversos trabalhos como estratégia para o atraso no processo de amadurecimento de frutos e senescência em folhas e folhas destacadas (Li *et al.*, 2014). No caso da ação do NO durante o amadurecimento de frutos, um dos mecanismos de ação sugeridos envolve a inibição da produção de etileno. Um desses mecanismos parece consistir na ligação do NO com o precursor do etileno, o ácido 1-aminociclopropano-1-carboxílico (ACC) e a enzima ACC oxidase, formando um complexo estável (Zaharah & Singh, 2011a). Além disso, o NO parece reprimir o acúmulo de transcritos de diferentes enzimas chaves da via de biossíntese de etileno, bem como controlar a produção de etileno por meio da S-nitrosilação da enzima metionina adenosiltransferase, inibindo sua atividade (Lindermayr *et al.*, 2006).

De forma similar, durante a senescência foliar e de peças florais, outro processo promovido pelo etileno, o NO também apresenta um forte efeito inibitório (Leshem *et al.*, 1998). No entanto, alguns desses estudos se valem da aplicação exógena de NO, que em determinadas concentrações é tóxico e está envolvido na sinalização de morte celular programada (Procházková & Wilhelmová, 2011).

6. Luz e metabolismo do NO

A percepção da luz pelas plantas é uma forma de otimizar as reações fotossintéticas e regular seu crescimento e desenvolvimento. Muitos processos tais como a germinação de sementes, inibição do alongamento de hipocótilos e caules, bem como a diferenciação dos cloroplastos são controlados pela luz (Beligni &

Lamattina, 2000). Essas respostas são essencialmente mediadas por fotorreceptores específicos para determinadas faixas de comprimento de onda do espectro luminoso.

São conhecidas ao menos quatro classes de fotoreceptores em plantas: os sensíveis à luz azul (criptocromos e fototropinas) e UV-A (criptocromos), os sensíveis ao UV-B (proteína UVR8, em *Arabidopsis*) e os fitocromos, os quais respondem aos comprimentos de onda vermelho (V) e vermelho-extremo (VE) (Fankhauser & Chory, 1997). Ainda que o conhecimento acerca da percepção da luz em vegetais tenha avançado significativamente, os mecanismos pelos quais esses fotorreceptores traduzem a informação luminosa em sinais bioquímicos ainda necessitam ser melhor esclarecidos. Dentre esses mecanismos, sabe-se, por exemplo, que o NO é capaz de participar do controle de diversas respostas fisiológicas moduladas pela luz via fitocromos, tais como a germinação de sementes fotoblásticas, alongamento caulinar, diferenciação plastidial e síntese de clorofilas (Giba *et al.*, 1998; Beligni & Lamattina, 2000; Melo, 2014).

Além dos receptores de luz clássicos, a presença da atividade fotossintética devido à presença de cloroplastos funcionais também consiste numa importante fonte de informação do contexto luminoso em que a planta se encontra, e parece influenciar a presença de NO nas células vegetais (Ördög *et al.*, 2013). Em contrapartida, estudos têm demonstrado que o NO estimula a formação e diferenciação de cloroplastos bem como a síntese de clorofilas (Tewari *et al.*, 2013; Melo *et al.*, 2014), apresentando, ainda, um importante papel na regulação da atividade fotossintética (Galatro *et al.*, 2013). De modo interessante, fortes indícios de correlação entre a percepção da luz via fitocromos e a produção de NO em plantas também foram recentemente obtidos em nosso laboratório, por meio do uso de mutantes de tomateiro deficientes na síntese desse fotorreceptor (Zuccarelli & Freschi, 2010). Nesse trabalho, verificou-se que a

condição de escuro era capaz de induzir aumentos de até cinco vezes na emissão de óxido nítrico por plântulas selvagens e de mutantes fotomorfogênicos, porém, essa emissão massiva de NO era fortemente inibida sob diversas condições de luz, mesmo sob baixas intensidades luminosas ($5 \mu\text{moles de fótons m}^{-2} \text{ s}^{-1}$). Entretanto, no mutante deficiente para fitocromos *yellow green 2* o comportamento de produção de NO sob luz vermelha (630-650 nm) de baixa intensidade ($5 \mu\text{moles de fótons m}^{-2} \text{ s}^{-1}$) foi semelhante à condição de escuro, indicando a possível participação dos fitocromos. Neste mesmo mutante, luz vermelha de maior intensidade ($200 \mu\text{moles de fótons m}^{-2} \text{ s}^{-1}$) foi capaz de inibir a emissão de NO, ainda que os níveis tenham se mantido acima daqueles encontrados na variedade selvagem sob mesma condição luminosa.

7. O tomateiro como modelo para estudos sobre a fotomorfogenese vegetal

O tomateiro (*Solanum lycopersicum* L.) é uma espécie de grande importância não apenas em termos agronômicos, mas também como modelo de estudo para diversos aspectos fisiológicos não presentes em outras plantas modelos tais como *Arabidopsis*, arroz, tabaco, etc. Uma das características mais marcantes desse modelo é a presença de um fruto carnoso, com amadurecimento tipicamente climatérico. Dentre os recursos genéticos disponíveis para essa espécie, encontramos em *S. lycopersicum* uma grande variedade de cultivares, mutantes e transgênicas com alterações em diversos aspectos metabólicos e de desenvolvimento (Gray *et al.*, 1994). Existem, por exemplo, mutantes descritos para a síntese de fotorreceptores (Kendrick, 1996), em vias de transdução de sinais luminosos (Kendrick *et al.*, 1997), padrões de amadurecimento (Lanahan *et al.*, 1994; Wang *et al.*, 2005), entre outros. Existem também diversos mutantes com alterações na produção ou sensibilidade a

diversos hormônios vegetais como auxinas (Daniel *et al.*, 2014), citocininas (Pino-Nunes, 2005), giberelinas (George-Jones, 1987), etileno (Lanahan *et al.*, 1994) e ácido abscísico (Burbidge *et al.*, 1999). Tais recursos genéticos representam uma ferramenta de grande valor para pesquisas diversas.

Quando comparado com o modelo clássico *Arabidopsis thaliana*, a cultivar miniatura de tomateiro Micro-Tom, proposta como modelo genético apresenta diversas vantagens frente às variedades comerciais de tomateiro (Meissner *et al.*, 1997), tais como possuir tamanho reduzido, ser capaz de produzir frutos e sementes em vasos de pequeno volume (50-150 mL) e completando seu ciclo de vida bastante curto (em 70-90 dias). Suas sementes e plântulas apresentam tamanho maior do que as de *Arabidopsis* o que facilita a separação de porções como cotilédones, hipocótilos ou raízes para análises bioquímicas e moleculares (Lombardi-Crestana *et al.*, 2012). Assim como *Arabidopsis*, o tomateiro apresenta um genoma de tamanho relativamente pequeno e com poucas sequências repetitivas de DNA (Breeding & Hall, 1988), o qual já se encontra totalmente sequenciado (Sato *et al.*, 2012).

Diante das interessantes evidências já obtidas em nosso laboratório acerca da influência da luz sobre os teores endógenos de NO em plântulas de tomateiro (Zuccarrelli & Freschi 2010; Melo, 2014) esse material de estudo parece representar um sistema interessante para estudos mais aprofundados acerca dos mecanismos bioquímicos e fisiológicos responsáveis pela produção, conjugação e degradação desse composto sinalizador. Por exemplo, a avaliação detalhada e simultânea dos conteúdos de NO e de sua degradação, ao longo de diferentes condições luminosas constitui por si só numa estratégia interessante para avaliar a possível interdependência entre diferentes aspectos do metabolismo desse radical livre com vistas à proporcionar a manutenção de seus níveis endógenos em patamares

adequados. Por outro lado, por se tratar de uma espécie modelo para estudos sobre a formação, desenvolvimento e amadurecimento de frutos carnosos climatéricos, o tomateiro representa um material bastante interessante não apenas para estudos sobre o metabolismo do NO em tecidos vegetativos, mas também em tecidos do fruto, cujo amadurecimento encontra-se sob forte influência do NO e que apresenta grande importância nutricional e econômica. Ademais, essa espécie consiste ainda num material de estudo bastante interessante para se investigar a importância de cloroplastos funcionais sobre o metabolismo de NO, uma vez que apresenta não apenas a clássica conversão de etioplastos em cloroplastos durante o desestiolamento de suas plântulas, mas também apresenta a conversão de cloroplastos em cromoplastos em seus frutos carnosos, sendo que ambos esses processos encontram-se sobre forte influência de sinais luminosos. Estudos sobre o metabolismo e sinalização do NO durante esses eventos de diferenciação plastidial, poderiam, portanto, proporcionar evidências interessantes sobre o papel dessas organelas no ainda pouco elucidado cenário das interações entre luz e NO em tecidos vegetais.

Objetivos

O presente trabalho buscou investigar a influência da luz sobre o metabolismo de NO durante a conversão de etioplastos em cloroplastos em plântulas de tomateiro e a conversão de cloroplastos em cromoplastos durante o amadurecimento dos frutos carnosos dessa espécie.

Capítulo 1

Light influence on NO production and degradation during tomato seedling de-etiolation

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ABSTRACT

The gaseous free radical nitric oxide (NO) has emerged as a very important signaling compound controlling many developmental and adaptive responses in plants and, not surprisingly, environmental cues strongly influence the metabolism of this signaling molecule. Although still scarcely characterized, light quality, intensity and duration seem to significantly impact NO production, conjugation and degradation in plants. Significant advances in the understanding of the diverse biochemical processes responsible for NO metabolism in plants have been achieved in recent years, however, the relevance of particular biochemical routes and systems in controlling NO availability and toxicity still deserves further investigation. The conjugation of NO with thiols groups have been characterized as a possible route for NO degradation. Among the distinct thiol-containing plant compounds, reduced glutathione (GSH) seems to be a central player not only in NO conjugation, storage and transport but also in the NO removal from plant tissues due to the existence of the enzyme S-nitrosogluthathione reductase (GSNOR). In this work, we have investigated the influence of light on NO production and degradation during the light-evoked greening of tomato seedlings (*Solanum lycopersicum*), particularly focusing on clarifying the importance of S-nitrosylation in this context. First, NO endogenous levels and degradation rates were analyzed in seedlings of wild-type (Micro-Tom, MT) and photomorphogenic tomato mutants (*aurea* and *high pigment-1*) undergoing the transition due to exposure to monochromatic red (RL) or blue light (BL). Such analyses revealed a progressive increase in endogenous NO release during the dark-to-light transition, which positively correlated with a parallel increase in NO removal capacity at these same tissues. The exaggerated responses to light characteristic of the

tomato *high pigment* mutant revealed that light signaling promotes both NO production and degradation rates in de-etiolating tomato seedlings. On the other hand, the phytochrome-deficient *aurea* mutant, which remains partially etiolated under RL, exhibited significantly reduced endogenous NO levels and degradation rates under these circumstances. Treatments with S-nitrosylation inhibitors successfully indicated a significant contribution of thiol nitrosylation as a NO scavenging mechanism in light-grown de-etiolated tomato seedlings. The light-dependent increments in GSNOR activities also indicated this enzyme as possible player in the light-evoked increases in NO removal rates in de-etiolating tomato seedlings. Finally, evidence obtained also suggested the existence of a route shared between the reactive oxygen species (ROS) and NO removal/detoxification in tomato seedlings. Altogether, these data demonstrate that light profoundly impacts NO metabolism and in de-etiolating tomato seedlings opening up a window of opportunities for further characterizations of the light influence on particular elements involved in NO production, conjugation and degradation in this plant model system.

1. Introduction

During the last two decades, the gaseous free radical nitric oxide (NO) has been implicated in diverse plant responses (Baudouin, 2011; Mur *et al.*, 2013) and, accordingly, increasing interest has been devoted to clarify the mechanisms responsible for NO production, conjugation and degradation in plant tissues (Igamberdiev *et al.*, 2006; Yamamoto-Katou *et al.*, 2006; Hebelstrup and Jensen, 2008; Malik *et al.*, 2011; Chaki *et al.*, 2011; Corpas *et al.*, 2013). Studies have revealed that NO biosynthesis in plants is conspicuously more diverse than in animal systems (Wendehenne *et al.*, 2001) and involves basically two main categories: (I) the nitrite-dependent reductive routes and (II) the L-arginine-dependent oxidative pathways (Crawford, 2006). Among the nitrite-dependent routes, the enzyme nitrate reductase (NR), primarily responsible for the reduction of nitrate to nitrite, seems to play a key role in the further reduction of nitrite to NO in plants under many physiological contexts (Yamasaki & Sakihama, 2000; Meyer *et al.*, 2005; Yamamoto-Katou *et al.*, 2006; Kolbert *et al.*, 2008; Seligman *et al.*, 2008; Salgado *et al.*, 2013). In agreement with its central position in the plant nitrogen metabolism, NR activity as well as protein and transcript abundances are strictly regulated by a wide range of environmental (*e.g.*, light, temperature, nitrate availability) (Beevers *et al.*, 1965; Huber *et al.*, 1992; Lillo, 1994; Saroop *et al.*, 1998; Tucker & Ort, 2002) and endogenous (*e.g.*, circadian clock, plant hormones, sugar) cues (Aslam *et al.*, 1976; Deng *et al.*, 1990; Lillo, 1991; Neill *et al.*, 2003; Tucker *et al.*, 2004; Yang & Midmore, 2005).

Other possible routes of nitrite reduction to NO include the mitochondrial electron chain transport, the reaction in the thylakoid membranes and in the peroxisomes (Tischner *et al.*, 2004; Jasid *et al.*, 2006; Gupta & Igamberdiev, 2011;

Tewari *et al.*, 2013; Corpas & Barroso, 2014). The mitochondrial electron chain transport has been indicated to generate NO mainly under low oxygen availability conditions, during which the reduction of nitrite to NO by cytochrome C oxidase seems to help to maintain, at least partially, the energetic status of plant cells (Gupta & Igamberdiev, 2011). The reduction of nitrite to NO in the chloroplast thylakoid membranes is much less understood; however, it is worth mentioning that this organelle is an important source of O_2^- radicals, and the formation of peroxynitrite ($ONOO^-$) from NO seems to be associated to the peroxidation of lipids and proteins, both important for the modulation of chloroplast functioning (Jasid *et al.*, 2006). In addition, *in vitro* studies have indicated that under anaerobiosis peroxisomes may also produce NO from nitrite under anaerobiosis via the action of the enzyme xanthine oxidoreductase (XOR) (Gupta *et al.*, 2011).

In contrast, relatively fewer L-arginine-dependent oxidative pathways have been detected in plants so far. Although the production of NO from L-arginine has already been extensively demonstrated in plants (Zhao *et al.*, 2007; Issue, 2009; Fröhlich & Durner, 2011), the occurrence of a plant NOS enzyme, homologous or non-homologous to the animal NOS, has remained elusive (Yamasaki & Cohen, 2006; Fröhlich & Durner, 2011). Besides this NOS-like pathway, NO also seems to be produced in plants during polyamine synthesis and from hydroxylamine (Gupta *et al.*, 2011), but the physiological relevance of these two potential biochemical sources of NO is currently unknown.

The multiple potential NO sources in plants together with the ubiquitous and highly diffusible nature of this molecule can be interpreted as challenging features for a signaling compound involved in so many plant responses. In this sense, the presence of diverse and highly efficient NO removal systems in plant cells seems a valid

alternative to facilitate controlling the levels, distribution, reactivity and signaling function of this molecule. At least three distinct NO degradation or inactivation mechanisms have already been described in plants (Liu *et al.*, 2001; Beligni & Lamattina, 2002; Perazzolli *et al.*, 2004; del Río *et al.*, 2006; Hebelstrup & Jensen, 2008).

Non-symbiotic hemoglobins (nsHb), for instance, are known to modulate NO homeostasis in plant tissues both via oxidation and via S-nitrosylation reactions (Crawford & Guo, 2005; Perazzolli *et al.*, 2006). Under hypoxic conditions, nsHb converts NO to nitrate, which in turn can be converted to nitrite via NR, restarting the inorganic nitrogen assimilation cycle (Gupta & Igamberdiev, 2011). Another important pathway through which NO can be degraded is by interacting with reactive oxygen species (ROS). Depending on the concentration and radical involved, NO may act either as an antioxidant (Beligni *et al.*, 2002; Hung & Kao, 2004) or a source of reactive species of even greater oxidizing potential (Vandelle & Delledonne, 2011). For example, the reaction between NO and superoxide ($O_2^{\cdot -}$) gives rise to peroxynitrite ($ONOO^-/ONOOH$), whose production is promoted under low NO concentration ($NO/O_2^{\cdot -} < 1$). However, when exceeding the superoxide concentration ($NO/O_2^{\cdot -} > 1$), NO and peroxynitrite molecules begin reacting among themselves, thereby removing these radical species and inhibiting the peroxidation of other molecules (O'Donnell *et al.*, 1997). In plants, ROS are also important signaling molecules in acclimation and resistance responses to biotic and abiotic stress conditions (Dat *et al.*, 2000; Baxter *et al.*, 2014), and, not surprisingly, many NO-ROS-derived compounds seem to be implicated in the regulation of these responses (Wrzaczek *et al.*, 2010; Vandelle & Delledonne, 2011).

A third mechanism of NO conjugation and/or removal in plants involves the enzyme S-nitrosogluthathione reductase (GSNOR), which has been indicated as one of main routes controlling NO and NO-derived molecules levels under diverse physiological contexts (Barroso *et al.*, 2006; Neill *et al.*, 2008; Corpas *et al.*, 2008; Malik *et al.*, 2011; Chaki *et al.*, 2011; Hancock *et al.*, 2011; Kubienová *et al.*, 2014). NO is known to react with reduced glutathione (GSH) by reversibly binding to its thiol group giving rise to S-nitrosogluthathione (GSNO), which in turn might act as a potential mobile NO reservoir (Barroso *et al.*, 2006; Corpas *et al.*, 2013). Such reversible binding of NO to the GSH thiol group means that the equilibrium of the reaction $[\text{NO} + \text{GSH} \leftrightarrow \text{GSNO}]$ directly depends on the relative concentration of GSH, NO and GSNO inside the plant cells. Besides being spontaneously converted back to GSH and NO, GSNO might also be efficiently removed from the cellular environment by GSNOR, resulting in the formation of GSSG and NH_3 (Corpas *et al.*, 2013).

The influence of environmental factors on the plant NO production and removal has been analyzed by numerous studies (Lillo, 1994; Ohwaki *et al.*, 2005; Foo *et al.*, 2006; Zhao *et al.*, 2007; Chaki *et al.*, 2011). Given the widespread signaling roles played by NO in both biotic and abiotic stress responses, the effects of stressful environmental conditions on NO metabolism have been the main focus of many investigations (Dordas, 2003; Zhang *et al.*, 2006a, 2007; Tian & Lei, 2006; Qiao & Fan, 2008). Considerably much less is known about the influence of environmental cues on NO metabolism under less stressful external conditions. Light, for instance, seem to be an important environmental factor controlling NO production not only under stressful situations (*e.g.*, high light, UV radiation) (Soheila *et al.*, 2001; Beligni & Lamattina, 2002; An *et al.*, 2005; Wang *et al.*, 2006), but also under

growth-promoting conditions, regulating many photomorphogenic responses such as seed germination, hypocotyl elongation and leaf and cotyledon greening (Beligni & Lamattina, 2000; Zhang *et al.*, 2006b; Lozano-Juste & León, 2011; Melo, 2014). During tomato seedling de-etiolation, for instance, exogenous NO has been shown to stimulate chloroplast formation and photosynthetic pigment accumulation via a complex signaling cascade involving phytochromes and phytohormones (Melo, 2014). During the de-etiolation of these seedlings, a marked increase in NR-dependent NO production temporally coincides with the conversion of etioplasts to chloroplasts and the impairment in NO production strongly disturbed the normal light-driven de-etiolation process in this species (Melo, 2014).

Taking advantage of this clear light-dependent modulation in endogenous NO levels in de-etiolating tomato seedlings, the present study investigated the light-driven changes in NO metabolism, particularly focusing on the NO scavenging mechanisms involving S-nitrosylation reactions. Here, we show for the first time that a strict balance is maintained between the NO endogenous levels and removal rates, which is significantly controlled by the plant light perception and signaling and probably involves the reaction of NO with ROS and/or the degradation of GSNO by GSNOR. Both NO production and scavenging systems are especially active in green, de-etiolated cotyledon tissues, thereby indicating that comparisons between distinct seedling regions might help to clarify the importance of enzymes, metabolites and organelles as potential players in the light-driven changes in plant NO metabolism.

2. Material and methods

2.1. Plant material

Seeds of tomato (*Solanum lycopersicum* L.) cv. Micro-Tom (MT) and the near-isogenic lines (NILs) harboring the mutations *aurea* (*au*) and *high pigment-1* (*hp-1*) were obtained from the tomato mutant collection maintained at ESALQ, University of São Paulo (USP), Brazil (<http://www.esalq.usp.br/tomato/>) (Carvalho *et al.*, 2011).

2.2. Growth conditions and treatments

Seeds were surface sterilized as described in Lombardi-Crestana *et al.* (2012) and directly sown in Magenta[®] (Sigma-Aldrich) vessels (approximately 50 seed per vessel) containing sterile medium composed of half-strength Murashige and Skoog (MS) salts and 2% (w/v) Phytagel[®] (Sigma-Aldrich). After seven days of 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. BL or RL were continuously 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 samples were harvested either under the specific light conditions used for seedling growth or under dim green light (0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$), for the continuous darkness treatment.

For the treatments with S-nitrosylation inhibitors, seedlings were obtained as described above and the inhibitors N-ethylmaleimide (NEM) or S-methyl methanethiosulfonate (MMTS) were added to the growth medium to a final concentration of 100 mM two days prior the start of the light treatment (5th day of germination). Treatments with reduced glutathione (GSH) were initiated at the 1st day

of germination. The optimal concentration of NEM, MMTS or GSH was determined based on previous experiments using a concentration range of these compounds (*e.g.* 10, 100 and 1000 mM). NEM and MMTS concentrations higher than 100 mM and GSH concentrations higher than 2 mM resulted in severe perturbation in seedling development (data not shown). In all cases, MMTS-, NEM- or GSH-treated seedlings were thoroughly rinsed with distilled water prior endogenous NO content and degradation analysis. Individual samples were composed of at least 50 seedlings harvested from one or more Magenta[®] vessel. All measurements were conducted in fresh samples immediately after harvesting.

2.3. NO measurements

For fluorometric NO determination, the cell-impermeant fluorophore diaminorhodamine-4M (DAR-4M) was used as described in Melo (2014). Seedlings were gently fragmented into small pieces (typically 5 mm in length) and immediately weighted (~300 mg) and incubated with 1 mL of 50 mM phosphate buffer (pH 7.2) containing 37.5 μ M DAR-4M for 30 min at 25 °C in dim green light, 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). At least, five independent samples were analyzed for each sampling time. 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).

2.4. H₂O₂ measurements

The production of H₂O₂ was detected by the oxidation of 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) to its quinone form by the peroxidase as described by Van Gestelen *et al.* (1998), with some modifications. Basically, seedlings were gently fragmented into small pieces (typically 5 mm in length), immediately weighted (~300 mg) and incubated for 3 h at room temperature with 2 mL of a solution containing 1 mM DHBS, 0.1 mM 4-aminoantipyrine (AAP) and 0.5 mg/ml horseradish peroxidase. The molecular complex formed by the reaction between DHBS and AAP was measured in the supernatant by using a spectrometer at 510 nm. The H₂O₂ concentration was estimated by comparison with a calibration curve (0 to 2.3 mM) and was expressed as $\mu\text{mol H}_2\text{O}_2 / \text{g DW/h}$.

2.5. GSNOR activity assay

GSNOR activity was assayed according to Sakamoto *et al.* (2002), with some modifications. Fresh seedling samples were ground in a cold mortar and pestle in the presence of buffer (~500 mg fresh tissue/mL) composed of 50 mM Tris-HCl (pH 7.5), 0.05% triton X100, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM dithiothreitol (DTT). After centrifugation (13,000 g, 10 min, 4°C), 100 μL of the supernatant was added to 1900 μL of reaction buffer composed of 50 mM Tris-HCl (pH 7.5), 0.05% Triton X100, 1 mM PMSF, 5 mM DTT, 400 μM GSNO and 2 mM NADH. The reactions were incubated for 30 min at 40 °C, and at 0, 10 and 30 minutes 100 μL -aliquots of the reaction medium were collected and the reaction was stopped by freezing with liquid nitrogen. GSNOR enzymatic activity was determined by the rate of consumption of GSNO detected according to Fang *et al.* (1998). The concentration of the GSNO was measured by chemiluminescence detection of NO

released by reductive decomposition of GSNO (Rogers *et al.*, 2013). The reactions aliquots were defreezed and diluted into 1 mL in 50 mM potassium phosphate (pH 6.5) buffer and injected into a purge chamber maintained at room temperature and in the presence of 2 mL 50 mM potassium phosphate (pH 6.5) buffer containing 100 μ M (saturated) cuprous chloride (I) and 100 μ M L-cysteine. A continuous flow (100 mL/min) of NO-free gaseous nitrogen was bubbled through the purge chamber, thereby carrying the released gas to NO analyzer (CLD 88ep; Eco Physics). The GSNO concentration was estimated by comparison with a calibration curve (0 to 400 μ M GSNO) and GSNOR activity was expressed in nmol GSNO reduced/g protein/min.

2.6. NO degradation assay

NO degradation rates were determined by monitoring the kinetics of disappearance of a known concentration of NO (300 ppm), which was exogenously applied to seedlings kept inside a 3 L sampling chamber maintained in absolute darkness and constant temperature of 25 ± 1 °C. The atmosphere inside each sampling chamber was maintained under continuous closed air circulation of 300 ml/min for assuring a complete homogenization of the gas mixture over time. About 150 seedlings were simultaneously analyzed in each sampling chamber, and at least two independent experiments were assayed for each sampling time. Every 30 minutes, samples of approximately 0.8 mL of the internal atmosphere of each sampling chamber were automatically injected into the NO analyzer (CLD 88ep; Eco Physics) until reaching undetectable NO levels (*i.e.*; complete degradation of the NO molecules initially injected into the chamber). The series of values obtained were used to find an exponential regression curve. The rate of degradation (R_c) was determined according

to the formula: $[R_c = 0.5 * I_c / t_{0.5}]$, in which (I_c) represents the 50% value of initial NO concentration (in nmol of NO) and ($t_{0.5}$) represents the time (in hours) required to achieve the 50% reduction in the initial NO concentration (Fig. 3). The non-biological spontaneous degradation of NO was subtracted, using empty chambers as reference (Fig. 3). All values were expressed in nmol NO/g DW/h.

Figure. 3 Schematic representation of the data sampling and determination of NO degradation rates. In this hypothetical example, the blue line (example 2) represents a situation of higher degradation rate than the green line (example 1) (t_1 and t_2 represents the time point in each 50% of the initial NO concentration was consumed in each of these hypothetical degradation curves 1 and 2, respectively). The red line indicates the spontaneous NO disappearance in an empty sampling chamber.

3. Results

3.1. Tomato seedling de-etiolation temporally coincides with a progressive increase in both NO production and degradation

The light-hypersensitive mutant *hpl* is known to vigorously de-etiolate when exposed to either BL or RL whereas the phytochrome-deficient mutant *au* displays completely normal de-etiolation under BL, but fails to assume photomorphogenic growth when exposed to RL (Georghiou & Kendrick, 1991). Taking advantage of this system, we first compared the rates of both NO production and degradation in seedlings of MT, *hpl* and *au* exposed to distinct light conditions.

As can be seen in Figure 1, dark-grown seedlings of all genotypes exhibited reduced endogenous NO release rates at all sampling times. Such reduced endogenous NO levels (Figs. 1A, C and F) were associated with low levels of NO degradation (Fig. 1B, D and E).

On the other hand, noticeable differences in endogenous NO release and NO degradation were observed in MT, *au* and *hpl* seedlings exposed to either RL or BL. In MT seedlings, endogenous NO progressively increased under RL or BL treatments, reaching more than twice the values observed under dark conditions (Fig. 4A). Interestingly, these RL- or BL-exposed MT seedlings also exhibited NO degradation rates exciding in up to two times those detected in dark-grown, etiolated seedlings (Fig. 4B). Among the three genotypes analyzed, *hpl* seedlings exhibited the highest values of both endogenous NO release and NO degradation regardless the light condition (Figs. 4C and D).

In contrast, *au* seedlings only presented increased endogenous NO levels and NO degradation under BL (Fig. 4 F and E). As expected for a phytochrome-deficient mutant, *au* seedlings remained partially etiolated under RL, and the values of endogenous NO

release and NO degradation rates under this light condition were similar to those observed in *au* seedlings continuously kept under continuous darkness.

Figure 4. Increases in NO levels during light-driven tomato seedling greening temporally coincide with the rise in NO degradation rates. Fluorometric quantification of endogenous NO release (A, C and E) and chemiluminescence analysis of NO degradation (B, D and F) in wild type (Micro-Tom, MT) seedlings (A and B) and in the photomorphogenic mutants *high pigment-1* (C and D) and *aurea* (E and F) maintained under continuous darkness (D) or transferred from darkness to continuous blue light (BL) or red light (RL) treatments. Means $\pm$ SE.

3.2. Cotyledons are the main site of NO production and degradation in de-etiolating tomato seedlings.

During tomato seedlings de-etiolation, cotyledons seem to represent the main source of NO production and degradation as evidenced by the marked differences illustrated in Figure 5. In cotyledons of wild-type (MT) seedlings, either BL or RL

exposure resulted in increases of up to four times in both endogenous NO release and NO degradation rates (Figs. 5A and B). On the other hand, no differences in either NO production or NO degradation were observed when MT hypocotyls and roots were exposed to BL or RL since these seedling regions continued presenting NO production and degradation levels as low as those observed under continuous dark (D) conditions (Figs. 5 C and D).

Figure 5. Cotyledons are the primary sites of NO production and degradation in de-etiolating tomato seedlings. Fluorometric quantification of endogenous NO release (A and C) and chemiluminescence analysis of NO degradation (B and D) in isolated cotyledons (A and B) or hypocotyl plus roots (C and D) of wild-type (MT) seedlings. Seedlings were maintained under continuous darkness (D) or transferred from darkness to continuous blue light (BL) or red light (RL) treatments. Means $\pm$ SE.

3.3. Increased NO degradation in green cotyledons is associated with higher levels of hydrogen peroxide and GSNOR activity

As observed for the NO degradation rates, cotyledons of BL- or RL-exposed tomato seedlings presented higher levels of H_2O_2 than those observed in dark-grown, etiolated seedlings (Fig. 6A). Interestingly, under the same light treatments, GSNOR

activity was also found at significantly higher levels in cotyledon tissues of light-exposed than in dark-grown MT seedlings (Fig. 6B).

Figure 6. De-etiolating tomato cotyledons exhibit increased levels of hydrogen peroxide and GSNOR activity. H₂O₂ content (A) and GSNOR activity (B) in cotyledons of wild-type (MT) seedlings maintained under continuous darkness (D) or transferred from darkness to continuous blue light (BL) or red light (RL) treatments. Means $\pm$ SE.

3.4. S-nitrosylation inhibitors partially block NO degradation only under light, but not under dark conditions

Suggesting S-nitrosylation as an important mechanism of NO conjugation and subsequent degradation in de-etiolated tomato seedlings, the application of inhibitors of thiol groups S-nitrosylation significantly reduced NO degradation in tomato seedlings (Fig. 7). Interestingly, though, such inhibition in NO degradation was only observed light-exposed seedlings (BL), having no effect on seedlings continuously

kept under dark conditions (Fig. 7B). Among the S-nitrosylation inhibitors analyzed, MMTS application resulted in the strongest impairment in the NO degradation rates of light-grown tomato seedlings, exhibiting degradation rates less than half of those observed in control seedlings kept under continuous darkness or exposed to BL. No significant changes were observed in endogenous NO release in seedlings treated with S-nitrosylation inhibitors, except for a slight, but statistically significant increase in dark-grown MT seedlings treated with MMTS (Fig. 7A).

Figure 7. S-nitrosylation inhibitors block NO degradation only under light conditions. Fluorometric quantification of endogenous NO release (A) and chemiluminescence analysis of NO degradation (B) in wild type (MT) seedlings treated with the S-nitrosylation inhibitors NEM and MMTS. Seedlings were maintained under continuous darkness (D) or transferred from darkness to continuous blue light (BL) treatment for 48h. Different letters indicate significant differences between the S-nitrosylation inhibitor treatments ($P < 0.05$ %; Tukey test). Means $\pm$ SE.

3.5 Increased availability in thiol groups apparently does not affect endogenous NO levels or degradation rates

Wild-type seedlings were also treated with GSH to offer an ample supply of thiol groups since this tripeptide is suggested as one of the most important target of NO conjugation in plants (Corpas *et al.*, 2013). Despite this, on the concentration used (2 mM), no differences were found between control and GSH-treated seedlings both in terms of NO removal rates and endogenous levels (Fig. 8). Higher GSH concentrations (data not shown) disturbed the seedling development and were not used in this study.

Figure 8. Increased thiol availability does not affect NO degradation rates or endogenous levels. Chemiluminescence analysis of NO degradation and fluorometric quantification of endogenous NO release in wild-type (MT) seedlings treated with GSH in the presence or absence of blue light (BL). Seedlings were maintained under continuous darkness (D) or transferred from darkness to continuous blue light (BL) for 48h. Different letters indicate significant differences between the GSH treatments ($P < 0.05$ %; Tukey test). Means $\pm$ SE.

4. Discussion

So far, scarce methodological approaches have been developed for accurate estimations of plant tissue NO removal/degradation capacities (Soegiarto *et al.*, 2003). In this study we have developed a relatively simple and robust system based on offering a precise concentration of NO to intact or dissected plant tissues maintained

enclosed in a constant air circulation apparatus and continuously monitoring the removal of NO molecules from the internal atmosphere. By using a sensitive gas phase chemiluminescence detection system, we have shown here that the NO removal can be continuously monitored *in vivo* when plant tissues are treated with physiologically relevant NO concentrations and small air aliquots of atmosphere surrounding the tissues are analyzed over time. This apparatus allowed high reproducibility in determining the global *in vivo* removal rates of NO and made possible to access virtually any biochemical pathway involved in NO conjugation and/or degradation by measuring a simple variable: the NO removal from the closed atmosphere.

By using such a system and a plant developmental process marked by clear changes in endogenous NO levels (*i.e.*, de-etiolation) we have demonstrated the occurrence of a strict balance between the NO endogenous content and removal rates. As observed in other studies, either in tomato (Melo, 2014) or other plant species (Zhang *et al.*, 2006b; Liu *et al.*, 2013), a clear light-evoked increase in endogenous NO levels was observed in the present study (Fig. 4) and, quite surprisingly, the same occurred in terms of NO removal rates. Further confirming that light perception and signal transduction somehow activate biochemical pathways involved not only in NO production but also in NO degradation, de-etiolating seedlings of the light-hypersensitive tomato mutant *hp1* have presented higher levels of both NO production and removal than those observed in wild-type ones (Fig. 4C and D). In this mutant, the absence of functional proteins of the UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1) repressor (Carvalho *et al.*, 2011) probably keeps active some light downstream signaling even under the absence of this environmental signal and, as a consequence, *hp1* seedlings presented increased levels of both NO content and

degradation rates even under absolute darkness (Fig. 4). A distinctive features of *hp1* (Melo, 2014), and some other light-hypertensive tomato mutants (Mustilli *et al.*, 1999), is the partial differentiation of chloroplasts under complete darkness and the increased number and size of these organelles in light-grown vegetative and reproductive tissues of adult plants. Interestingly, chloroplasts are recognized as important sites for both NO production and degradation in plants (Jasid *et al.*, 2006). However, although the exaggerated and precocious development of chloroplasts in this mutant might possibly be associated with the pronounced NO degradation rates in *hp1*, it seems less likely that this distinctive phenotype would be main responsible for the increased production of NO in this mutant since in tomato seedlings NR apparently is the main NO biosynthetic source (Melo, 2014). Interestingly, though, *hp1* seedlings are known to present up to three times more NR activity than wild type ones (Coud & Sharma, 1994; Melo, 2014) and, therefore, its increased NO production seems to be associated to this exaggerated light-driven NR induction.

As expected, *au* seedlings retained a partially etiolated phenotype under red light, thereby emphasizing the central importance of phytochromes for the acquisition of many photomorphogenic traits. The RL-triggered NR activity induction is also strongly impaired in *au* seedlings (Becker *et al.*, 1992; Coud & Sharma, 1994; Melo, 2014), and this may account for the low NO endogenous levels observed under these experimental conditions (Fig. 4). Similar trends in NO endogenous levels and degradation rates were observed either in D- or RL-treated *au* seedlings, indicating that RL perception via phytochromes is essential for modulating NO production and degradation in tomato seedlings. In this sense, the data obtained in RL-treated *au* seedlings confirm that other possible physicochemical impacts of light, such as, light-

catalyzed redox reactions, do not significantly contribute to the NO scavenging rates observed in this plant material.

Interestingly, all light-driven impacts on NO metabolism were restricted to cotyledon tissues, not being observed in hypocotyls or roots (Fig. 5). Since the vast majority of the light-driven etioplast-to-chloroplast conversion takes place at the cotyledons and taking into consideration the key role played by NO in this organelle differentiation (Melo, 2014), it seems plausible to hypothesize that such strict light-dependent control of NO production and degradation in cotyledons might possibly be associated with the development of fully functional chloroplasts. The exact nature of the NO and chloroplast interaction is far from being fully characterized, however, one possible NO action mechanism during the plant tissue greening seem to be via an up-regulation in the expression of proteins and hormone production associated with increased photosynthetic activity (Chen *et al.*, 2014).

In plants, S-nitrosylation of thiol groups is currently considered one of the most relevant mechanisms of NO conjugation and degradation under normoxia (Corpas *et al.*, 2013), and among the distinct plant S-nitrosylation reactions, the reversible conjugation of NO with GSH followed by the GSNO catabolism via GSNOR seems to play a central role (Barroso *et al.*, 2006; Malik *et al.*, 2011; Xu *et al.*, 2013; Leterrier *et al.*, 2014). Here, we have demonstrated that feeding S-nitrosylation inhibitors to pre-germinated tomato seedlings strongly impaired NO degradation rates in light-exposed, de-etiolated seedlings, but have no effect in seedlings maintained in etiolated state (Fig. 7). Since the photosynthetic process is recognized as an important source of ROS (Asada, 2006) and glutathione is considered key player in the redox metabolism homeostasis in plants (Meyer & Hell, 2005; Gill *et al.*, 2013), most of remaining GSH not blocked by the chemical

inhibitors might have been diverted to buffer the higher production of ROS in the light-grown, photosynthetically active cotyledons. In fact, as suggested by Foyer and Shigeoka (2011), despite the relatively high GSH concentration (mM) present in plant chloroplasts, the antioxidant defenses in this organelle are not sufficient to remove all ROS produced by photosynthesis. In agreement, *in vivo* estimations of hydrogen peroxide (H_2O_2), a fairly stable and commonly produced ROS in plants, revealed significantly more abundant levels of this ROS in cotyledons of light-grown seedlings than in those maintained under dark conditions (Fig. 6). Due to a potentially lower GSH demand for buffering the limited ROS production under dark conditions, the GSH molecules remained not affected by the S-nitrosylation inhibitors might have been enough for maintained NO removal rates as high as those observed for control etiolated seedlings (Fig. 7). It's worth mentioning that higher dosages of S-nitrosylation inhibitors (higher than 100 mM) disturbed the normal development of tomato seedlings either in dark or light conditions (data not shown) and, therefore, such high concentrations were not used in this study. Similarly, feeding S-nitrosylation inhibitors as soon as at seed imbibitions phase strongly blocked germination in all tested dosages (data not shown), thereby indicating the effectiveness of these inhibitors and also highlighting the central importance of S-nitrosylation for the correct NO homeostasis during seed germination (Kopyra & Gwóźdz 2003; Libourel *et al.*, 2006; Arc *et al.*, 2013a,b). On the other hand, when applied from the 5th day germination onwards, the concentration of both S-nitrosylation inhibitors (*i.e.*, NEM and MMTS) used in this study did not disturb seedling development, which might indicate that enough GSNO might have been produced at this point to allow the normal completion of tomato seedling development.

In agreement with a recent study (Kubienová *et al.*, 2014) we have demonstrated that light exposure significantly induces GSNOR activity in tomato cotyledons (Fig. 6). This enzyme is able to consume GSNO producing GSSG and NH_3 , thereby effectively removing the NO from the cellular environment (Corpas *et al.*, 2013). Whether this light-evoked increased in GSNOR activity is associated with the higher NO degradation rates observed in light-exposed tomato seedlings remains to be determined.

In general, endogenous NO content was not affected by application of S-nitrosylation inhibitors (Fig. 7). Increased endogenous NO levels observed in MMTS-treated seedlings maintained under complete darkness might perhaps be a consequence of the thiol buffer system, in which the NO and GSNO concentrations must exist in an equilibrium, and the presence of the inhibitor probably dislocated the equilibrium constant to the direction of NO formation by reducing the GSH availability in the reaction $[\text{NO} + \text{GSH} \leftrightarrow \text{GSNO}]$. Despite the GSH-treated seedlings have shown no clear differences in NO endogenous content or degradation rates (Fig. 8), the resulting phenotype of the seedlings (data not shown) resembled those of ethylene-treated seedlings, with the classical triple response (Guzmán & Ecker, 1990) and the germination was delayed.

Similarly to the treatment with S-nitrosylation inhibitors, the presence of higher dosages of GSH in the culture media disturbed the tomato seed germination and seedling development (data not shown). Accumulating evidence indicates an intense cross-talk between NO and ethylene in germination and de-etiolation (Arc *et al.*, 2013b; Melo, 2014), therefore, more sophisticated experimental designs and tools are required to elucidate the impacts of modulating the GSH pools on the NO

metabolism given the conflicting data regarding the effects of inhibition or stimulation of GSH synthesis on plant NO metabolism (Creissen *et al.*, 1999; Xiang *et al.*, 2001).

Altogether, the data obtained indicated the existence of a clear fine-tuned balance between NO endogenous levels and removal rates in tomato de-etiolating seedlings, which is significantly influenced by light and probably involves the reaction of NO with ROS and/or the degradation of GSNO by GSNOR. Further studies using de-etiolating tomato cotyledons might help to elucidate the light influence on enzymes, metabolites and organelles associated with NO production, conjugation and removal in plant systems.

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Capítulo 2

Light signaling influences NO metabolism during tomato fruit ripening

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Short running title

Light and NO during fruit ripening.

ABSTRACT

Fruit ripening is characterized by a set of physiological changes controlled by many environmental and endogenous signals. New evidence indicates nitric oxide (NO) as an important molecule coordinating external signals and plant development. Although still poorly understood, the mechanisms controlling the endogenous NO concentration and distribution in vegetative plant tissues seem to rely on numerous biochemical pathways for the production, conjugation and degradation of this key signaling molecule. In recent years, exogenous NO has been shown to strongly modulate ripening onset and progression in flesh fruits, but the pathways responsible for regulating the endogenous production and degradation of this signaling compound in fruit tissues have remained largely unexplored. In this work, we have investigated the role of nitrate reductase (NR) on NO production during fruit ripening using tomato (*Solanum lycopersicum*) photomorphogenic mutants as a tool to elucidate a possible influence of light on NO metabolism in climacteric fruits. In parallel, we have also estimated the NO degradation rates at distinct tomato fruit ripening stages and have also pharmacologically accessed the relevance of S-nitrosylation for the NO removal system in flesh fruit tissues. In general, the onset of ripening in tomato fruits was marked by a concomitant reduction in both NO endogenous levels and degradation rates. NO degradation was maintained at very low and stable levels all over the ripening process, whereas endogenous NO increased soon after the beginning of the ripening process and only reduced again when the red ripe stage was achieved. Therefore, marked changes in the endogenous NO metabolism were observed during the climacteric ripening phase of tomato fruits. In addition, light signaling was shown to positively modulate NO production and NR activity in mature green fruits and promoted NO degradation in red ripe fruit tissues. Mature green fruits treated with S-nitrosylation inhibitors presented a clear reduction in NO degradation rates, thereby indicating that the conjugation of NO with thiol groups might represent an important mechanism for the homeostasis of this signaling molecule during tomato fruit ripening. Altogether, these results demonstrate that marked changes in the endogenous NO production and degradation are triggered during tomato fruit ripening and light represents an important environmental factor controlling NO metabolism in this reproductive organ.

Keywords: tomato; ripening; nitric oxide; nitrate reductase, S-nitrosylation.

1. Introduction

The onset and progression of ripening in flesh, climacteric fruits are characterized by distinct structural and biochemical changes, which are orchestrated by a multitude of signaling molecules. Among these signaling compound, ethylene plays a central role, triggering a cascade of events that includes modification in the fruit composition (*e.g.*, carbohydrates, acids, carotenoids, flavonoids and volatile compounds profiles) and structural features (*e.g.*, cell walls and plastids structure). In this process, ethylene is known to closely interact with other plant hormones such as auxins, abscisic acid and brassinosteroids (Garay-Arroyo *et al.*, 2012; Kumar *et al.*, 2014). More recently, new players were identified to interact with ethylene during fruit ripening. Among these signaling molecules, the gaseous free radical nitric oxide (NO) has been demonstrate to counteract ethylene action during the ripening process of distinct flesh fruits (Leshem *et al.*, 1998; Leshem & Wills, 1998; Leshem & Pinchasov, 2000). In this sense, exogenous NO application has been suggested as an potential strategy to increase the post-harvest quality of fruits by delaying the ripening and senescence processes (Zaharah & Singh, 2011a; Li *et al.*, 2014). Moreover this approach has been suggested as an alternative post-harvest preservation method to ameliorate the senescence resistance in broccoli (*Brassica oleracea*), green bean (*Phaseolus vulgaris*) and bok choy (*Brassica chinensis*) (Choy *et al.*, 2004) indicating a broader function of NO in antagonizing ethylene effects.

Decreasing NO emission has been observed during the ripening of both climacteric and non-climateric fruits (Zhu *et al.*, 2006; Zaharah & Singh, 2011b; Manjunatha *et al.*, 2012), and exogenous NO application has been shown to reduce

ethylene production during the ripening phase (Zhu & Zhou, 2007). Such antagonistic relationship seems to be mainly centered on the inhibition of ethylene biosynthesis by NO, involving the repression of ethylene biosynthetic enzymes at transcriptional and post-translation levels. In tomato, for instance, NO has been shown to decrease and delay the expression of ACC oxidase (ACO) genes without affecting the ACC synthase (ACS) genes (Eum *et al.*, 2009). In addition, NO also seems to regulate ACS and methionine adenyltransferase (MAT) via nitrosylation, resulting in both cases in a reduction in the enzymatic activity (Lindermayr *et al.*, 2006). Finally, NO also seems to interact directly with ACC and the enzyme ACO, forming a stable complex and decreasing the formation of ethylene by this enzyme (Zaharah & Singh, 2011b).

Contrasting with the detailed characterization of the ethylene biosynthetic route in plants, much less is known about the mechanisms responsible for NO production, conjugation and degradation in plants, and particularly flesh fruits (Manjunatha *et al.*, 2010). In vegetative plant tissues, NO production involves basically two main precursors: nitrite and L-arginine. Among the nitrite-dependent NO production reactions, the enzyme nitrate reductase (NR) seems to be of central importance under many physiological contexts (Bright *et al.*, 2006; Stöhr & Stremlau, 2006; Seligman *et al.*, 2008; Tewari *et al.*, 2013). Besides converting nitrate to nitrite, NR can further reduce nitrite to NO, and accumulating evidence indicate this NR side reaction as the main biosynthetic source of NO in plants (Meyer *et al.*, 2005). Examples of physiological responses controlled by NR-derived NO include stomatal closure, lateral root formation, water deficit responses, pathogen infection responses, among others (Garcı & Lamattina, 2003; Yamamoto-Katou *et al.*, 2006; Kolbert *et al.*, 2008). Under specific physiological contexts, the mitochondrial electron chain

transport and the thylakoid membranes have also been reported as other possible sources of NO from nitrite (Jasid *et al.*, 2006; Gupta & Igamberdiev, 2011). Moreover, although not yet associated to a particular enzymatic entity, NO can also be produced from L-arginine (Corpas *et al.*, 2009); however, the actual relevance of L-arginine-dependent NO production for plants still remains to be elucidated.

Reported as probably the main sources of NO in plants, NR is strictly controlled by several endogenous (*e.g.*, plant hormones, circadian rhythm) (Suty *et al.*, 1993; Tucker *et al.*, 2004) and exogenous (*e.g.*, light, temperature, nitrate) factors (Lillo & Appenroth, 2001; Kaiser *et al.*, 2002; Yaneva *et al.*, 2002). NR regulation by light seems to involve both the perception of this environmental signal via phytochromes and the production of carbohydrates via photosynthesis (Rajasekhar *et al.*, 1988; Lillo, 1994). Interestingly, NO has also been implicated in many plant responses controlled by light such as seedling de-etiolation, leaf greening, stomatal closure, and others (Beligni & Lamattina, 2000; Xiao-ping *et al.*, 2004; Bright *et al.*, 2006; Zhang *et al.*, 2006*b*). In some of these light-controlled responses, NO seems to be produced via NR activity (Desikan *et al.*, 2002; Garcı́ & Lamattina, 2003). The relevance of NR as biosynthetic source of NO in fruit tissues is, however, much less understood. In tomato, Teitel *et al.*, (1986) demonstrated that NR activity decreases during the tomato fruit ripening. On the other hand, some limited evidence has indicated that NR activity increases in leaves of plants during the early stages of fruit development, decreasing along the fruit ripening process (Amaral *et al.*, 2001; Reis *et al.*, 2009).

The control of NO in plant metabolism involves not only its production but also a set of systems capable of degrading and/or storing this signaling molecule. Like many other reactive molecules produced by living organism, NO interacts with many

defense systems. In plants, non-symbiotic hemoglobins (Hb) are indicated as one possible route for NO removal (Perazzolli *et al.*, 2006). The process involves the oxidation of NO by $\text{Hb(Fe}^{2+})\text{O}_2$ producing NO_3^- and $\text{Hb(Fe}^{3+})$. The $\text{Hb(Fe}^{3+})$ are regenerated in $\text{Hb(Fe}^{2+})\text{O}_2$ by consumption of NAD(P)H and subsequent association with O_2 . Another potential mechanism of NO degradation via Hbs involves the consumption of the NO-derived molecule S-nitrosoglutathione (GSNO) with the formation of the intermediate S-nitrosoHb and the subsequent production of NO_3^- and $\text{Hb(Fe}^{3+})$.

The tripeptide glutathione, present in the plant cell cytosol at mM concentration, has a paramount importance in the protection against oxidative cellular damage as well as in NO metabolism (Meister & Anderson, 1983). The thiol group of reduced glutathione (GSH) reversibly binds to NO forming GSNO. This compound represents a mobile NO reservoir and has a central importance in the NO metabolism in plants (Barroso *et al.*, 2006; Corpas *et al.*, 2013). Besides representing a NO reservoir, GSNO can also participate in NO degradation when reduced by the enzyme GSNO reductase (GSNOR), giving rise to NH_3 and dimeric oxidated glutathione (GSSG). In fact, the removal of GSNO via GSNOR is currently considered the most important route for NO degradation in plants (Barroso *et al.*, 2006; Neill *et al.*, 2008; Corpas *et al.*, 2008; Malik *et al.*, 2011; Chaki *et al.*, 2011; Hancock *et al.*, 2011; Kubienová *et al.*, 2014). Interestingly, light, as well other abiotic factors, can modulate the expression of GSNOR (Kubienová *et al.*, 2014); however, the actual relevance of such light-induced modulation in GSNOR transcript abundance remains elusive.

Representing a site for both NO production and degradation, the presence and abundance of functional chloroplasts might also be of relevance for the general NO

metabolism in plant tissues (Jasid *et al.*, 2006; Tewari *et al.*, 2013; Misra *et al.*, 2014). In many fruits, such as tomato, the ripening process is marked by the conversion of chloroplasts into chromoplasts, which will be responsible for the accumulation of pigments such as carotenoids (Shi & Le Maguer, 2000). This light-driven event is mainly controlled via phytochromes (Gupta *et al.*, 2014) and marks the loss of the fruit capacity to perform photosynthetic activity, to cope with oxidative damage and to perform many other chloroplast-dependent reactions (Mondal *et al.*, 2004; Renato *et al.*, 2014). Interestingly, some light-hypersensitive tomato mutants such as *high pigment 1* (*hp1*) and *high pigment 2* (*hp2*) present increased number and size of chloroplasts in fruit tissues whereas certain phytochrome-deficient tomato mutants (*e.g.*, *aurea* and *yellow-green*) show lower abundance and/or smaller fruit plastids (Yen *et al.*, 1997; Caspi *et al.*, 2008; Kendrick, 1996; Muramoto *et al.*, 2005). Therefore, such plant material seems particularly attractive for studies on the influence of light and plastid abundance and development on fruit NO metabolism and homeostasis.

By analyzing fruits from distinct tomato photomorphogenic mutants, the present study have revealed that marked changes in NO production and degradation take place during the onset and progression of tomato fruit ripening, and light perception and signaling can modulate different aspects of NO metabolism in this particular plant organ.

2. Material and methods

2.1 Plant material

Tomato (*Solanum lycopersicum* L.) cv. Micro-Tom (MT) and the near-isogenic lines (NILs) harboring the mutations *aurea* (*au*) or *high pigment-2* (*hp2*) were obtained from the tomato mutant collection maintained at ESALQ, University of São Paulo (USP), Brazil (<http://www.esalq.usp.br/tomato/>) (Carvalho *et al.*, 2011).

2.2. Growth conditions and treatments

Tomato plants were grown in 6-L pots in greenhouse under automatic irrigation (twice a day) at an average mean temperature of 25°C; 11.5 h/13 h (winter/summer) photoperiod and 250-350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR irradiance. Fruits at mature green (MG), breaker (BR) and red ripe (RR) stages were harvested at approximately 35, 38 and 55 days after anthesis, respectively. Prior the experiments, the fruits were quickly rinsed in 0.1 % solution of sodium hypochlorite for 15 minutes, thoroughly washed in distilled water and maintained in clear plastic boxes in the presence of wet filter paper. The plastic boxes were kept inside a temperature-controlled growth chamber maintained at 25±1°C and constant white light at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by an array of SMD5050 Samsung LEDs. All stages were assayed at least 24 h after harvesting to minimize manipulation injury artifacts.

Treatments with S-nitrosylation inhibitors were performed by injecting approximately 400 μL of a 100 mM solution of the inhibitors N-ethylmaleimide (NEM) or S-methyl methanethiosulfonate (MMTS) in each mature green (MG) fruit

approximately 24 hours prior the start of the NO degradation assays. Fruits injected with 400 μ L distilled water were used as control.

2.3. NO measurements

For fluorometric NO determination, the cell-impermeant fluorophore diaminorhodamine-4M (DAR-4M) was used. Fruit pericarp were dissected and gently fragmented into small pieces (typically 5x5 mm) and immediately weighted (~300 mg) and incubated with 1 mL of 50 mM phosphate buffer (pH 7.2) containing 37.5 μ M DAR-4M for 30 min at 25 °C in dim green light, 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). At least five independent samples composed of mixed fragments of five fruits were analyzed for each sampling time. 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).

2.4. NR activity assay

In vivo NR activity was assayed according to Jaworski (1971) with some modifications. Briefly, fruit pericarp samples were fragmented into small pieces (typically 5x5 mm) and immediately weighted (~ 1 g) and incubated with 2 mL of 100 mM phosphate buffer (pH 7.2) containing 100 mM KNO₃ and 3% (v/v) isopropanol . Tissue fragments were vacuum-infiltrated three times (500 mmHg for 30 s each) and then incubated at 30 °C for approximately 1 hour. After incubation, 1 mL of the

solution was collected and centrifuged for 10 min x 16.100g and the supernatant was used for nitrite quantification. Nitrite ions produced were quantified by the reaction with 0.7 ml 1% (m/v) sulfanilamide-HCl and 0.7 ml of 0.2% (m/v) *N*-(1-Naphthyl)ethylenediamine. After 20 minutes of incubation, nitrite concentration was spectrophotometrically determined at 540nm.

2.5. NO degradation assay

NO degradation rates were determined by monitoring the kinetics of disappearance of a known concentration of NO (300 ppm) exogenously applied to intact fruits kept inside a 3-L sampling chamber, maintained under absolute darkness, temperature of 25 ± 1 °C and continuous closed air circulation (300 mL / min) for assuring a complete homogenization of the gas mixture over time. About 20 g of fruits (9 to 20 intact fruits) were simultaneously analyzed in each sampling chamber and, at least two independent experiments were assayed for each sampling time.

Samples of approximately 0.8 mL of the internal atmosphere of each sampling chamber were automatically injected into the NO analyzer (CLD 88ep; Eco Physics) every 30 minutes until reaching undetectable NO levels (*i.e.*; complete degradation NO initially injected into the chamber). The series of values obtained were used to find an exponential regression curve. The rate of degradation (R_c) was determined by the 50% value of initial NO concentration (I_c) divided by the time required to achieve this reduction in NO concentration ($t_{0.5}$) (Fig. 3) according to the formula $R_c = 0.5 * I_c / t_{0.5}$. The non-biological, spontaneous degradation of NO was subtracted, using an empty chamber as reference (Fig. 3). The values were expressed in nmol NO/gDW/h.

2.6. Results

Marking the start of the ripening process, the breaker (BR) stage comprises drastic developmental, metabolic and signaling modifications (Alexander & Grierson, 2002; Klee & Giovannoni, 2011). In this work, we have observed a transitory reduction in endogenous NO release specifically at the BR stage (Fig. 9A). As soon as two days after BR, endogenous NO levels returned to values close to those observed at the mature green (MG) stage and were maintained at these higher levels until the 6th day after BR. Twenty one days after BR, when the ripening process is already completed in Micro-Tom, endogenous NO release returned to levels as low as those observed at BR stage.

Interestingly, NO degradation rates sharply decreased on BR stage and were maintained at low and fairly stable levels until the complete ripening of Micro-Tom (MT) fruits (Fig. 9B).

Figure 9. The start of tomato fruit ripening temporally coincides with decreases in NO content and degradation rates. Fluorometric quantification of endogenous NO release (A) and chemiluminescence analysis of NO degradation (B) during Micro-Tom (MT) fruit ripening. Fruits were analyzed at the stages mature green (MG), breaker (BR), breaker + 1 d (BR+1), breaker + 2 d (BR+2), breaker + 3 d (BR+3) breaker + 6 d (BR+6), and breaker + 21 d (BR+21). Means $\pm$ SE.

Fruits of the light-hypersensitive tomato mutant *high pigment* (*hp2*) is known to exhibit increased abundance of chloroplasts, pigments and antioxidants when compared to the wild type (Peters *et al.*, 1992; Yen *et al.*, 1997) and the opposite occurs in the phytochrome-defective mutant *aurea* (*au*) (Muramoto *et al.*, 2005). Taking advantage of these clear differences in fruit composition and physiology, we decided to analyze the impact of these photomorphogenic mutations on NO metabolism during tomato fruit ripening.

Since the NR has been identified as one of main sources of NO in plants (Planchet & Kaiser, 2006; Gupta *et al.*, 2011), we decided to analyze the *in vivo* NR activity in fruit pericarp tissues of wild-type and the *hp2* and *au* tomato photomorphogenic mutants (*hp2* and *au*) in order to gain more information about the light influence on the NO production during fruit ripening. As shown in Figure 10, regardless of the genotype analyzed, significantly higher levels of NR activity were observed in fruits at MG than in those at RR stage. Moreover, suggesting a positive influence of light on the NR activity levels in tomato fruit tissues, MG or RR pericarp samples of *hp2* exhibited approximately twice more NR activity than wild type (MT) ones. On the other hand, NR activity in MG or RR pericarp samples of the phytochrome-defective *aurea* mutant were not significantly different from that detected in MT (Fig. 10)

Figure 10: NR activity in tomato pericarp tissues is positively regulated by light and strongly decreases during fruit ripening. *In vivo* NR activity was determined in mature green (MG) and red ripe (RR) pericarp samples of wild type (Micro-Tom, MT) and of the photomorphogenic mutants *high pigment-2* (*hp2*) and *aurea* (*au*) immediately after harvesting. Means $\pm$ SE. Distinct capital and small letters indicate differences between genotypes at the same ripening stage and between distinct ripening stages at the same genotype, respectively ($P < 0.05$ %; Tukey test).

In terms of NO degradation rates, fruits of both wild type (MT) and *au* mutant exhibited significantly lower rates of NO degradation at the RR stage than at the MG stage (Fig. 11). Interestingly, though, NO degradation levels in MG and RR fruits were not significantly distinct in the light-hypersensitive *hp2* mutant (Fig. 11).

Figure 11. NO degradation is influenced by the fruit ripening stage and differs among tomato photomorphogenic mutants. Chemiluminescence analysis of NO degradation in mature green (MG) and red ripe (RR) fruits of the wild type (Micro-Tom MT) and of the photomorphogenic mutants *high-pigment-2* (*hp2*) and *aurea* (*au*). Means $\pm$ SE. Distinct capital and small letters indicate differences between genotypes at the same ripening stage and between distinct ripening stages at the same genotype, respectively ($P < 0.05$ %; Tukey test).

Evidencing an important role played by thiol groups in NO metabolism, MG fruits of wild-type plants treated with S-nitrosylation inhibitors exhibited significantly reduced NO degradation rates than control ones (Fig. 12). Among the S-nitrosylation inhibitors analyzed, MMTS triggered the most conspicuous reduction in tomato fruit NO degradation rates, exhibiting values about half of those observed in control fruits.

Figure 12. S-nitrosylation inhibitors block NO degradation in the mature green tomato fruits. Chemiluminescence analysis of NO degradation of wild type MG fruits treated for 24 h with 400 μ L of a 100 mM solution of the S-nitrosylation inhibitors NEM and MMTS. Fruits were maintained under continuous white light. Means $\pm$ SE. Distinct letters indicate significant differences between the treatments ($P < 0.05$ %; Tukey test).

4. Discussion

Accumulating evidence indicates NO as an important element in the signaling networks controlling fruit development and ripening (Wills *et al.*, 2000; Zhu & Zhou, 2007; Manjunatha *et al.*, 2010; Zaharah & Singh, 2011*b*). Since the seminal studies of Leshem *et al.*, (1998), which have demonstrated a progressive reduction in NO emission levels during fruit ripening, many reports have been published on the repressor influence of exogenous NO on the ripening process of fruits as diverse as tomato, strawberry (*Fragaria ananassa* L.), mango(*Mangifera indica* L.) and plum (*Prunus salicina* L.) (Bruggink *et al.*, 1988; Zhu & Zhou, 2007; Singh *et al.*, 2009; Manjunatha *et al.*, 2010; Zaharah & Singh, 2011*b*). However, the biochemical mechanisms controlling NO homeostasis in fruits have remained fairly uncharacterized.

In the present work, we have demonstrated that endogenous NO exhibits an abrupt reduction at the breaker (Fig. 9A), which is a stage marked by the start in the climacteric peak of ethylene production and the onset of many structural and biochemical modifications in tomato fruits (Alexander & Grierson, 2002; Cara & Giovannoni, 2008; Tatsuki, 2010).

At breaker stage, climacteric fruits as tomato display the activation of autocatalytic ethylene burst (system 2 of ethylene production) (Lelievre *et al.*, 1997; Alexander & Grierson, 2002; Cara & Giovannoni, 2008) and, therefore, such abrupt reduction in endogenous NO levels might have alleviated the antagonistic influence of this free radical on the ethylene biosynthetic pathway, thereby facilitating the establishment of the climacteric ripening phase. Due to the relevant repressor influence of NO on ethylene biosynthetic enzymes such as MET, ACS and ACO (Eum *et al.*, 2009; Manjunatha *et al.*, 2010; Zaharah & Singh, 2011b), it seems plausible to suggest that such reduction in endogenous NO levels might have de-repressed the accumulation of transcription and/or activity of these enzymes specifically at this ripening stage, facilitating the onset of the autocatalytic ethylene burst. It is relevant to mention that the breaker stage in tomato fruits is also marked by drastic changes in other important signaling molecules such as auxins and cytokinins (Davey & Van Staden, 1978; Trainotti *et al.*, 2007; Kumar *et al.*, 2014), whose reduction in endogenous levels also seem to be associated with the onset of the ripening process. In the tomato ripening-impaired mutant *ripening inhibitor (rin)*, the endogenous levels of both auxins and cytokinins at the breaker stage are maintained higher than those observed in wild-type fruits (Davey & Van Staden, 1978), thereby suggesting that the reduction in these signaling compounds might be associated with the start in the ripening process. Moreover, many recent studies have also established

the importance both, exogenous auxins (Trainotti *et al.*, 2007; Zaharah *et al.*, 2011; Böttcher *et al.*, 2013) and exogenous NO (Manjunatha *et al.*, 2010, 2012; Zaharah & Singh, 2011b; Li *et al.*, 2014) in the control of the fruit ripening process. In fact, feeding auxins (Ma *et al.*, 2014) or NO (Li *et al.*, 2014) have been suggested as alternative strategies for increasing the post-harvest quality of fruits by delaying their ripening.

Curiously, NO degradation rates in tomato fruits have also drastically dropped at the breaker stage and were maintained at low levels during the rest of the ripening period (Fig. 9), which might suggest that the NO removal in fruit tissues are somehow fine-tuned with the production and endogenous levels of this free radical. It seems relevant to mention that the breaker stage in tomato is marked by the conversion of functional chloroplasts into chromoplasts (Egea *et al.*, 2010, 2011) and recent studies have established the importance of functional chloroplasts for both the production and degradation of NO (Jasid *et al.*, 2006; Tewari *et al.*, 2013; Misra *et al.*, 2014). Moreover, functional chloroplasts are also widely recognized as important sites for the production of diverse oxidants (Saed-Moucheshi *et al.*, 2014) and antioxidants (Halliwell, 1987; Okamoto *et al.*, 2001; Foyer & Shigeoka, 2011), which are capable of interacting and removing NO from the plant tissues (O'Donnell *et al.*, 1997; Hung & Kao, 2004; Misra *et al.*, 2014). For example, chloroplasts are responsible for a significant part of the plant cell production of GSH, which is stimulated by NO (Innocenti *et al.*, 2007) and light (Bielawski & Joy, 1986). As discussed elsewhere this tripeptide is a key player in the NO conjugation and degradation systems in plants (Corpas *et al.*, 2008; Zechmann, 2014). Therefore, the conversion of chloroplasts into chromoplasts during the fruit transition to the ripe stage may implicate in reductions in the GSH-dependent NO scavenging system. In agreement with this hypothesis,

studies have shown that GSH and GSSG levels significantly decrease during the ripening of tomato fruits (Mondal *et al.*, 2004; Torres & Andrews, 2006).

Interestingly, light-hypersensitive tomato mutants such as *hp1* and *hp2* are known to retain higher contents of GSH, GSSH and other antioxidants through their fruit ripening process (Torres & Andrews, 2006). These mutants are also known to exhibit increased plastid abundance and size both in leaves and fruits (Yen *et al.*, 1997). In these sense, the retention of higher NO degradation rates in red ripe *hp2* fruits than in wild-type fruits (Fig. 11) positively correlates with the higher levels of GSH, GSSG and other antioxidants classically observed in this light-hypersensitive mutant (Andrews *et al.*, 2004; Lenucci *et al.*, 2006; Torres & Andrews, 2006). Curiously, in early stages of development of tomato fruits GSH levels do not differ between *high pigment* and wild-type fruits (Torres & Andrews, 2006), which might help to explain the similar NO degradation rates observed in *hp2* and MT fruits at MG stage (Fig. 11). Curiously, green and ripen fruits of the phytochrome-deficient tomato mutant *aurea* presented NO degradation rates similar to those observed in wild-type fruits (Fig. 11). The GSH, GSSG and antioxidant pools in this phytochrome-deficient mutant remain to be determined.

In agreement with results obtained in vegetative tissues of *high pigment* mutants (Rajasekhar *et al.*, 1988), we have observed that mature green *hp2* fruits exhibited higher NR activity level than wild-type ones. Although the light signaling influence on NR gene expression and activity in leaf tissues has already been established long ago (Rajasekhar *et al.*, 1988; Lillo & Appenroth, 2001), the light signaling impacts on fruit NR have remained uncharacterized. In green and ripen fruits of the *aurea* mutant, however, NR activities was similar to those detected in wild-type fruits (Fig. 10), which might be explained by the presence of up to 30%

functional phytochromes in mature tissues of this mutant when grown under greenhouse conditions (Parks *et al.*, 1987; Goud & Sharma, 1994). Therefore, besides the normal perception of light signal via other photoreceptors (*e.g.*, cryptochromes, phototropins), *aurea* mature tissues still display significant red light perception via these remaining functional phytochromes. In agreement with our finding in fruit tissues, the light-induction of NR activity and gene expression has also been observed in leaf tissues of *aurea* plants growing under white light conditions. Moreover, Becker *et al.*, (1992) also observed no significant differences in the diurnal cycle of NR gene expression between wild type and *au* seedlings growing under white light. This same work also reported that lower abundance NR transcript levels in *aurea* were only detected when seedlings of this mutant were grown under red light conditions. Altogether, these results indicated that although light signaling plays an important role on NR regulation in tomato fruits, even low levels of functional phytochromes may be sufficient to promote NR activity at levels as high as those observed in wild-type fruits.

Pericarp tissues of all genotypes consistently showed lower NR activity levels in the ripe stage (Fig. 10). This result seems to be in agreement with the fact that pre-climateric fruits usually present higher NO emission rates than those at advanced ripening (Leshem *et al.*, 1998; Laurenzi *et al.*, 1999; Manjunatha *et al.*, 2010, 2012), thereby suggesting that this enzyme may play a role in the NO production not only in vegetative tissues (Yamasaki & Sakihama, 2000; Rockel *et al.*, 2002; Yamamoto-Katou *et al.*, 2006) but also in fruits. The reduction in NR activity followed by a reduction in endogenous NO production might, therefore, facilitate the onset of tomato fruit ripening by alleviating the repressor influence of this free radical on the ethylene biosynthetic pathway.

As important as its production, NO degradation seems also to be key in the control of the potential toxic effects of this radical and may also help to explain how such small and ubiquitous molecule can regulate so diverse and sometimes contrasting plant responses. Here, the abrupt reduction in the total capacity of NO removal in fruit tissues may be interpreted as a compensatory mechanism to allow some balance in NO homeostasis even when its production dramatically decreases. The marked reduction in the NO degradation rates in fruits treated with S-nitrosylation inhibitors suggests that S-nitrosylation might represent a relevant biochemical pathway for NO scavenging, storage and possibly degradation in fruits. Recent studies have shown the importance of the S-nitrosylation pathway in plant developmental and defense responses by the control of the activity of the enzyme GSNOR (Malik *et al.*, 2011; Chaki *et al.*, 2011; Leterrier *et al.*, 2014). In *Arabidopsis*, for example, the production of nitrosative stress by down-regulating GSNOR activity establishes a link between this enzyme and NO homeostasis during specific plant responses such as thermotolerance (Lee *et al.*, 2008) and overall control of redox metabolism (Xu *et al.*, 2013).

The presence of a relatively large concentration of GSH in the plant cellular media is important not only because of its antioxidant properties but also as a potential reservoir site for NO storage in the GSNO. This system forms a NO buffer that may be capable of controlling and coordinating the different NO production sites in one central source and also allow NO removal from tissues via GSNOR activity. This system is of a particular importance due to the gaseous nature of NO, and the fact that this molecule is produced by other organisms in the environment (*e.g.*, soil microorganisms) (Ludwig *et al.*, 2001), therefore, an efficient system capable of

coping with fluctuations in exogenously and endogenously produced NO are of great importance to the signaling function of this singular molecule.

In conclusion, this work demonstrate that marked changes in NO metabolism can be observed during tomato fruit ripening and such metabolism can be influenced by light perception and signal transduction. Moreover, evidence also indicated NR as a potential biosynthetic source of NO and S-nitrosylation of thiol residues as candidate route for NO degradation in ripening tomato fruits. Finally, the temporal correlation between the fluctuation in NO levels and the start in fruit ripening also suggested that transitory reduction in endogenous NO might be associated with the induction of the tomato fruit ripening process.

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Conclusões

No presente trabalho constatamos uma correlação positiva entre os conteúdos endógenos e as taxas de degradação de NO, indicando que a disponibilidade tecidual desse radical livre estaria sob controle preciso de diferentes mecanismos de conjugação e/ou degradação.

De modo geral, tecidos portadores de cloroplastos funcionais (*e.g.*, plântulas desestioladas, frutos imaturos) apresentaram maiores taxas tanto de produção quanto de remoção de NO dos tecidos, denotando um possível papel chave para essa organela no metabolismo de NO.

Tanto a produção quanto a degradação de NO em tomateiro parecem estar sob regulação da luz, uma vez que incrementos em ambos processos foram observados em plântulas expostas à luz, bem como em tecidos cotiledonares e de frutos de mutantes hipersensíveis à esse sinal ambiental.

Os dados obtidos também sugerem que a S-nitrosilação consistiria num importante mecanismo de remoção do NO tanto em plântulas quanto em frutos de tomateiro. Embora os resultados obtidos indiquem a enzima GSNOR de tomateiro seja estimulada pela luz, estudos adicionais ainda serão necessário para esclarecer os mecanismos pelos quais esse sinal ambiental influenciaria a capacidade de remoção de NO nos tecidos vegetativos e reprodutivos de tomateiro.

Resumo

Ao longo dos últimos anos, o radical livre gasoso óxido nítrico (NO) vem ganhando destaque como uma importante molécula sinalizadora em respostas fotomorfogênicas em plantas. Sua produção e degradação parecem incluir uma diversificada gama de rotas bioquímicas, entretanto, a importância relativa de cada um dos sistemas capazes de regular sua disponibilidade e toxicidade nos tecidos vegetais ainda permanece pouco compreendida. Dentre as possíveis rotas de conjugação e degradação do NO em tecidos vegetais, postula-se que a glutatona (GSH) desempenhe um papel de destaque no armazenamento desse radical livre por meio da formação reversível da S-nitrosoglutationa (GSNO), sendo possível sua subsequente degradação através da ação da enzima S-nitrosoglutationa redutase (GSNOR). No presente trabalho investigamos a influência da luz sobre o metabolismo de NO em duas etapas de desenvolvimento vegetal caracterizados pela ocorrência de eventos de diferenciação plastidial: (I) o desestiolamento de plântulas e (II) o amadurecimento de frutos carnosos de tomateiro (*Solanum lycopersicum*). Além do genótipo selvagem Micro-Tom (MT), também foram utilizados os mutantes fotomorfogênicos *aurea* (*au*) e *high pigment 1* e *2* (*hp1* e *hp2*). Durante o desestiolamento das plântulas de tomateiro constatou-se um incremento progressivo tanto nos teores endógenos quando nas taxas de degradação de NO, bem como na atividade da enzima GSNOR. Sob condições luminosas similares, mutantes com respostas exageradas à luz apresentaram incrementos ainda mais evidentes nesses parâmetros do que aqueles observados no genótipo selvagem. A aplicação de inibidores de S-nitrosilação de proteínas, bem como a avaliação do conteúdo de espécies reativas de oxigênio (ROS) indicaram que tanto a formação de S-nitrosotiois quanto a interação do NO com ROS contribuíram para a determinação da capacidade de remoção de NO nos tecidos fotossinteticamente ativos de tomateiro. Em frutos, observou-se uma correlação positiva entre a atividade da enzima nitrato redutase (NR) e o padrão temporal de produção de NO, uma vez que ambos os parâmetros apresentaram maiores níveis em frutos imaturos. O amadurecimento desses frutos foi acompanhado por uma diminuição transitória dos conteúdos de NO ao passo que as taxas de degradação de NO mantiveram-se bastante reduzidas durante todo o processo de amadurecimento, sugerindo a existência de um estoque de NO na forma de GSNO ou algum outro S-nitrosotiol. A sinalização

luminosa influenciou positivamente tanto a produção quanto a degradação de NO em frutos imaturos de tomateiro. Em conjunto, os resultados obtidos permitem concluir que o metabolismo do NO em tomateiro é fortemente controlado pela luz, a qual é capaz de modular conjuntamente as taxas de produção e degradação desse importante composto sinalizador.

Abstract

In recent years, the gaseous free radical nitric oxide (NO) has emerged as an important signaling molecule in plant photomorphogenic response. NO production and degradation seems to include a wide range of biochemical routes; however, the relative importance of which one of the systems capable of regulating NO availability and toxicity in plant tissues remains elusive. Among all potential NO degradation and conjugation routes in plant tissues, it has been suggested that glutathione (GSH) plays a key role in NO storage due to the formation of S-nitrosogluthathione (GSNO), being possible its subsequent degradation by the action of enzyme S-nitrosogluthathione reductase (GSNOR). In this work, we have investigated the light influence on NO metabolism during two plant developmental events characterized by the occurrence of plastidial differentiation: (I) seedling de-etiolation and (II) fruit ripening of tomato (*Solanum lycopersicum*). Besides the wild-type Micro-Tom (MT) genotype, the tomato photomorphogenic mutants *aurea* (*au*) and *high pigment 1* and *2* (*hp1* and *hp2*) were also employed in this study. During the de-etiolation of tomato seedlings, a progressive increment was observed in the NO endogenous levels and degradation rates as well as in the GSNOR activity. Under similar light conditions, light hypersensitive mutants exhibited more conspicuous increases in these parameters than those detected in the wild-type genotype. Feeding protein S-nitrosylation inhibitors and measurements of reactive oxygen species (ROS) production indicated that both S-nitrosothiols formation and NO interaction with ROS may to contribute for determining the NO removal capacity in photosynthetically active tissues of tomato. In fruits, a positive correlation was observed between nitrate reductase (NR) activity and the temporal pattern of NO production since both parameters exhibited increased levels in immature fruits. The ripening of theses fruits was accompanied by a transitory reduction in endogenous NO levels whereas its degradation rates were maintained reduced all over the ripening process, thereby suggesting the existence of a more stable NO reservoir such as GSNO or some other S-nitrosothiol. In general light signaling positively influenced both NO production and degradation in mature green tomato fruits. Altogether, the data obtained indicated that tomato NO

metabolism is significantly influenced by light, which is able to simultaneously modulate both the production and degradation of this important signaling compound.

Perspectivas

Devido suas características químicas intrínsecas e a grande diversidade de rotas pelas quais o NO pode ser produzido em plantas, estudos acerca da função fisiológica do óxido nítrico em sistemas vegetais apresentam uma série de desafios de natureza metodológica.

Um dos grandes desafios consiste na necessidade de pormenorizar a localização das rotas de biossíntese, conjugação e degradação do NO nos diferentes compartimentos celulares durante a indução de eventos fisiológicos particulares. Nesse sentido, a utilização de diferentes técnicas de microscopia atualmente disponíveis associada ao emprego de fluoróforos específicos para detecção do NO e técnicas imunocitoquímicas para a detecção das enzimas envolvidas no metabolismo do NO poderiam auxiliar na determinação dos mecanismos responsáveis pela especificidade do NO e de seus derivados.

A avaliação da importância relativa das diferentes vias de biossíntese e degradação de NO durante os eventos fotomorfogênicos analisados no presente trabalho, por exemplo, poderiam auxiliar na elucidação dos mecanismos pelos quais os níveis desse sinalizador são mantidos em níveis adequados para desencadear eventos de sinalização particulares, sem atingir patamares tóxicos ou acarretar em respostas inespecíficas. Nesse sentido, além de explorar em maior detalhe o papel da nitrato redutase NR na produção de NO durante a indução de respostas fotomorfogênicas so análise, também seriam necessárias maiores investigações sobre outras vias potenciais de síntese desse composto nessas respostas fisiológicas. Da mesma forma, a contribuição relativa das diferentes rotas de biossíntese e degradação de NO também necessitariam de um estudo mais pormenorizado durante a indução de respostas fotomorfogênicas, incluindo, por exemplo, a quantificação dos teores endógenos de S-nitrosotiois e GSNO, determinação da abundância de proteínas e transcritos das enzimas GSNOR, hemoglobinas não simbióticas, entre outras. Por exemplo, a avaliação dos conteúdos de espécies S-nitrosiladas e de GSNO, bem como GSH e GSSG, ao longo do desenvolvimento dos frutos, ou durante a desestiolamento de plântulas de tomateiro, acompanhada da determinação da atividade da enzima GSNOR seriam estratégias interessantes para elucidar a importância dessa rota de conjugação e degradação de NO durante esses eventos fisiológicos.

Além disso, a produção de plantas transgênicas de tomateiro, nas quais a expressão de enzimas chaves tais como a GSNOR ou as hemoglobinas não simbióticas seriam manipuladas em tecidos ou momentos específicos do desenvolvimento poderiam também proporcionar dados relevantes no que concerne ao papel dessas rotas específicas de degradação de NO durante a sinalização de respostas vegetais induzidas pela luz. De forma similar, manipulações genéticas com vistas à alteração na disponibilidade de resíduos tióis, tais como mudanças na disponibilidade de glutatona, poderiam também ser bastante informativas desde que realizadas de maneira seletiva, seja espacial ou temporalmente.

Outro aspecto que ainda merece ser explorado em maior detalhe, consiste na interação do NO com outras espécies reativas de oxigênio e de nitrogênio. Uma caracterização detalhada da importância das ROS, por exemplo, sobre o metabolismo, disponibilidade e sinalização do NO poderiam contribuir para esclarecer se as possíveis interações entre o metabolismo de diferentes espécies reativas de oxigênio e nitrogênio teriam importância fisiológica durante a indução de respostas vegetais por sinais luminosos. Tendo em vista que a produção de ROS possui íntima relação com a produção e degradação de NO, manipulações farmacológicas ou genéticas na produção ROS seguidas de uma caracterização dos impactos sobre o metabolismo de NO também poderiam auxiliar na compreensão da complexa rede bioquímica em que o NO está envolvido durante eventos de sinalização em tecidos vegetativos e reprodutivos.

Ademais, o oferecimento de NO aos modelos vegetais em patamares e formas mais próximas àquelas encontradas em condições fisiológicas, como por exemplo, em sistemas com preciso controle de fluxo e concentração desse gás, permitiriam uma precisão muito mais adequada sobre a dosagem e continuidade do tratamento farmacológico quando comparado com os doadores químicos de NO comumente utilizados (*e.g.*, nitroprussiato de sódio). Além de estar livre de substâncias e efeitos indesejados produzidos por esses tratamentos clássicos, esse tipo de estratégia poria vir a eliminar o conflito que existe na literatura, com resultados de difícil reprodutibilidade, sobre o papel do NO na sinalização de respostas fisiológicas específicas. No caso dos impactos do NO sobre o amadurecimento dos frutos de tomateiro, por exemplo, o tratamento contínuo de frutos ligados ou destacados da planta-mãe com reduzidas concentrações de NO permitiria avaliar os efeitos farmacológicos desse radical livre sobre o metabolismo de etileno e outros hormônios

vegetais, sobre o progresso do amadurecimento e de seus processos associados (*e.g.*, carotenogênese, síntese de compostos voláteis), ou ainda, sobre a diferenciação de cloroplastos em cromoplastos.

Por fim, estudos sobre a integração entre as rotas de sinalização do NO e de fitormônios também parecem ser uma etapa lógica na elucidação dos mecanismos regulatórios responsáveis pela transdução do sinal luminoso em respostas fotomorfogênicas particulares, tais como o desestiolamento de plântulas ou o amadurecimento e a carotenogênese em frutos de tomateiro.

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