Rafael Zuccarelli

Manipulação dos níveis de óxido nítrico e seus impactos sobre a fisiologia e qualidade nutricional de frutos de tomateiro

Manipulation of nitric oxide levels and its impacts on tomato fruit physiology and nutritional quality

São Paulo

2022

Rafael Zuccarelli

Manipulação dos níveis de óxido nítrico e seus impactos sobre a fisiologia e qualidade nutricional de frutos de tomateiro

Manipulation of nitric oxide levels and its impacts on tomato fruit physiology and nutritional quality

Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Botânica.

Orientador: Luciano Freschi
Co-Orientadora: Maria Magdalena Rossi

São Paulo

2022

Ficha Catalográfica

Zuccarelli, Rafael
Manipulação dos níveis de óxido nítrico e seus impactos sobre a fisiologia e qualidade nutricional de frutos de tomateiro/ Rafael Zuccarelli ; orientador Luciano Freschi ; coorientadora Maria Magdalena Rossi -- São Paulo, 2022.
100 p.

Tese (Doutorado) -- Instituto de Biociências da Universidade de São Paulo. Programa de Pós-Graduação em Botânica.

1. Óxido Nítrico 2. Antioxidantes 3. Amadurecimento

Comissão Julgadora:

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Dedicatória

Dedico este trabalho
a todos que lutaram e sofreram
em nome da liberdade de pensamento

Epígrafe

"Todas as coisas complexas estão condenadas à decadência."

Siddhārtha Gautama

Agradecimentos

Ofereço meu sentimento de gratidão a todos que contribuíram para o progresso do meu doutorado e meu desenvolvimento pessoal durante o período. Esta emoção em geral não é apresentada na língua franca da ciência, em que é enunciada *thanks* ou *acknowledgments*, cujas origens remetem a pensar, entender, (*to thank – to think*, *acknowledgments - knowledge*).

Por meio dessa herança, reconheço e enalteço as agências de fomento a pesquisa de nosso país. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil – (CAPES) – Código de Financiamento 001. A Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) também contribuiu com minha bolsa de Doutorado, através do processo número 2016/02033-1. Agradeço também a Universidade de São Paulo por seu apoio financeiro e institucional que contribuiu direta ou indiretamente para a viabilização deste trabalho. Adiciono aqui meu sentimento, por isso agradeço também, a todas as pessoas que representam essas instituições, que graças ao seu trabalho, presenteiam nosso país com o progresso da ciência.

Agradeço também a todos os companheiros de trabalho, alunos de todos os níveis, egressos, funcionários da universidade, contratados terceirizados e prestadores de serviços, que ultrapassaram por vezes muito suas atribuições oficiais e deveres, e que ofereceram sempre ajuda, seja de ordem objetiva, ou naquilo que é intangível. O afeto permanecerá, tal qual o som de um instrumento de corda, reverberando por um tempo duradouro, para além da ação que lhe deu causa.

Agradeço especialmente ao meu orientador e co-orientadora, Luciano Freschi e Maria Magdalena Rossi, por seu ensino inspirador, sempre demonstrada por sua dedicação tenaz ao trabalho e acolhimento generoso. O empenho dos meus orientadores também evoca o nível mais profundo da gratidão, aquilo que dialoga com o senso de dever e nos compele a oferecer alguma retribuição que nos torne digno de tal benefício e oportunidade. Por isso, tão belamente expresso em nosso idioma, ofereço também meu muito obrigado a ambos e é minha esperança que esse trabalho de algum modo seja parte dessa retribuição.

Agradeço também aos meus amigos mais íntimos Pedro Rodriguez e Natália Galli, que residem hoje no além-mar, em Portugal. Mesmo a distância, mantiveram sua vigília constante, como um faroleiro que cuida da luz que guia as embarcações. Suas amizades mantiveram vivos meu senso de família, lazer, e tudo aquilo que nos faz sentir amado e respeitado.

Por fim agradeço a minha companheira Dora Takyia Bonadio, por dividir comigo um lar e o seu otimismo. Agradeço-lhe, seu olhar agudo que encontra o belo, revela o valor e a perfeição. Por sua caminhada paralela à minha, em sua carreira na pesquisa. Agradeço-lhe sua companhia amorosa, que manifesta permanentemente a vida que vale a pena ser vivida. Agradeço-lhe sua lucidez e razão, todas as razões.

Índice

Resumo 8

Abstract 9

Introdução Geral 10

Capítulo Único. Multifaceted roles of nitric oxide in tomato fruit ripening: NO-induced metabolic rewiring and consequences for fruit quality traits 44

Abstract 44

1.1. Introduction 45

1.2. Materials and Methods 48

1.3. Results 54

1.4. Discussion 69

1.5 Conclusions 76

1.6 References 78

Discussão Geral e Conclusões 90

Anexos e Apêndices 93

Resumo

O amadurecimento de frutos carnosos é um fenômeno controlado por uma complexa rede de hormônios e moléculas sinalizadoras, e o óxido nítrico (NO) tem sido apontado como um importante mediador desse processo. No entanto, ainda são pouco conhecidos os mecanismos moleculares associados à ação do NO nos frutos, bem como suas consequências nos eventos responsáveis por dar prosseguimento as mudanças de cor, sabor aroma e valor nutricional que ocorrem durante o amadurecimento. Por isso, neste trabalho, conduzimos uma detalhada caracterização do transcriptoma e metaboloma em frutos de tomateiro (*Solanum lycopersicum*), tratados com concentrações fisiologicamente relevantes de NO. Esse tratamento foi efetuado por meio do oferecimento de uma atmosfera enriquecida com NO, de fluxo contínuo e em normóxia, no estágio pré-climatérico de frutos destacados. Após o tratamento, os frutos exibiram um fenótipo de maturação atrasada, com a preservação da coloração esverdeada da região pedicelar em estágios mais avançados do amadurecimento. Essas mudanças ocorreram sem causar injúrias ou danos a aparência dos frutos tratados. Aproximadamente um terço dos transcritos detectados durante o amadurecimento sofreu alteração em sua abundância em função do tratamento com NO, com relevante repressão de diversos genes reguladores do amadurecimento, o que acarretou na diminuição da produção e sensibilidade dos frutos ao etileno. Ensaios *in vitro* da atividade de enzimas-chaves do metabolismo antioxidante e análises dos níveis de peróxido de hidrogênio revelaram uma alteração significativa no metabolismo redox dos frutos tratados com NO. Esse desbalanço no estado redox dos frutos resultou, por sua vez, na intensificação do estresse nitro-oxidativo, o qual pôde ser verificado pelo aumento dos eventos de *S*-nitrosação e nitração de proteínas detectadas ao longo do amadurecimento. Foi observado também, interessantemente, o aumento da atividade da *S*-nitrosoglutationa redutase (GSNOR), enzima responsável por remover os estoques endógenos de *S*-nitrosoglutationa (GSNO), um dos principais metabólitos derivados do NO. Sofreram impacto, em simultâneo, a biossíntese de flavonoides e ascorbato, com incrementos de 60% e 25% nas suas concentrações, respectivamente. Esse incremento foi acompanhado, consistentemente, pelo aumento da abundância de transcritos de genes relacionados com a biossíntese dessas duas classes de compostos. Os carotenoides, por outro lado, apresentaram nos frutos tratados com NO apenas 70% da concentração encontrada no grupo controle, com a concomitante supressão dos transcritos de diversos genes relacionados com a biossíntese de licopeno, o principal carotenoide responsável pela cor vermelha do tomate. Por fim, os demais compostos relacionados com o sabor e aroma, tais como açucares, ácidos orgânicos, aminoácidos e compostos voláteis, sofreram apenas uma discreta alteração dos seus conteúdos. Em conjunto, nossos resultados indicam que o tratamento de NO, nos estágios iniciais do amadurecimento, foi capaz de promover o aumento da durabilidade do fruto, com alterações na composição antioxidante, e poucos impactos em atributos relacionados ao sabor e aroma do fruto.

Abstract

The ripening of fleshy fruits is a phenomenon controlled by a complex network of hormones and signaling molecules, and nitric oxide (NO) has been recognized as an important mediator of this process. However, the molecular mechanisms behind NO action in fruits and their consequences on the events leading to ripening-associated changes in fruit color, flavor, aroma and nutritional value remain poorly elucidated. Therefore, in this work, we conducted a detailed characterization of the transcriptome and metabolome in NO-treated tomato (*Solanum lycopersicum*) fruits under physiologically relevant concentrations. The treatment was performed in a NO-enriched atmosphere under continuous flow and normoxia during the pre-climacteric stage of detached fruits. The NO-treated fruits exhibited a late-ripening phenotype, with the preservation of the greenish coloration of the pedicellar region at advanced stages of ripening. These changes occurred without causing injuries or damage to the external appearance of the fruits. Approximately one-third of the transcripts detected during ripening displayed alterations in their abundance in response to NO, with relevant repression of several genes encoding ripening master regulators. This resulted in repression in both the fruit production and sensitivity to ethylene. *In vitro* activity assays of key antioxidant enzymes and quantification of hydrogen peroxide content revealed significant changes in the redox metabolism of NO-treated fruits. Such imbalance in fruit redox status resulted, in turn, in nitro-oxidative stress, as evidenced by the higher frequency of *S*-nitrosation and nitration events in proteins. Interestingly, increased *S*-nitrosoglutathione reductase (GSNOR) activity was also observed, which is the enzyme responsible for removing *S*-nitrosoglutathione (GSNO), one of the main metabolites derived from NO. Furthermore, flavonoids and ascorbate accumulation in NO-treated fruits presented an increase of 60% and 25%, respectively. Consistently, the abundance of gene transcripts related to flavonoid and ascorbate biosynthesis was also up-regulated. On the other hand, the carotenoid content in NO-treated fruits was only 70% of the concentration found in the untreated control group. In agreement, NO treatment repressed the transcript abundance of several genes related to lycopene biosynthesis, which is the main carotenoid responsible for giving the red color of tomato fruits. Finally, other compounds responsible for flavor and aroma, such as sugars, organic acids, amino acids and volatile compounds, were only marginally affected by the NO treatment. Together, our results indicated that the NO treatment at the early stages of ripening can prolong fruit shelflife, with significant impacts on the antioxidant composition and minimal changes in attributes associated with fruit flavor and aroma.

Introdução Geral

Amadurecimento de frutos carnosos

Os frutos são estruturas reprodutivas presentes nas angiospermas, os quais contribuem para a dispersão de sementes em diversos contextos ecológicos (Tiffney, 1984; Tiffney and Mazer, 1995). Durante a evolução das angiospermas os frutos carnosos apareceram, de forma convergente, em vários grupos filogenéticos e estão relacionados principalmente com a dispersão através de animais, também conhecida como dispersão zoocórica. Em virtude desta relação ecológica, os frutos carnosos apresentam importantes modificações na coloração, aroma e sabor, alterações de textura, além do acúmulo de substâncias nutritivas que os tornam proveitosos aos animais dispersores de sementes (Bolmgren and Eriksson, 2010; Eriksson, 2016). Essas mudanças fisiológicas são reguladas por diversos fitormônios e outros sinalizadores, de modo que os frutos geralmente manifestam sua atratividade aos animais dispersores quando as sementes já se encontram completamente desenvolvidas.

Além de sua importância ecológica, os frutos constituem uma componente fundamental na nutrição humana equilibrada. Por esse motivo, respondem por grande parte da atividade agrícola (Pomerleau *et al.*, 2004), e desde a antiguidade a humanidade empenhou esforços na domesticação e melhoramento genético de culturas produtoras de frutos, proporcionando o aumento na produtividade, refinamento de sabor e aparência. No entanto, o aumento da durabilidade e o controle do amadurecimento permanecem, ainda, um importante desafio. Parte dessa

dificuldade decorre da íntima relação bioquímica, molecular e evolutiva existente entre os processos relacionados a senescência e deterioração, com aqueles responsáveis pelo acúmulo de nutrientes e características organolépticas, especialmente durante os estágios finais do amadurecimento.

Etileno e amadurecimento de frutos carnosos

Dentre os hormônios vegetais, o etileno (ET) ocupa lugar central como agente indutor e mantenedor do processo de amadurecimento. Essa relação foi observada em trabalhos seminais do início do século passado que utilizaram a aplicação exógena de ET (ou propileno, um análogo) com o objetivo de induzir ou acelerar o amadurecimento de frutos (Vinson, 1910; Newton and Cook, 1927; Wolfe, 1931). Posteriormente, a correlação entre a produção endógena de ET e a indução do amadurecimento foi demonstrada pela medição da emissão desse hormônio gasoso, após o advento da cromatografia em fase gasosa (Burg and Burg, 1962). Essa correlação, por sua vez, ganhou robustez com trabalhos em frutos de plantas transgênicas de tomateiro com níveis diminuídos da produção endógena de ET (Oeller *et al.*, 1991; Picton *et al.*, 1993), bem como pela descoberta e aplicação de substâncias com efeito repressor sobre a síntese ou percepção de ET como, por exemplo, o 1-metilciclopropeno (1-MCP) (Golding *et al.*, 1998; Watkins, 2006) (Golding *et al.*, 1998; Watkins, 2006).

Essas descobertas resultaram em aplicações tecnológicas, nas quais ambas estratégias, estímulo ou repressão do ET, são amplamente utilizadas em produtos agrícolas hoje disponíveis no mercado, tanto com objetivo de obter melhor controle do amadurecimento durante o transporte e armazenamento, bem como o aumento do tempo de prateleira nos centros de venda (Saltveit, 1999; Khan and Singh, 2009; Sdiri

et al., 2012; Golding and Singh, 2017; Ma *et al.*, 2017; Saraiva *et al.*, 2018; Cai *et al.*, 2018; Mendes *et al.*, 2018).

Tradicionalmente, os frutos carnosos são classificados em dois tipos principais quanto ao padrão de resposta ao ET, a saber: climatéricos e não-climatéricos. No caso dos primeiros, como, por exemplo, o tomate (*Solanum lycopersicum*), o principal modelo experimental para frutos carnosos, o início do amadurecimento é acompanhado por um súbito e volumoso aumento da emissão de ET, com o concomitante aumento das taxas respiratórias. Esses eventos coincidem com o momento em que o fruto alcança seu máximo desenvolvimento em tamanho, e a partir deste estágio, diversas mudanças fisiológicas o tornam mais sensível ao ET, e consequentemente, muito mais responsivo à sua aplicação exógena (Cherian *et al.*, 2014). Neste caso, uma vez iniciado o disparo da produção elevada de ET, o amadurecimento é inexoravelmente conduzido a termo. Esse processo ocorre mesmo quando o fruto é destacado da planta mãe, contanto que o máximo desenvolvimento pré-maturação do fruto tenha sido atingido (Fukano and Tachiki, 2021).

Já nos frutos não-climatéricos, como por exemplo o pimentão (*Capiscum annuum*), outro importante modelo para frutos carnosos, as taxas respiratórias e emissão de ET permanecem em níveis baixos, com pouca variação ao longo das diferentes fases de amadurecimento. A aplicação exógena de ET, no caso destes, apesar de promover ou acelerar certos aspectos da maturação, resulta em pouco ou nenhum incremento em sua produção endógena (Goldschmidt, 1998; Fuentes *et al.*, 2019). Além disso, na maioria dos frutos não-climatéricos, o amadurecimento só ocorre até sua plenitude se o fruto permanecer conectado à planta mãe (Fukano and Tachiki, 2021).

A despeito dessa classificação tradicional, uma enorme variação biológica é encontrada em relação padrão de emissão de ET, das taxas respiratórias, e da resposta à sua aplicação exógena em diferentes espécies e cultivares de frutos (Paul *et al.*, 2012). Essa variação biológica é reveladora da notável evolução convergente que ocorreu, ao menos três vezes independentes, em angiospermas. As evidências genômicas e moleculares que estão hoje disponíveis para diversas espécies apontam para reorganização de diferentes circuitos genéticos relacionados a senescência, identidade de órgãos florais e regulação epigenética no surgimento dos frutos carnosos (Lü *et al.*, 2018). Além disso, o comportamento apresentado por frutos climatéricos e não climatéricos conduzem à hipótese de diferentes estratégias adaptativas voltadas a animais dispersores específicos, e são observados casos de difícil classificação, como, por exemplo, frutos que apresentam fenótipo de climatério menos pronunciado (*i.e.* aumento das taxas de emissão de ET menos conspícua) (Fukano and Tachiki, 2021). Ademais, importantes alterações metabólicas possivelmente ocorreram em muitas cultivares durante o processo de domesticação (Lü *et al.*, 2018). Por isso, nos estudos que buscam elucidar os fenômenos nos quais o ET e outros sinalizadores interatuam em frutos, como, por exemplo, o óxido nítrico (NO), os resultados obtidos num dado fruto poderão não ser comparáveis a outro, e a relação de homologia dos processos deverá ser sempre destacada.

É válido salientar, no entanto, que a via de produção de ET está inequivocamente mapeada em angiospermas (Yang and Hoffman, 1984; Kende, 1993; Wang *et al.*, 2002; Argueso *et al.*, 2007). Sua biossíntese tem início com a metionina e dentro das células vegetais cerca de 80% da produção deste aminoácido é convertida em S-adenosilmetionina (SAM) pela ação da S-adenosilmetionina transferase (MAT) (Ravanel *et al.*, 1998). Na segunda etapa bioquímica, o SAM é

convertido em ácido 1-aminociclopropano 1-carboxílico (ACC) pela enzima ACC sintase (ACS). Por fim, a última etapa corresponde a oxidação da ACC pela ACC oxidase (ACO) produzindo ET, CO_2 e cianeto.

A percepção do ET pelas células vegetais, por sua vez, envolve a participação de receptores que estão presentes nas membranas do retículo endoplasmático e estes se dividem em duas subfamílias: (i) ETR1 (*Ethylene response 1*) e ETRS1 (*Ethylene Response Sensor 1*), (ii) ETR2 (*Ethylene response 2*), ERS2 (*Ethylene response sensor 2*) e EIN4 (*Ethylene insensitive 4*) (Liu *et al.*, 2015). Esses receptores são reguladores negativos de resposta, sendo que na ausência de ET mantêm ativa uma proteína quinase, a CTR1 (*Constitutive Triple-Response 1*), responsável por fosforilar o domínio C-terminal da proteína EIN2 (*Ethylene insensitive 2*). Essa fosforilação induz a ubiquitinação da EIN2 pelo complexo E3 ubiquitina ligase, contendo as proteínas ETP1 e ETP2 (EIN2-Targueting protein 1 e 2, respectivamente) marcando-a para degradação via proteossoma 26S. Esses eventos removem a EIN2, mantendo-a em níveis baixos e a ação a jusante (*downstream*) desta é mantida reprimida.

Na presença de ET, por outro lado, os receptores inibem a ação da CTR1. Neste caso a EIN2 é mantida na forma desfosforilada, permanece em níveis maiores e ocorre sua migração ao núcleo. Em seguida, a EIN2 inibe a ubiquitinação de outros TF presentes no núcleo, tais como o EIN3 (Ethylene Insensitive 3), EIL1 (EIN3-Like1) e EIL (EIN3-Like2), pela proteína EBS (EIN3-binding F-box). Uma vez suprimida a ação da EBS, o EIN3, o EL2 e o EIL3 deixam de ser marcados para degradação e seus níveis aumentam como consequência. Por fim, os EIL1, EIL2 e EIN3 induzem a expressão de um outro grande grupo de TF, os ERFs (*Ethylene Response Factors*), associados ao controle de uma vasta gama de genes e respostas mediadas pelo ET (Binder, 2020) (Binder, 2020).

Papel de outros fitormônios e sinalizadores

Apesar de o ET ser o sinal dominante, existem evidências da participação de outros fitormônios e moléculas sinalizadoras, coexistindo e coordenando o amadurecimento (Barry and Giovannoni, 2007). Um dos aspectos fundamentais dessa interação é a substituição da alça de regulação negativa (sistema 1), tipicamente presente nos tecidos vegetativos e que controla os níveis de ET durante os estágios iniciais do desenvolvimento do fruto, por uma alça de regulação positiva de biossíntese de ET (sistema 2), responsável pelo brusco incremento de sua produção, típico do climatério. Em tomateiro já foram identificados os genes que codificam para diferentes isoformas das enzimas especificamente necessárias para o sistema 1 (*ACS1A, ACS3,ACS6, ACO1* e *ACO4*) e sistema 2 (*ACS2*, *ACS4*, *ACO1*, e *ACO4*) (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000) de produção de ET. No entanto, ainda é recente e incerto o papel específico de outros fitormônios, TFs e outros sinalizadores durante essa transição.

Por exemplo, é sabido que a produção de ácido abscísico (ABA), fitormônio fundamental para o desenvolvimento e dormência de sementes, apresenta um incremento que precede a produção de etileno climatérico. A progressão desse aumento tem início nas sementes, quando estas atingem seu máximo desenvolvimento e depois progride nos demais tecidos do fruto de tomate e pêssego (*Prunus persica*). Nessas mesmas espécies foi demonstrada a capacidade do ABA induzir a expressão de genes relacionados com a produção de ET, e também diversos fatores de transcrição relacionados com o controle do amadurecimento. Além disso, tratamentos que interferem com o aumento do ABA promovem o atraso no amadurecimento (Zhang *et al.*, 2009; Sun *et al.*, 2012; Ji *et al.*, 2014; Mou *et al.*, 2016).

As giberelinas (GA), por sua vez, apresentam uma relação inversa com as concentrações de ET em tomate, exibindo um marcado declínio que antecede o início do climatério. A queda das concentrações desse hormônio é acompanhada pelo aumento da expressão de genes de enzimas responsáveis pela inativação de GA, e a diminuição daqueles relacionados com sua biossíntese (Li *et al.*, 2019). Sua aplicação exógena também demonstra ser capaz de reprimir a expressão de genes das vias de biossíntese de ET, e de fatores de transcrição relacionados ao amadurecimento, o que acarreta em seu atraso no amadurecimento. De forma consistente, a indução e aceleramento do amadurecimento é encontrado em experimentos utilizando inibidores da ação das GA, em frutos portadores de deficiência na produção de GA, ou ainda em plantas transgênicas com sobre-expressão de genes de inativação de GA (Li *et al.*, 2019).

Alguns avanços já foram obtidos também no caso das auxinas, cujos níveis endógenos sofrem um incremento que acompanha o aumento (e subsequente queda) dos níveis de ET durante o climatério em tomate e pêssego (Trainotti *et al.*, 2007). Neste caso, a subsequente diminuição dos níveis endógenos de auxinas é necessária para o prosseguimento do amadurecimento (Jones *et al.*, 2002; Trainotti *et al.*, 2007; Kumar *et al.*, 2012; Devoghalaere *et al.*, 2012). Essa relação é atestada pelo tratamento de frutos de tomate com auxinas, que resulta no atraso na transição do sistema 1 para sistema 2, com a supressão da expressão de diversos genes relacionados com o metabolismo de carotenoides, degradação da parede celular e metabolismo energético (Li *et al.*, 2016).

Além disso, estudos tambem indicam que alguns ARFs (A*uxin response factors*) responsáveis por coordenar a ação das auxinas sobre a expressão de diversos genes, também atuam no amadurecimento de frutos em tomateiro.

O ARF4, por exemplo, apresenta uma marcada diminuição da sua expressão durante o amadurecimento de frutos de tomateiro e em linhagens transgênicas com silenciamento de *ARF4* apresentam frutos com alterações significativas em atributos de qualidade (Sagar *et al.*, 2013). Por outro lado, o ARF2, sobre-expressão de ARF2 em tomateiro, resulta na formação de zonas irregulares de amadurecimento acelerado, com emissão precoce de ET e indução de componentes da cascata de sinalização de ET e outros reguladores do amadurecimento. De forma condizente, o silenciamento de *ARF2* gera frutos com atraso no amadurecimento, partenocarpia e perfil hormonal alterado (Breitel *et al.*, 2016).

Diversos outros Tfs atuam como reguladores mestres no processo de amadurecimento, muitas vezes com ação a montante aos fitormônios e outas moléculas sinalizadoras. Muitos desses Tfs foram identificados inicialmente a partir do isolamento de mutantes de tomateiro cujos frutos apresentavam bloqueio e/ou alterações no amadurecimento. Alguns exemplos de mutantes que merecem destaque nesse contexto são o *rin* (*ripening-inhibitor*)*, nor* (*non-ripening*) e Cnr (*Colorless non-ripening*). A identificação dos genes e proteínas afetados por tais mutações levou à descoberta de diversos outros TFs que atuam tanto a montante, como a jusante da ação do ET e outros fitormônios, com uma topologia e interação complexa nos frutos (Wang *et al.*, 2020).

Sabe-se, atualmente que o TF MADS-RIN, possuí como alvo um grande número de genes, incluindo *ACS2* e *ACS4*, o receptor de ET *NR* (*Never ripe*), os

genes responsivos ao ET *E4* e *E8*, além de muitos outros envolvidos no amadurecimento em tomateiro (Martel, 2010; Zhong *et al.*, 2013). A princípio, a demonstração da ação do MADS-RIN sobre esses genes sugere que este seja indispensável para o amadurecimento, em virtude do forte fenótipo de inibição exibido pelo mutante natural *rin.* No entanto, outros estudos utilizando o silenciamento ou a deleção deste gene indicam que, apesar de apresentar um papel importante sobre as emissões de ET, este não é fundamental para o prosseguimento do amadurecimento (Ito *et al.*, 2017; Wang *et al.*, 2019). Um cenário semelhante é observado para outros TF como o NAC-NOR, o qual atua a montante do MADS-RIN, e regula um número ainda maior de genes relacionados ao ET. Linhagens transgênicas com deleção para esse gene também apresentam, diferentemente do mutante natural *nor*, um fenótipo de inibição apenas parcial do amadurecimento (Wang *et al.*, 2019).

Ressalta-se, ainda, a existência de um grande número de outros TF que atuam conjuntamente, exibindo, muitas vezes interações proteína-proteína que resultam na formação de complexos que atuam na repressão ou promoção da expressão de diversos genes (Leseberg *et al.*, 2008; Bemer *et al.*, 2012; Shima *et al.*, 2013). Chama atenção que esses complexos proteicos tem como alvo, além de outros genes e fitormônios, a região promotora de outros TF também relacionados ao amadurecimento (Liu *et al.*, 2020).

O óxido nítrico como sinalizador em frutos

Além dos já citados TF e fitormônios, descobertas recentes indicam que as espécies reativas de oxigênio e nitrogênio (ROS e RNS, respectivamente), classicamente estudadas em fenômenos relacionados ao estresse, defesa e senescência, são peças

chaves na regulação do amadurecimento de frutos, em especial o óxido nítrico (NO) (Corpas *et al.*, 2018).

O NO é capaz de agir tanto de forma coordenada com o ET e outros fitormônios, bem como através de outros mecanismos independentes que atuam nos níveis bioquímico e molecular (Domingos *et al.*, 2015). Por se tratar de um gás, de natureza lipofílica, ter uma alta reatividade química e meia vida relativamente longa em baixas concentrações, o NO merece destaque como um sinalizador com características singulares e está presente de forma universal em seres vivos. Todavia, sua presença em certas circunstâncias pode acarretar danos aos componentes da maquinaria celular e, por esse motivo, estão presentes mecanismos altamente eficientes para sua degradação em sistemas biológicos. Além disso, o NO pode ser conjugado à um grande número de moléculas orgânicas presentes no ambiente celular, as quais podem atuar como reservatórios estáveis, ou agir como intermediários em eventos de sinalização, entre outras funções (Mur *et al.*, 2013).

Em plantas superiores, a produção de NO se dá por diferentes rotas e estas podem ser divididas em dois grupos principais: (i) vias redutivas, nas quais o NO é produzido a partir da redução do nitrito e (ii) vias oxidativas, onde a produção de NO é dependente de L-arginina como substrato. Dentre as vias redutivas, a mais importante e bem caracterizada é a participação da enzima nitrato redutase (NR), que além de sua atividade principal na redução do nitrato a nitrito, possui também é capaz de gerar NO. Esta atividade secundária ocorre no mesmo sítio ativo da primeira e é capaz de converter nitrito a NO com cerca de 1% da atividade exibida na redução do nitrato. No entanto, essa segunda atividade é inibida pelo próprio nitrato, exige uma alta concentração de nitrito, e é favorecida em anóxia (Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000; Rockel *et al.*, 2002).

Em trabalhos posteriores foi demonstrada a existência de outra proteína, a NOFNiR (N*itric oxide-forming nitrite reductase*) capaz de interagir com a NR e que permite a produção de NO a partir de nitrito. Neste caso, a reação ocorre em normóxia e não é inibida por nitrato (Yang *et al.*, 2015; Chamizo-Ampudia *et al.*, 2016, 2017). Outras vias redutivas são sugeridas, com determinação incerta acerca de seu papel na produção de NO em frutos como, por exemplo, a ação da cadeia de transporte de elétrons mitocondrial sob anóxia (Gupta and Igamberdiev, 2011; Salgado *et al.*, 2013), ou a produção de NO por uma enzima nitrito redutase ligada a membrana (Ni:NOR), capaz de produzir NO no espaço apoplástico, mas neste caso essa atividade é restrita a raízes (Stöhr and Stremlau, 2006).

Ainda mais incerta é a produção de NO a partir de L-arginina, pois ainda que já tenha sido demonstrada bioquimicamente em plantas, carece, até o presente momento, da descoberta de uma enzima, homóloga ou não à NO-sintase (NOS) presente em animais. A atividade detectada *in vitro* neste caso corresponde à produção de NO e L-citrulina, dependente de calmodulina, semelhante àquela produzida pela NOS de animais e por esse motivo, é chamada NOS-like. No entanto, sua importância, ou mesmo sua existência *in vivo* em plantas superiores é objeto de controvérsia (Santolini *et al.*, 2017; Astier *et al.*, 2018).

Espécies S-nitrosadas

Tão importante quanto as vias de produção, o controle da homeostase do NO depende de mecanismos eficientes para sua remoção ou imobilização, permitindo o controle espaço-temporal preciso da sua função sinalizadora. O mecanismo bioquímico que se revelou como o mais relevante e bem estudado é a interação do NO, de forma reversível, com resíduos tióis reduzidos (-SH) presentes em proteínas e peptídeos.

Essa ligação induz a formação de espécies S-nitrosadas (SNO) e esta modificação é responsável pela regulação da atividade e função de uma ampla gama de fenômenos biológicos em plantas, tais como morte celular programada, germinação de sementes, resposta ao estresse, fotossíntese e sinalização hormonal (Terrile *et al.*, 2012; Feng *et al.*, 2013; Hu *et al.*, 2015; Albertos *et al.*, 2015; Iglesias *et al.*, 2018; Huang *et al.*, 2019).

Nesse contexto, principal alvo do NO é a ligação com a glutationa (GSH), um importante tripetídeo, que possuí uma cisteína na sua posição central e portanto contém um resíduo tiol. Em sua forma reduzida (GSH) esta pode participar de processos antioxidantes, gerando sua forma oxidada, que se apresenta como um dímero, com os resíduos tióis ligados um ao outro (GSSG). Uma vez que a GSH está presente em grandes concentrações no citosol (escala milimolar), a presença de NO livre prontamente induz a formação da S-nitrosoglutationa (GSNO) (Airaki *et al.*, 2011; Corpas *et al.*, 2013*a*). Esta representa um reservatório reversível de NO e, desse modo, a GSH funciona como uma barreira, protegendo a célula de concentrações elevadas de NO livre produzidas sob certas condições. Além disso, a GSNO pode ser utilizada como substrato para a transferência de resíduos nitrosados, doando o grupo nitroso de um resíduo tiol a o outro (transnitrosação), tanto por via enzimática como não-enzimática. Por esse motivo, a GSNO é um dos metabólitos mais diretamente relacionado com a especificidade da função sinalizadora do NO, possuindo o potencial de induzir via transnitrosação, alterações na conformação, atividade, estabilidade e/ou localização celular de diversas proteínas (Astier *et al.*, 2011; Lamotte *et al.*, 2015; Feng *et al.*, 2019).

Essa função bioquímica do NO é facilitada pela participação do sistema tioredoxina-tioredoxina redutase, o qual é responsável, além de outras funções, por

mediar a transferência dos radicais NO de um resíduo tiol a outro, que tanto pode ser uma molécula de GSH (resultando na formação de GSNO) como outra proteína, efetivamente promovendo a desnitrosação de alvos específicos (Jedelská *et al.*, 2020). A atividade desse sistema ocorre mesmo em situações desfavoráveis, como, por exemplo, em circunstâncias nas quais o balanço químico da reação de nitrosação de um dado resíduo tiol esteja particularmente elevado, ou ainda em proteínas cujos resíduos tióis possuam valor de pKa e acessibilidade estérica que dificultam a remoção espontânea dos radicais NO previamente ligados a esses resíduos.

O controle dos conteúdos endógenos dos estoques de GSNO, por sua vez, é assegurado por meio da enzima altamente conservada S-nitrosoglutationa reductase (GSNOR), a qual é capaz de degradar a GSNO gerando GSSG e NH_3 como produtos (Liu *et al.*, 2001). Portanto, sua ação reduz o reservatório celular de espécies S-nitrosadas, diminuindo o potencial de liberação de NO livre no ambiente intracelular ou a transferência direta de SNO a outras moléculas. De modo interessante, a GSNOR também está sujeita à regulação por meio de S-nitrosação (Guerra *et al.*, 2016; Zhan *et al.*, 2018) e diversos trabalhos demonstram a importância da GSNOR e do sistema tioredoxina-tioredoxina redutase em diferentes fenômenos biológicos tais como desenvolvimento vegetal, respostas ao estresse (a)biótico, fertilidade, produção de sementes e sinalização de auxinas e ABA (Leterrier *et al.*, 2011; Lindermayr, 2018; Jahnová *et al.*, 2019; Hussain *et al.*, 2019).

Em frutos, já foi demonstrado que os conteúdos endógenos de espécies S-nitrosadas apresentam uma correlação com o início e progressão dos amadurecimento. Em pimentão, por exemplo, foi observado o acúmulo de espécies S-nitrosadas e a diminuição dos conteúdos de NO nos estágios finais do

amadurecimento e esse aumento é acompanhado pela diminuição da atividade da GSNOR (Corpas *et al.*, 2018).

Nitração de aminoácidos e outros compostos

Outro mecanismo bem estabelecido pelo qual o NO atua ocorre através da modificação pós-traducional irreversível de aminoácidos presentes em proteínas, a qual, e semelhante a S-nitrosação, exerce papel regulatório na função de diversas enzimas, Tfs e outras proteínas. Já foram publicados muitos trabalhos demonstrando à sua dessas reações irreversíveis em processos como germinação de sementes, desenvolvimento e amadurecimento de frutos, bem como mecanismos de resposta a estresses (a)biótico (Cellini *et al.*, 2011; Chaki *et al.*, 2015; Airaki *et al.*, 2015; Signorelli and Considine, 2018).

Dentre os resíduos presentes em proteínas, a tirosina (Tyr) e o triptofano (Trp) são observados como os mais relevantes alvos para a adição de um grupo nitro ($-NO_2$) (Corpas *et al.*, 2013*b*, 2021) Essa reação ocorre por meio da ação do peroxinitrito ($ONOOH/ONOO^-$), formado a partir do NO e do superóxido ($O_2^{\bullet-}$) (Ferrer-Sueta *et al.*, 2018) ou pela ação do dióxido de nitrogênio ($\bullet NO_2$), que pode ser produzido a partir de nitrito (NO_2^-) e peróxido de hidrogênio (H_2O_2), na presença de hemoperoxidase (HPO) (Souza *et al.*, 2008). Esses compostos podem dar origem a diferentes derivados, e são citados o 3-nitroso-tirosina, o 4-nitro-triptofano e o 6-nitro-triptofano como os mais importantes, havendo correlação entre os níveis endógenos de proteinas nitradas e o prosseguimento do amadurecimento em pimentão (Chaki *et al.*, 2015; Corpas *et al.*, 2021).

É sugerido ainda que além dos aminoácidos, outros compostos também possam ser modificados pelo NO (e seus derivados) como lipídios e oligonucleotídios

(livres ou em ácidos nucleicos) (Desel *et al.*, 2007; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2019). No caso dos lipídios, a insaturação presente em algumas cadeias de ácidos graxos pode ser modificada e receber um grupo nitro (-NO_2) pela ação do NO_2 ou do peroxinitrito, levando a formação de ácidos nitro-graxos (NO_2-FAs). A função sinalizadora dos NO_2-FAs se dá predominantemente por meio da modificação pós-traducional de proteías, uma vez que sua porção nitroalqueno os torna suscetíveis ao ataque nucleofílico de cisteínas, histidinas ou lisinas. Desse modo, ocorre a conjugação da proteína com uma molécula de ácido graxo de forma reversível. Essa ligação se dá no carbono β do adjacente aquele ligado ao resíduo -NO_2 e a adição do NO_2-FA pode atuar na regulação da atividade ou função destas proteínas (Mata-Pérez *et al.*, 2018). Alternativamente, os NO_2-FAs podem reagir com a cisteína da GSH e formar adutos GSH-NO_2-Fas, também envolvidos na regulação pós traducional de proteínas (Baker *et al.*, 2007).

A função dos NO_2-FAs já foi demonstrada em plantas em respostas a estresse abiótico, formação de raízes em plântulas e fechamento estomático (Mata-Pérez, 2016; Palma, 2020). Além disso, já foram identificadas diversas proteínas contendo alvos para essa modificação em plantas e animais, e tem se mostrado relevante o interesse nesses compostos, pois além de sua função sinalizadora em plantas, a presença destes em alimentos, como, por exemplo, no caso do azeite de oliva extra-virgem contribui na prevenção de doenças cardiovasculares e apresenta quantidades significativas de ácido linolênico conjugado a NO_2 (NO_2-LA) (Sánchez-Calvo *et al.*, 2013; Mata-Pérez *et al.*, 2017).

Observa-se ainda que os NO_2-FAs podem atuar como um reservatório de NO, e devido ao fato dos fosfolipídios presentes nas membranas possuírem distribuição, organização e concentração variada de cadeias saturadas/insaturadas nos diferentes

compartimentos e regiões celulares, a liberação NO a partir dos NO_2-FAs podem ocorrer a partir dos lipídios presentes nas membranas, em organelas ou zonas subcelulares específicas, concentrando e contendo sua ação (Mata-Pérez, 2016; Su *et al.*, 2019).

Outra modificação promovida pelo NO é a nitração de nucleotídeos, como a guanidina, presente tanto em ácidos nucléicos como no sinalizador monofosfato de guanosina cíclica (cGMP). Esta molécula é amplamente conhecida por sua função sinalizadora em animais, e vem ganhando destaque em plantas (Isner and Maathuis, 2018). A modificação deste nucleotídeo ocorre pela ação do peroxinitrito, levando à formação de 8-nitroguanosina 3', 5' monofosfato cíclico (8-nitro-cGMP). O conhecimento de sua função em plantas ainda é limitado, ainda que já tenha sido implicada no fechamento estomático e tenha sido sugerido que possa participar em outras respostas relacionadas ao estresse (Joudoi *et al.*, 2013; Petřivalský, 2020). Além disso, a nitração de guaninas em moléculas de RNA já foi descrita na regulação de morte celular programada (PCD) em resposta ao ataque de patógenos e quebra de dormência de sementes (Izbiańska *et al.*, 2018; Andryka-Dudek *et al.*, 2019).

Metabolismo antioxidante em frutos

O processo de amadurecimento de frutos carnosos é considerado homólogo, e funcionalmente semelhante à senescência e morte celular programada, visto que parte significativa das vias, processos bioquímicos e genes envolvidos parecem ter sofrido neofuncionalização durante a evolução das angiospermas (Lü *et al.*, 2018). De forma semelhante ao observado durante a senescência de tecidos vegetativos, o amadurecimento de frutos é também marcado por um pronunciado aumento da concentração endógena de ROS. Tais compostos são tóxicos em níveis elevados,

contudo, geralmente estão presentes em concentrações baixas e controladas como parte do metabolismo normal. De modo interessante, esses mesmos compostos possuem também diversas funções sinalizadoras (Considine and Foyer, 2014).

Isso é ilustrado, por exemplo, nos eventos associados ao estresse nos quais pertubações físico-químicas são impostas aos sistemas bioquímicos. Disso decorre a consequência termodinâmica inevitável do favorecimento de reações secundárias e indesejadas que promovem o aumento da produção de ROS e a inibição das reações que contribuem com a sobrevivência da célula (Garg and Manchanda, 2009). Por esse motivo, o aumento da concentração de ROS representa um sinal informativo e coerente das condições deletérias que a célula está submetida. Além disso, esse tipo de sinal tem o potencial de ser propagado célula a célula rapidamente por longas distâncias e ativar respostas de defesa em regiões distantes da planta, por meio do aumento da produção endógena de ROS em cada célula individualmente ao longo do trajeto, podendo alcançar velocidades de até 8,4 cm/min em algumas situações (Mittler *et al.*, 2011).

Nos eventos relacionados ao desenvolvimento ou reprodução, por outro lado, uma série de eventos endógenos e sinais externos são concatenados e estimulam o aumento da concentração de ROS, com ação local, temporalmente específica, regulada e não destrutiva (Mittler *et al.*, 2011). Ainda assim, em muitos casos, isso pode também induzir programas de desenvolvimento que levam *posteriormente* à perda da integridade das estruturas e morte celular. Um exemplo desse fenômeno pode ser observado durante a diferenciação de vasos xilemáticos, em que as células do parênquima que estão adjacentes àquelas que estão se diferenciando em vasos xilemáticos são fonte do H_2O_2 necessário para biossíntese e polimerização da lignina, sendo esse evento acompanhado pela concomitante produção local de NO (Gabaldón

et al., 2004; Ros Barceló, 2005). De modo comparável, as transformações que ocorrem no amadurecimento causam posteriormente a perda da homeostase e morte celular, como, por exemplo, na desmontagem dos polissacarídeos das paredes celulares, resultando no amolecimento tipicamente observado em muitos frutos maduros. Esse processo conta, além de diversas enzimas, com processos bioquímicos não enzimáticos promovidos por ROS (Cheng *et al.*, 2008; Duan *et al.*, 2011).

As ROS são um vasto grupo de moléculas que podem se dividir, de modo aproximado, em ROS livres, moléculas de tamanho pequeno, constituídas apenas por átomos de oxigênio e hidrogênio, e ROS incorporados, moléculas derivadas dos primeiros pela ligação destes com outros compostos. Em sistemas biológicos dois dos ROS livres que mais se destacam são o oxigênio singleto ($^1O_2$) e o radical ânion de superóxido ($O2^{\bullet -}$). A partir desses primeiros, outros ROS livres podem ser produzidos, como o peróxido de hidrogênio (H_2O_2), o radical hidroperoxila ($HO_2^{\bullet}$) e o radical hidroxila ($HO^{\bullet}$) (Khorobrykh *et al.*, 2020).

Dentre as espécies reativas derivadas que merecem destaque são as RNS, tais como o já citado $NO^{\bullet}$, o dióxido de nitrogênio ($NO^{\bullet}_2$), o peroxinitrito ($ONOO^-$), o trióxido de dinitrogênio (N_2O_3), o tetróxido de dinitrogênio (N_2O_4) e o nitroperoxicarbonato ($ONOOCO_2^-$); e diversos radicais orgânicos tais como radical peroxil de lipídios, ($LOO^{\bullet}$), radical peroxil ($ROO^{\bullet}$), peróxidos orgânicos (ROOH), radical alcoxila ($RO^{\bullet}$), radical peroxinitrito de alquila (ROONO) (Decros *et al.*, 2019).

As três principais fontes de ROS em plantas são a atividade fotossintética no cloroplasto, a respiração mitocondrial e o ciclo de fotorrespiração peroxissômica. No caso dos tecidos fotossinteticamente ativos, o ânion superóxido é o principal

subproduto da reação do oxigênio com componentes reduzidos da cadeia de transporte de elétrons fotossintética, principalmente a ferrodoxina. O superóxido pode exercer diretamente funções sinalizadoras ou ser quimicamente reduzido em H_2O_2. Essa reação é acelerada pela ação da enzima superóxido dismutase (SOD) e é responsável por manter o superóxido em níveis controlados (Bhattacharjee, 2019). O H_2O_2 gerado por esse e outros processos é então removido pela ação da enzima catalase (CAT) e adicionalmente, pela ação do ciclo ascorbato-glutationa.

O ciclo ascorbato-glutationa opera no citosol, mitocôndria, plastídios e peroxissomos. Este tem início com a redução do H_2O_2 pela ascorbato peroxidase (APX), que consome ascorbato como doador de elétrons. Essa reação produz H_2O e monodehidroascorbato, que pode ser espontaneamente desprotonado em dehidroascorbato. O ascorbato é regenerado então pela ação das enzimas dehidroascorbato redutase (DHAR) e monodehidroascorbato redutase (MDHAR) as custas de GSH, produzindo GSSG. Finalmente, a GSH é regenerada a partir da GSSG através da glutationa redutase (GR) usando NADPH como doador de elétrons. Desse modo, o conteúdo tanto de ascorbato quanto de GSH, é mantido estável, e os elétrons são efetivamente transferidos do NADPH para o H_2O_2 (Pang and Wang, 2010).

De modo interessante, nos tecidos imaturos de muitos frutos, como no caso do tomate, estão presentes cloroplastos fotossinteticamente ativos. No entanto, as evidências presentes na literatura sugerem que essa atividade possui pouca contribuição para assimilação de carbono, ainda que seja importante para o desenvolvimento das sementes e acúmulo do conteúdo total de ascorbato (Lytovchenko *et al.*, 2011; Ntagkas *et al.*, 2019). Esses cloroplastos sofrem uma transição fisiológica, convertendo-se em cromoplastos, organela responsável por acumular pigmentos que conferem a cor característica do fruto maduro. O início

dessa conversão em cromoplastos coincide com o amadurecimento e os sistemas de defesa antioxidante como a atividade da SOD, e do sistema do ciclo ascorbato-glutationa apresentam um incremento, especificamente nos plastídios, o que sugere uma elevação nos processos geradores de ROS (Martí *et al.*, 2009).

Outro processo que é potencial fonte de ROS e que sofre um importante aumento no fruto é a respiração mitocondrial, responsável pelo expressivo aumento das emissões de CO_2 que precedem e intensificam-se durante o climatério. Nas mitocôndrias de plantas está presente um sistema alternativo de oxidases terminais, resistente ao cianeto e antimicina, capaz de manter o transporte de elétrons provenientes da ubiquitina, e contorna os complexos III e IV (responsáveis pelo bombeamento de prótons). Isso garante que o estado reduzido dos componentes da cadeia de transporte de elétrons mitocondrial seja mantido sob controle e prevenindo a produção excessiva de ROS. Essa atividade oxidase alternativa (AOX) permite também que a glicólise e o ciclo do ácido cítrico mantenham seu funcionamento, com a regeneração dos estoques de NADH e NADPH, e produção de ATP. No entanto, como o bombeamento de prótons é contornado na mitocôndria, a produção de ATP por essa via fica comprometida (Vanlerberghe, 2013). Além de estar associados a eventos de estresse e funcionar como uma via secundária e protetiva, a ativação da via AOX é implicada também no início e progressão do amadurecimento, com relevante papel na transição entre o sistema 1 e 2 de ET (Arnholdt-Schmitt *et al.*, 2006; Saha *et al.*, 2016; Hewitt and Dhingra, 2019). Isso é consistente, por exemplo, com o fenótipo de plantas transgênicas de tomateiro com o sistema AOX reduzido, as quais produzem frutos com a supressão do pico climatérico de respiração e emissão de ET, redução do acúmulo de carotenoides, e supressão de genes relacionados ao amadurecimento (Xu *et al.*, 2012).

Estresse oxidativo e durabilidade dos frutos

Durante o amadurecimento de muitos frutos o estresse oxidativo aumenta progressivamente, conforme indicado pelo acúmulo gradativo de H_2O_2 (Martí *et al.*, 2009; Pilati *et al.*, 2014; Kumar *et al.*, 2016). De fato, em frutos climatéricos, podem ser observados dois picos de aumento de ROS. O primeiro precedendo imediatamente o incremento de ET e associado com sua função sinalizadora; e outro nos estágios finais, onde o fruto inicia o processo de degeneração e senescência, tornando-se mais vulnerável a danos físico-químicos e ao ataque de patógenos. No entanto, esse processo final pode ser relevante na dispersão de sementes em muitas espécies (Jimenez *et al.*, 2002; Muñoz and Munné-Bosch, 2018). É bem estabelecido, não surpreendentemente, que as diferenças encontradas na durabilidade de frutos tem íntima relação com a manutenção da capacidade e atividade dos sistemas antioxidantes, especialmente nos estágios mais próximos ao fim do amadurecimento, como é notado, por exemplo, em variedades de tomate com tempo de prateleira mais curto, os quais apresentam menor atividade antioxidante e maiores sinais de estresse oxidativo quando comparadas com variedades com tempo de prateleira mais longo (Cocaliadis *et al.*, 2013).

Uma das estratégias mais utilizadas, nesse sentido, é o armazenamento e transporte dos frutos em baixas temperaturas, pois a diminuição da velocidade das reações químicas resulta, dentro de certos limites, na repressão da respiração e diversos processos metabólicos, incluindo a produção de ROS. No entanto, esse procedimento frequentemente acarreta injúrias e perda de qualidade de frutos, especialmente quando esses são recondicionados para temperatura ambiente (Wang, 1989). Chama atenção que diversos tratamentos que apresentaram sucesso em aumentar a durabilidade dos frutos expostos a baixas temperaturas promovem a

ativação ou incremento de enzimas antioxidantes e o acúmulo de substâncias correlatas (Shi *et al.*, 2020; Loayza *et al.*, 2021; Hou *et al.*, 2021).

Outra estratégia importante é o controle da composição gasosa que circunda os frutos durante o transporte e armazenamento. Na maior parte dos protocolos, utiliza-se baixas concentrações de oxigênio e altas concentrações de CO_2, as quais contribuem para a repressão da respiração e produção de ROS (Qu *et al.*, 2022).

Mais recentemente, uma vasta gama de outros procedimentos está sendo explorada visando melhorar a durabilidade de frutos, com especial destaque para o uso de substâncias como fitormônios e outros sinalizadores, de modo a atuar diretamente nas vias e processos biológicos relacionados ao amadurecimento e senescência. Já foi demonstrado, por exemplo, que a exposição ao NO e outras ROS e RNS, em concentrações adequadas, consegue ativar os sistemas antioxidantes, resultando numa capacidade superior de enfrentar agentes estressores (Lai *et al.*, 2011; Jiménez-Muñoz *et al.*, 2021). O desafio atual, no caso do NO, consiste em encontrar uma concentração adequada e método de oferecimento para cada contexto de armazenamento e transporte, espécie ou cultivar. Parte desse desafio decorre da natureza reativa do NO, que em presença de oxigênio converte-se em NO_2, composto muito mais estável, e apresenta potencial de ser tóxico (Kasten *et al.*, 2017). Outra limitação importante, também relacionada a essa questão, é a carência de estudos padronizados, com a entrega de concentrações fisiologicamente relevantes NO a frutos, mantidos sob normóxia e nos quais seja mantida um fluxo constante de renovação da mistura gasosa (ar + NO), de modo a minimizar a exposição ao NO_2 (Kasten *et al.*, 2017). Tais estudos poderiam aprofundar nossa compreensão dos

processos regulados especificamente pelo NO e gerar oportunidades de explorar aplicações tecnológicas mais precisas e vantajosas.

Com o intuito de contribuir para reduzir essa lacuna de conhecimento, o presente trabalho teve como objetivo central realizar uma detalhada caracterização do transcriptoma e metaboloma durante o amadurecimento de frutos de tomateiro (*Solanum lycopersicum*) tratados com concentrações fisiologicamente relevantes de NO.

Referências Introdução Geral

Airaki M, Leterrier M, Valderrama R, Chaki M, Begara-Morales JC, Barroso JB, del Río LA, Palma JM, Corpas FJ. 2015. Spatial and temporal regulation of the metabolism of reactive oxygen and nitrogen species during the early development of pepper (*Capsicum annuum*) seedlings. Annals of Botany **116**, 679–693.

Airaki M, Sanchez-Moreno L, Leterrier M, Barroso JB, Palma JM, Corpas FJ. 2011. Detection and quantification of S-Nitrosoglutathione (GSNO) in pepper (*Capsicum annuum* L.) plant organs by LC-ES/MS. Plant and Cell Physiology **52**, 2006–2015.

Albertos P, Romero-Puertas MC, Tatematsu K, Mateos I, Sánchez-Vicente I, Nambara E, Lorenzo O. 2015. S -nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. Nature Communications **6**, 8669.

Andryka-Dudek P, Ciacka K, Wiśniewska A, Bogatek R, Gniazdowska A. 2019. Nitric oxide-induced dormancy removal of apple embryos is linked to alterations in expression of genes encoding ABA and JA biosynthetic or transduction pathways and RNA nitration. International Journal of Molecular Sciences **20**, 1007.

Arasimowicz-Jelonek M, Floryszak-Wieczorek J. 2019. A physiological perspective on targets of nitration in NO-based signaling networks in plants. Journal of Experimental Botany **70**, 4379–4389.

Argueso CT, Hansen M, Kieber JJ. 2007. Regulation of ethylene biosynthesis. journal of plant growth regulation **26**, 92–105.

Arnholdt-Schmitt B, Costa JH, de Melo DF. 2006. AOX – a functional marker for efficient cell reprogramming under stress? Trends in Plant Science **11**, 281–287.

Astier J, Gross I, Durner J. 2018. Nitric oxide production in plants: an update. Journal of Experimental Botany **69**, 3401–3411.

Astier J, Rasul S, Koen E, Manzoor H, Besson-Bard A, Lamotte O, Jeandroz S, Durner J, Lindermayr C, Wendehenne D. 2011. S-nitrosylation: An emerging post-translational protein modification in plants. Plant Science **181**, 527–533.

Baker LMS, Baker PRS, Golin-Bisello F, Schopfer FJ, Fink M, Woodcock SR, Branchaud BP, Radi R, Freeman BA. 2007. Nitro-fatty acid reaction with glutathione and cysteine. Journal of Biological Chemistry **282**, 31085–31093.

Barry CS, Giovannoni JJ. 2007. Ethylene and fruit ripening. Journal of Plant Growth Regulation **26**, 143–159.

Barry CS, Llop-Tous MI, Grierson D. 2000. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant Physiology **123**, 979–986.

Bemer M, Karlova R, Ballester AR, Tikunov YM, Bovy AG, Wolters-Arts M, Rossetto P de B, Angenent GC, Maagd RA de. 2012. The tomato FRUITFULL Homologs TDR4/FUL1 and MBP7/FUL2 regulate ethylene-independent aspects of fruit ripening. The Plant Cell **24**, 4437–4451.

Bhattacharjee S. 2019. ROS and Regulation of Photosynthesis. Reactive Oxygen Species in Plant Biology. New Delhi: Springer India, 107–125.

Binder BM. 2020. Ethylene signaling in plants. Journal of Biological Chemistry **295**, 7710–7725.

Bolmgren K, Eriksson O. 2010. Seed mass and the evolution of fleshy fruits in angiosperms. Oikos **119**, 707–718.

Breitel DA, Chappell-Maor L, Meir S, *et al.* 2016. AUXIN RESPONSE FACTOR 2 intersects hormonal signals in the regulation of tomato fruit ripening. PLOS Genetics **12**, e1005903.

Burg SP, Burg EA. 1962. Role of ethylene in fruit ripening. Plant Physiology **37**, 179–189.

Cai H, An X, Han S, Jiang L, Yu M, Ma R, Yu Z. 2018. Effect of 1-MCP on the production of volatiles and biosynthesis-related gene expression in peach fruit during cold storage. Postharvest Biology and Technology **141**, 50–57.

Cellini A, Corpas FJ, Barroso JB, Masia A. 2011. Nitric oxide content is associated with tolerance to bicarbonate-induced chlorosis in micropropagated *Prunus* explants. Journal of Plant Physiology **168**, 1543–1549.

Chaki M, Álvarez de Morales P, Ruiz C, Begara-Morales JC, Barroso JB, Corpas FJ, Palma JM. 2015. Ripening of pepper (*Capsicum annuum*) fruit is characterized by an enhancement of protein tyrosine nitration. Annals of Botany **116**, 637–647.

Chamizo-Ampudia A, Sanz-Luque E, Llamas A, Galvan A, Fernandez E. 2017. Nitrate reductase regulates plant nitric oxide homeostasis. Trends in Plant Science **22**, 163–174.

Chamizo-Ampudia A, Sanz-Luque E, Llamas Á, Ocaña-Calahorro F, Mariscal V, Carreras A, Barroso JB, Galván A, Fernández E. 2016. A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in Chlamydomonas. Plant, Cell & Environment **39**, 2097–2107.

Cheng G, Duan X, Shi J, Lu W, Luo Y, Jiang W, Jiang Y. 2008. Effects of reactive oxygen species on cellular wall disassembly of banana fruit during ripening. Food Chemistry **109**, 319–324.

Cherian S, Figueroa CR, Nair H. 2014. 'Movers and shakers' in the regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric fruit. Journal of Experimental Botany **65**, 4705–4722.

Cocaliadis MF, Fernández-Muñoz R, Pons C, Orzaez D, Granell A. 2013. Increasing tomato fruit quality by enhancing fruit chloroplast function. A double-edged sword? Journal of Experimental Botany **65**, 4589–4598.

Considine MJ, Foyer CH. 2014. Redox regulation of plant development. Antioxidants & Redox Signaling **21**, 1305–1326.

Corpas FJ, Alché JD, Barroso JB. 2013*a*. Current overview of S-nitrosoglutathione (GSNO) in higher plants. Frontiers in Plant Science **4**.

Corpas FJ, Freschi L, Rodríguez-Ruiz M, Mioto PT, González-Gordo S, Palma JM. 2018. Nitro-oxidative metabolism during fruit ripening. Journal of Experimental Botany, 1–15.

Corpas FJ, González-Gordo S, Palma JM. 2021. Protein nitration: A connecting bridge between nitric oxide (NO) and plant stress. Plant Stress **2**, 100026.

Corpas F, Palma J, del Río L, Barroso J. 2013*b*. Protein tyrosine nitration in higher plants grown under natural and stress conditions. Frontiers in Plant Science **4**, 29.

Decros G, Baldet P, Beauvoit B, Stevens R, Flandin A, Colombié S, Gibon Y, Pétriacq P. 2019. Get the balance right: ROS homeostasis and redox signalling in fruit. Frontiers in Plant Science **10**.

Desel C, Hubbermann EM, Schwarz K, Krupinska K. 2007. Nitration of γ-tocopherol in plant tissues. Planta **226**, 1311–1322.

Devoghalaere F, Doucen T, Guitton B, *et al.* 2012. A genomics approach to understanding the role of auxin in apple (*Malus x domestica*)fruit size control. BMC Plant Biology **12**, 7.

Domingos P, Prado AM, Wong A, Gehring C, Feijo JA. 2015. Nitric Oxide: A multitasked signaling gas in plants. Molecular Plant **8**, 506–520.

Duan X, Zhang H, Zhang D, Sheng J, Lin H, Jiang Y. 2011. Role of hydroxyl radical in modification of cell wall polysaccharides and aril breakdown during senescence of harvested longan fruit. Food Chemistry **128**, 203–207.

Eriksson O. 2016. Evolution of angiosperm seed disperser mutualisms: the timing of origins and their consequences for coevolutionary interactions between angiosperms and frugivores. Biological Reviews **91**, 168–186.

Feng J, Chen L, Zuo J. 2019. Protein S-Nitrosylation in plants: Current progresses and challenges. Journal of Integrative Plant Biology **61**, 1206–1223.

Feng J, Wang C, Chen Q, Chen H, Ren B, Li X, Zuo J. 2013. S-nitrosylation of phosphotransfer proteins represses cytokinin signaling. Nature Communications **4**, 1529.

Ferrer-Sueta G, Campolo N, Trujillo M, Bartesaghi S, Carballal S, Romero N, Alvarez B, Radi R. 2018. Biochemistry of peroxynitrite and protein tyrosine nitration. Chemical Reviews **118**, 1338–1408.

Fuentes L, Figueroa CR, Valdenegro M. 2019. Recent advances in hormonal regulation and cross-talk during non-climacteric fruit development and ripening. Horticulturae **5**, 45.

Fukano Y, Tachiki Y. 2021. Evolutionary ecology of climacteric and non-climacteric fruits. Biology Letters **17**, 20210352.

Gabaldón C, Gómez Ros LV, Pedreño MA, Ros Barceló A. 2004. Nitric oxide production by the differentiating xylem of *Zinnia elegans*. New Phytologist **165**, 121–130.

Garg N, Manchanda G. 2009. ROS generation in plants: Boon or bane? Plant Biosystems **143**, 81–96.

Golding JB, Shearer D, Wyllie SG, McGlasson WB. 1998. Application of 1-MCP and propylene to identify ethylene-dependent ripening processes in mature banana fruit. Postharvest Biology and Technology **14**, 87–98.

Golding JB, Singh SP. 2017. Use of 1-MCP in the storage life extension of fruit. Reference Module in Food Science. Elsevier, B9780081005965210000.

Goldschmidt EE. 1998. Ripening of citrus and other non-climacteric fruits: a role for ethylene. Acta Horticulturae, 335–340.

Guerra D, Ballard K, Truebridge I, Vierling E. 2016. S-Nitrosation of conserved cysteines modulates activity and stability of S-nitrosoglutathione reductase (GSNOR). Biochemistry **55**, 2452–2464.

Gupta KJ, Igamberdiev AU. 2011. The anoxic plant mitochondrion as a nitrite: NO reductase. Mitochondrion **11**, 537–543.

Hewitt S, Dhingra A. 2020. Beyond Ethylene: New Insights Regarding the Role of AOX in the Respiratory Climacteric. Front. Plant Sci. **11**:543958.

Hou Y, Li Z, Zheng Y, Jin P. 2021. Effects of CaCl2 Treatment alleviates chilling injury of loquat fruit (*Eribotrya japonica*) by modulating ros homeostasis. Foods **10**, 1662.

Hu J, Huang X, Chen L, Sun X, Lu C, Zhang L, Wang Y, Zuo J. 2015. Site-specific nitrosoproteomic identification of endogenously S-nitrosylated proteins in arabidopsis. Plant Physiology **167**, 1731–1746.

Huang D, Huo J, Zhang J, Wang C, Wang B, Fang H, Liao W. 2019. Protein S-nitrosylation in programmed cell death in plants. Cellular and Molecular Life Sciences **76**, 1877–1887.

Hussain A, Yun B-W, Kim JH, Gupta KJ, Hyung N-I, Loake GJ. 2019. Novel and conserved functions of S-nitrosoglutathione reductase in tomato. Journal of Experimental Botany **70**, 4877–4886.

Iglesias MJ, Terrile MC, Correa-Aragunde N, *et al.* 2018. Regulation of SCFTIR1/AFBs E3 ligase assembly by S-nitrosylation of Arabidopsis SKP1-like1 impacts on auxin signaling. Redox Biology **18**, 200–210.

Isner J-C, Maathuis FJM. 2018. cGMP signalling in plants: from enigma to main stream. Functional Plant Biology **45**, 93.

Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Shima Y, Nakamura N, Kotake-Nara E, Kawasaki S, Toki S. 2017. Re-evaluation of the *rin* mutation and the role of RIN in the induction of tomato ripening. Nature Plants **3**, 866–874.

Izbiańska K, Floryszak-Wieczorek J, Gajewska J, Meller B, Kuźnicki D, Arasimowicz-Jelonek M. 2018. RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans*. Frontiers in Plant Science **9**, 672.

Jahnová J, Luhová L, Petřivalský M. 2019. S-Nitrosoglutathione reductase—the master regulator of protein S-nitrosation in plant no signaling. Plants **8**, 48.

Jedelská T, Luhová L, Petřivalský M. 2020. Thioredoxins: Emerging players in the regulation of protein S-nitrosation in plants. Plants **9**, 1426.

Ji K, Kai W, Zhao B, *et al.* 2014. SlNCED1 and SlCYP707A2: key genes involved in ABA metabolism during tomato fruit ripening. Journal of Experimental Botany **65**, 5243–5255.

Jimenez A, Creissen G, Kular B, Firmin J, Robinson S, Verhoeyen M, Mullineaux P. 2002. Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. Planta **214**, 751–758.

Jiménez-Muñoz R, Palma F, Carvajal F, Castro-Cegrí A, Pulido A, Jamilena M, Romero-Puertas MC, Garrido D. 2021. Pre-storage nitric oxide treatment enhances chilling tolerance of zucchini fruit (*Cucurbita pepo* L.) by S-nitrosylation of proteins and modulation of the antioxidant response. Postharvest Biology and Technology **171**, 111345.

Jones B, Frasse P, Olmos E, Zegzouti H, Li ZG, Latché A, Pech JC, Bouzayen M. 2002. Down-regulation of DR12, an auxin-response-factor homolog, in the tomato results in a pleiotropic phenotype including dark green and blotchy ripening fruit. The Plant Journal **32**, 603–613.

Joudoi T, Shichiri Y, Kamizono N, Akaike T, Sawa T, Yoshitake J, Yamada N, Iwai S. 2013. Nitrated cyclic GMP modulates guard cell signaling in *Arabidopsis*. The Plant Cell **25**, 558–571.

Kasten D, Durner J, Gaupels F. 2017. Gas Alert: The NO_2 pitfall during NO fumigation of plants. Frontiers in Plant Science **8**:85.

Kende H. 1993. Ethylene biosynthesis. Annual review of plant biology **44**, 283–307.

Khan AS, Singh Z. 2009. 1-MCP application suppresses ethylene biosynthesis and retards fruit softening during cold storage of 'Tegan Blue' Japanese plum. Plant Science **176**, 539–544.

Khorobrykh S, Havurinne V, Mattila H, Tyystjärvi E. 2020. Oxygen and ROS in Photosynthesis. Plants **9**, 91.

Kumar R, Agarwal P, Tyagi AK, Sharma AK. 2012. Genome-wide investigation and expression analysis suggest diverse roles of auxin-responsive GH3 genes during development and response to different stimuli in tomato (*Solanum lycopersicum*). Molecular Genetics and Genomics **287**, 221–235.

Kumar V, Irfan M, Ghosh S, Chakraborty N, Chakraborty S, Datta A. 2016. Fruit ripening mutants reveal cell metabolism and redox state during ripening. Protoplasma **253**, 581–594.

Lai T, Wang Y, Li B, Qin G, Tian S. 2011. Defense responses of tomato fruit to exogenous nitric oxide during postharvest storage. Postharvest Biology and Technology **62**, 127–132.

Lamotte O, Bertoldo JB, Besson-Bard A, Rosnoblet C, Aimé S, Hichami S, Terenzi H, Wendehenne D. 2015. Protein S-nitrosylation: specificity and identification strategies in plants. Frontiers in Chemistry **2**: 114.

Leseberg CH, Eissler CL, Wang X, Johns MA, Duvall MR, Mao L. 2008. Interaction study of MADS-domain proteins in tomato. Journal of Experimental Botany **59**, 2253–2265.

Leterrier M, Chaki M, Airaki M, Valderrama R, Palma JM, Barroso JB, Corpas FJ. 2011. Function of S-nitrosoglutathione reductase (GSNOR) in plant development and under biotic/abiotic stress. Plant Signaling & Behavior **6**, 789–793.

Li J, Tao X, Li L, Mao L, Luo Z, Khan ZU, Ying T. 2016. Comprehensive RNA-Seq analysis on the regulation of tomato ripening by exogenous auxin. PLOS ONE **11**, e0156453.

Li H, Wu H, Qi Q, *et al.* 2019. Gibberellins play a role in regulating tomato fruit ripening. Plant and Cell Physiology **60**, 1619–1629.

Lindermayr C. 2018. Crosstalk between reactive oxygen species and nitric oxide in plants: Key role of S-nitrosoglutathione reductase. Free Radical Biology and Medicine **122**, 110–115.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. 2001. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature **410**, 490–494.

Liu M, Pirrello J, Chervin C, Roustan J-P, Bouzayen M. 2015. Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. Plant Physiology **169**, 2380–2390.

Liu Y, Tang M, Liu M, Su D, Chen J, Gao Y, Bouzayen M, Li Z. 2020. The molecular regulation of ethylene in fruit ripening. Small Methods **4**, 1900485.

Loayza FE, Brecht JK, Simonne AH, Plotto A, Baldwin EA, Bai J, Lon-Kan E. 2021. A brief hot-water treatment alleviates chilling injury symptoms in fresh tomatoes. Journal of the Science of Food and Agriculture **101**, 54–64.

Lü P, Yu S, Zhu N, *et al.* 2018. Genome encode analyses reveal the basis of convergent evolution of fleshy fruit ripening. Nature Plants **4**, 784–791.

Lytovchenko A, Eickmeier I, Pons C, *et al.* 2011. Tomato fruit photosynthesis is seemingly unimportant in primary metabolism and ripening but plays a considerable role in seed development. Plant Physiology **157**, 1650–1663.

Ma Y, Yang M, Wang J, Jiang C-Z, Wang Q. 2017. Application of exogenous ethylene inhibits postharvest peel browning of 'Huangguan' pear. Frontiers in Plant Science **7**.

Martel C. 2010. Characterization of the roles of rin and nor during tomato (*Solanum Lycopersicum*) fruit ripening.

Martí MC, Camejo D, Olmos E, Sandalio LM, Fernández-García N, Jiménez A, Sevilla F. 2009. Characterisation and changes in the antioxidant system of chloroplasts and chromoplasts isolated from green and mature pepper fruits. Plant Biology **11**, 613–624.

Mata-Pérez C. 2016. Nitric oxide release from nitro-fatty acids in Arabidopsis roots. Plant Signal Behaviour **11**:e1154255

Mata-Pérez C, Padilla MN, Sánchez-Calvo B, Begara-Morales JC, Valderrama R, Chaki M, Barroso JB. 2018. Biological properties of nitro-fatty acids in plants. Nitric Oxide **78**, 176–179.

Mata-Pérez C, Sánchez-Calvo B, Padilla MN, Begara-Morales JC, Valderrama R, Corpas FJ, Barroso JB. 2017. Nitro-fatty acids in plant signaling: New key mediators of nitric oxide metabolism. Redox Biology **11**, 554–561.

Mendes TDC, Pereira AM, Puiatti M, Cecon PR, Finger FL. 2018. Shelf life of gilo fruits treated with ethylene. Revista Brasileira de Ciências Agrárias - Brazilian Journal of Agricultural Sciences **13**, 1–9.

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F. 2011. ROS signaling: the new wave? Trends in Plant Science **16**, 300–309.

Mou W, Li D, Bu J, Jiang Y, Khan ZU, Luo Z, Mao L, Ying T. 2016. Comprehensive analysis of ABA effects on ethylene biosynthesis and signaling during tomato fruit ripening. PLOS ONE **11**, e0154072.

Muñoz P, Munné-Bosch S. 2018. Photo-oxidative stress during leaf, flower and fruit development. Plant Physiology **176**, 1004–1014.

Mur LAJ, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJM, Hebelstrup KH, Gupta KJ. 2013. Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plants **5**, pls052–pls052.

Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A. 1998. Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiology **118**, 1295–1305.

Newton R, Cook WH. 1927. The effect of ethylene on the respiration of bananas during ripening. Plant Physiology **2**, 357–360.

Ntagkas N, Woltering E, Nicole C, Labrie C, Marcelis LFM. 2019. Light regulation of vitamin C in tomato fruit is mediated through photosynthesis. Environmental and Experimental Botany **158**, 180–188.

Oeller PW, Lu MW, Taylor LP, Pike DA, Theologis A. 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254**, 437–439.

Palma AAD. 2020. Nitro-oleic acid triggers ROS production via NADPH oxidase activation in plants. A pharmacological approach. Journal of Plant Physiology, 9.

Pang C-H, Wang B-S. 2010. Role of ascorbate peroxidase and glutathione reductase in ascorbate–glutathione cycle and stress tolerance in plants. In: Anjum NA,, In: Chan M-T,, In: Umar S, eds. Ascorbate-glutathione pathway and stress tolerance in plants. Dordrecht: Springer Netherlands, 91–113.

Paul V, Pandey R, Srivastava GC. 2012. The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene. An overview. Journal of Food Science and Technology **49**, 1–21.

Petřivalský M. 2020. Nitrated nucleotides: new players in signaling pathways of reactive nitrogen and oxygen species in plants. Frontiers in Plant Science **11**, 8.

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D. 1993. Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. The Plant Journal **3**, 469–481.

Pilati S, Brazzale D, Guella G, Milli A, Ruberti C, Biasioli F, Zottini M, Moser C. 2014. The onset of grapevine berry ripening is characterized by ROS accumulation and lipoxygenase-mediated membrane peroxidation in the skin. BMC Plant Biology **14**, 87.

Pomerleau J, Lock K, McKee M, Altmann DR. 2004. The challenge of measuring global fruit and vegetable intake. The Journal of Nutrition **134**, 1175–1180.

Qu P, Zhang M, Fan K, Guo Z. 2022. Microporous modified atmosphere packaging to extend shelf life of fresh foods: A review. Critical Reviews in Food Science and Nutrition **62**, 51–65.

Ravanel S, Gakière B, Job D, Douce R. 1998. The specific features of methionine biosynthesis and metabolism in plants. Proceedings of the National Academy of Sciences **95**, 7805–7812.

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM. 2002. Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. Journal of Experimental Botany **53**, 103–110.

Ros Barceló A. 2005. Xylem parenchyma cells deliver the H_2O_2 necessary for lignification in differentiating xylem vessels. Planta **220**, 747–756.

Sagar M, Chervin C, Roustan J-P, Bouzayen M, Zouine M. 2013. Under-expression of the Auxin Response Factor *Sl-ARF4* improves post-harvest behavior of tomato fruits. Plant Signaling & Behavior **8**, e25647.

Saha B, Borovskii G, Panda SK. 2016. Alternative oxidase and plant stress tolerance. Plant Signaling & Behavior **11**, e1256530.

Salgado I, Carmen Martínez M, Oliveira HC, Frungillo L. 2013. Nitric oxide signaling and homeostasis in plants: a focus on nitrate reductase and S-nitrosoglutathione reductase in stress-related responses. Brazilian Journal of Botany **36**, 89–98.

Saltveit ME. 1999. Effect of ethylene on quality of fresh fruits and vegetables. Postharvest Biology and Technology **15**, 279–292.

Sánchez-Calvo B, Barroso JB, Corpas FJ. 2013. Hypothesis: Nitro-fatty acids play a role in plant metabolism. Plant Science **199–200**, 1–6.

Santolini J, André F, Jeandroz S, Wendehenne D. 2017. Nitric oxide synthase in plants: Where do we stand? Nitric Oxide **63**, 30–38.

Saraiva LA, Castelan FP, Gomes BL, Purgatto E, Cordenunsi-Lysenko BR. 2018. Thap Maeo bananas: Fast ripening and full ethylene perception at low doses. Food Research International **105**, 384–392.

Sdiri S, Navarro P, Monterde A, Benabda J, Salvador A. 2012. New degreening treatments to improve the quality of citrus fruit combining different periods with and without ethylene exposure. Postharvest Biology and Technology **63**, 25–32.

Shi F, Li X, Meng H, Wei W, Wang Y. 2020. Reduction in chilling injury symptoms by hot electrolyzed functional water treatment may function by regulating ROS metabolism in Satsuma orange fruit. LWT **125**, 109218.

Shima Y, Kitagawa M, Fujisawa M, Nakano T, Kato H, Kimbara J, Kasumi T, Ito Y. 2013. Tomato FRUITFULL homologues act in fruit ripening via forming MADS-box transcription factor complexes with RIN. Plant Molecular Biology **82**, 427–438.

Signorelli S, Considine MJ. 2018. Nitric oxide enables germination by a four-pronged attack on ABA-induced seed dormancy. Frontiers in Plant Science **9**, 296.

Souza JM, Peluffo G, Radi R. 2008. Protein tyrosine nitration—Functional alteration or just a biomarker? Free Radical Biology and Medicine **45**, 357–366.

Stöhr C, Stremlau S. 2006. Formation and possible roles of nitric oxide in plant roots. Journal of Experimental Botany **57**, 463–470.

Su Y-H, Wu S-S, Hu C-H. 2019. Release of nitric oxide from nitrated fatty acids: Insights from computational chemistry. Journal of the Chinese Chemical Society **66**, 41–48.

Sun L, Sun Y, Zhang M, *et al.* 2012. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. Plant Physiology **158**, 283–298.

Terrile MC, París R, Calderón-Villalobos LIA, Iglesias MJ, Lamattina L, Estelle M, Casalongué CA. 2012. Nitric oxide influences auxin signaling through S-nitrosylation of the Arabidopsis TRANSPORT INHIBITOR RESPONSE 1 auxin receptor: S-nitrosylation of the TIR1 auxin receptor. The Plant Journal **70**, 492–500.

Tiffney BH. 1984. Seed size, dispersal syndromes, and the rise of the angiosperms: evidence and hypothesis. Annals of the Missouri Botanical Garden **71**, 551.

Tiffney BH, Mazer SJ. 1995. Angiosperm growth habit, dispersal and diversification reconsidered. Evolutionary Ecology **9**, 93–117.

Trainotti L, Tadiello A, Casadoro G. 2007. The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. Journal of Experimental Botany **58**, 3299–3308.

Vanlerberghe G. 2013. Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. International Journal of Molecular Sciences **14**, 6805–6847.

Vinson AE. 1910. The stimulation of premature ripening by chemical means. Journal of the American Chemical Society **32**, 208–212.

Wang CY. 1989. Chilling injury of fruits and vegetables. Food Reviews International **5**, 209–236.

Wang R, Angenent GC, Seymour G, de Maagd RA. 2020. Revisiting the role of master regulators in tomato ripening. Trends in Plant Science **25**, 291–301.

Wang KL-C, Li H, Ecker JR. 2002. Ethylene biosynthesis and signaling networks. The Plant Cell **14**, S131–S151.

Wang R, Tavano EC da R, Lammers M, Martinelli AP, Angenent GC, de Maagd RA. 2019. Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis. Scientific Reports **9**, 1696.

Watkins CB. 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. Biotechnology Advances **24**, 389–409.

Wolfe HS. 1931. Effect of ethylene on the ripening of bananas. Botanical Gazette **92**, 337–366.

Xu F, Yuan S, Zhang D-W, Lv X, Lin H-H. 2012. The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. Journal of Experimental Botany **63**, 5705–5716.

Yamasaki H, Sakihama Y. 2000. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. Febs Letters **468**, 89–92.

Yamasaki H, Sakihama Y, Takahashi S. 1999. An alternative pathway for nitric oxide production in plants: new features of an old enzyme. Trends in plant science **4**, 128–129.

Yang J, Giles LJ, Ruppelt C, Mendel RR, Bittner F, Kirk ML. 2015. Oxyl and hydroxyl radical transfer in mitochondrial amidoxime reducing component-catalyzed nitrite reduction. Journal of the American Chemical Society **137**, 5276–5279.

Yang SF, Hoffman NE. 1984. Ethylene biosynthesis and its regulation in higher plants. Annual review of plant physiology **35**, 155–189.

Zhan N, Wang C, Chen L, *et al.* 2018. S-Nitrosylation targets GSNO reductase for selective autophagy during hypoxia responses in plants. Molecular Cell **71**, 142-154.e6.

Zhang M, Yuan B, Leng P. 2009. The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. Journal of Experimental Botany **60**, 1579–1588.

Zhong S, Fei Z, Chen Y-R, *et al.* 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. Nature Biotechnology **31**, 154–159.

Capítulo Único

Multifaceted roles of nitric oxide in tomato fruit ripening: NO-induced metabolic rewiring and consequences for fruit quality traits

This chapter is organized as published in the Journal of Experimental Botany:

Zuccarelli R, Rodríguez-Ruiz M, Lopes-Oliveira PJ, Pascoal GB, Andrade SCS, Furlan CM, Purgatto E, Palma JM, Corpas FJ, Rossi M, Freschi L. 2021. Multifaceted roles of nitric oxide in tomato fruit ripening: NO-induced metabolic rewiring and consequences for fruit quality traits. Journal of Experimental Botany 72, 941-958.

Supplementary information is available for this paper at:
https://academic.oup.com/jxb/article/72/3/941/5963948#supplementary-data

Highlights

Nitric oxide-induced transcriptional changes and metabolic rewiring at early ripening promote the accumulation of water-soluble antioxidants and preserve taste and aroma in tomato fruits.

Abstract

Nitric oxide (NO) has been implicated as part of the ripening regulatory network in fleshy fruits. However, very little is known about the simultaneous action of NO on the intertwined web of regulatory events and metabolic reactions behind ripening-related changes in fruit color, taste, aroma and nutritional value. Here, we performed an in-depth characterization of the concomitant changes in tomato (*Solanum lycopersicum*) fruit transcriptome and metabolome associated with the delayed-ripening phenotype caused by NO supplementation at the pre-climacteric stage. Approximately one-third of the fruit transcriptome was altered in response to NO, including a multilevel down-regulation of ripening regulatory genes, which in turn restricted the production and tissue sensitivity to ethylene. NO also repressed hydrogen peroxide-scavenging enzymes, intensifying nitro-oxidative stress and *S*-nitrosation and nitration events throughout ripening. Carotenoid, tocopherol, flavonoid and ascorbate biosynthesis were differentially affected by NO, resulting in overaccumulation of ascorbate (25%) and flavonoids (60%), and impaired lycopene production. In contrast, the biosynthesis of compounds related to tomato taste (sugars, organic acids, amino acids) and aroma (volatiles) was slightly affected by NO. Our findings indicate that NO triggers extensive transcriptional and metabolic rewiring at

the early ripening stage, modifying tomato antioxidant composition with minimal impact on fruit taste and aroma.

Keywords: Antioxidants, ascorbate, carotenoids, ethylene, flavonoids, fruit ripening, nitric oxide, reactive oxygen and nitrogen species, redox, *Solanum lycopersicum*.

1.1. Introduction

Fruit ripening is driven by multiple phytohormones in conjunction with a set of developmental non-hormonal signals, and involves extensive metabolic rewiring leading to changes in fruit composition, color, aroma, and texture (Gapper *et al.*, 2013; Liu *et al.*, 2015*a*). In climacteric fruits, including tomato (*Solanum lycopersicum*), ripening is intricately regulated by ethylene, with a burst in both ethylene production and respiratory rates at early ripening coinciding with massive transcriptional changes (Cherian *et al.*, 2014; Liu *et al.*, 2015*a*). Besides being one of the most important horticultural crops, tomato is a reference species for the elucidation of climacteric ethylene production, sensing and signal transduction. Ripening-associated developmental regulators (formerly known as master regulators of ripening), such as RIPENING-INHIBITOR (RIN), NON-RIPENING (NOR) and COLORLESS NONRIPENING (CNR), which act either upstream or in concert with ethylene, have also been first identified in this Solanaceous species (Li *et al.*, 2019; Wang *et al.*, 2020). Multiple signals converge to transcriptionally regulate ripening-associated ethylene biosynthetic genes in tomato fruits, particularly those encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), the latter responsible for the conversion of ACC into ethylene (Liu *et al.*, 2015*a*). After biosynthesis, ethylene is perceived by dedicated receptors (ETHYLENE RESPONSE, ETR) and initiates a linear transduction pathway that culminates in the activation of ETHYLENE RESPONSE FACTORs (ERFs), which in

turn transcriptionally regulate genes associated with ripening-related traits (Liu *et al.*, 2015*a*).

Without undermining the role of ethylene, seminal research by the Leshem group (Leshem and Wills, 1998; Leshem and Pinchasov, 2000) paved the way for the identification of nitric oxide (NO) as a component of the signaling cascades controlling fruit ripening. Since then, the action of this reactive nitrogen species (RNS) in fruit biology has been investigated under two complementary perspectives. From an agronomical point of view, NO has emerged as a promising new molecule to extend the post-harvest longevity while maintaining fruit quality traits, such as color, aroma, texture and nutritional value, with potential implications for a multi-billion dollar agro-industry sector worldwide (Manjunatha *et al.*, 2012; Corpas and Palma, 2018). From a fruit physiology perspective, the place occupied by NO in the complex regulatory modules controlling fruit development, growth, and ripening is now becoming clearer (Bodanapu *et al.*, 2016; Mukherjee, 2019; González-Gordo *et al.*, 2019; Palma *et al.*, 2019). Several studies have repeatedly implicated the negative impacts of NO on the ethylene biosynthetic pathway as the primary mechanism of action of this RNS during ripening of various climacteric fruits (Rudell and Mattheis, 2006; Zhu and Zhou, 2006, 2007; Eum *et al.*, 2009; Cheng *et al.*, 2009; Palma *et al.*, 2019), and also a few non-climacteric species (Zhu and Zhou, 2007), possibly via crosstalk with melatonin (Liu *et al.*, 2019*b*).

As fruits ripen, the synthesis and removal rates of reactive oxygen species (ROS) and RNS undergo significant changes (Jimenez *et al.*, 2002; Corpas and Barroso, 2013; Kumar *et al.*, 2016), thereby altering the fruit nitro-oxidative landscape with repercussions on NO-dependent post-translational modifications (NO-PTMs, e.g. Tyr-nitration and *S*-nitrosation) of target proteins (Chaki *et al.*, 2015;

Corpas *et al.*, 2018; Palma *et al.*, 2019). Given its capacity to interact both with anti- and pro-oxidants (Groß *et al.*, 2013), NO is considered a significant player in fruit nitro-oxidative metabolism (Chaki *et al.*, 2015; González-Gordo *et al.*, 2019; Palma *et al.*, 2019), acting at multiple levels. At the transcriptional level, NO influences the transcript abundance of genes involved in both enzymatic and non-enzymatic fruit antioxidant defenses (Boscari *et al.*, 2012; Huang *et al.*, 2018; Kolbert *et al.*, 2019). At the post-translational level, antioxidant isoenzymes expressed in fruit tissues have been identified as targets of both protein nitration and *S*-nitrosation eventsm (Chaki *et al.*, 2015; Corpas *et al.*, 2018; Rodríguez-Ruiz *et al.*, 2019). As non-enzymatic, lipid-soluble (e.g. carotenoids and tocopherols) and hydrophilic (e.g. ascorbate and phenolics) antioxidants that accumulate in fleshy fruits are relevant sources of nutrients for human diet (Moco *et al.*, 2006), the modulation of their biosynthetic routes in response to NO has been increasingly investigated (Manjunatha *et al.*, 2012). NO treatment was shown to limit lycopene accumulation in tomato (Eum *et al.*, 2009), cause the overaccumulation of phenolics in ripe peach and citrus (Zhou *et al.*, 2016; Li *et al.*, 2017), and ascorbate in sweet pepper and citrus fruits (Zhou *et al.*, 2016; Rodríguez-Ruiz *et al.*, 2017). Moreover, NO has been implicated in the accumulation of sugars, organic acids and amino acids in fleshy fruits (Deng *et al.*, 2013; Bodanapu *et al.*, 2016; Han *et al.*, 2018), which are key determinants of flavor and Brix (Tieman *et al.*, 2007).

The vast majority of studies on NO and fruit ripening have focused on specific metabolic routes (Eum *et al.*, 2009; Zhou *et al.*, 2016; Li *et al.*, 2017), with few exceptions (Tanou *et al.*, 2015; Kang *et al.*, 2016; González-Gordo *et al.*, 2019). In sweet pepper (*Capsicum annuum*), thousands of genes, many of which are linked to fruit quality traits, were modulated in response to NO (Corpas *et al.*, 2018; González-

Gordo *et al.*, 2019). Despite its worldwide importance, tomato has rarely been employed in research focused on RNS metabolism and action during climacteric ripening (Corpas *et al.*, 2018, and references therein). Here, we employed a controlled approach to offer NO to pre-ripe tomato fruits under physiologically- and post-harvest-relevant conditions, followed by a comprehensive screening of transcriptional and metabolic rearrangements triggered by this molecule. Our findings indicate that NO acts early in the molecular processes controlling tomato ripening, and differentially affects distinct biochemical routes involved in the definition of tomato fruit sensory and nutritional quality traits.

1.2. Material and Methods

Plant material and treatment conditions

Tomato (*Solanum lycopersicum* L.) 'Micro-Tom' plants were grown under greenhouse conditions, as described in (Cruz *et al.*, 2018). Fruits were harvested at mature green (MG, approximately 30 d after anthesis) stage and divided into two groups: (i) control fruits were continuously flushed with humidified air (300 ml min^{-1}); and (ii) fruits submitted to NO-enriched atmosphere (300 ppm in air, 300 ml min^{-1}) for 96 h and subsequently flushed with humidified air (300 ml min^{-1}) until sampling. In both cases, fruits were maintained in sealed transparent vessels under continuous white light (150 µmol m^{-2} s^{-1}) and constant temperature (25±1 °C) throughout the experiment. A continuous flow of the desired NO concentration was obtained by mixing a standard commercial mixture (10 000 ppm NO in nitrogen) with NO-free air at appropriate flow ratios, as described in (Melo *et al.*, 2016). Samples were harvested after reaching the following ripening stages: MG (immediately after 96 h of NO treatment), breaker (Bk, displaying the first yellowing signal), and Bk3 and Bk7 corresponding to 3 d and 7 d after Bk, respectively. Four biological samples

composed of the pericarp of at least five fruits each were harvested at each sampling time, snap frozen in liquid N_2, powdered and stored at –80 °C until further use.

Ethylene sensitivity assay

To test whether NO treatment interferes with ethylene perception, MG fruits treated with NO for 96 h were subsequently exposed to 100 ppm of ethylene for 6 h (Cruz *et al.*, 2018). Four biological samples composed of the pericarp of at least five fruits each were harvested at each sampling time, snap frozen in liquid N_2, powdered and stored at –80 °C until use in RT-qPCR analysis.

Detection of nitrosated and nitrated proteins

The biotin switch assay was performed as described by (Jaffrey *et al.*, 2001), with modifications. Frozen powdered fruit pericarp samples were homogenized with extraction buffer composed of 0.1 M Tris-HCl (pH 7.8), 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 5 mM N-ethylmaleimide (NEM) and 0.1 mM neocuproine. Aliquots containing approximately 80 µg protein were treated with 20 mM S-methyl methanethiosulfonate (MMTS) and 2.5% (w/v) sodium dodecyl sulfate (SDS) at 50 °C for 1 h with continuous vortexing and under dark conditions, to block free thiol groups of Cys residues. Residual MMTS was eliminated by precipitation with two volumes of ice-cold acetone. Samples were treated with 1.25 mM sodium ascorbate and 1 mM biotin-HPDP (Pierce, USA) at 25 °C for 1 h, which allowed the biotinylation of the endogenous *S*-nitrosated thiol residues. Next, the proteins were precipitated with two volumes of ice-cold acetone and dissolved in a non-reducing buffer for western blot analysis.

SDS-PAGE was carried out using 4–20% precast polyacrylamide gels and a Mini-Protean electrophoresis cell (Bio-Rad, Hercules, CA, USA). For immunoblot analysis, proteins were transferred onto a 0.45-µm nitrocellulose membrane and

blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 for 1 h. *S*-nitrosated proteins were detected by incubating with anti-biotin mouse monoclonal antibody (1:100) followed by goat anti-mouse IgG1 horseradish peroxidase-conjugated antibody (1:10 000). Nitrated proteins were detected by a polyclonal antibody against 3-nitrotyrosine (NO_2-Tyr, Sigma Aldrich, USA; 1:200) followed by goat anti-rabbit IgG horseradish peroxidase conjugate (1:8000). For immunodetection, an enhanced chemiluminescence kit (Clarity™ Western ECL Substrate, BioRad, USA) was used.

Antioxidant enzyme activity assays and hydrogen peroxide content analysis

For the analysis of antioxidant enzyme activity, approximately 300 mg FW (fresh weight) of frozen powdered fruit pericarp was extracted with a buffer composed of 0.1 M phosphate buffer (pH 7.5), 1 mM EDTA and 2% (w/v) PVPP. After centrifugation (18 000 × *g*, 4 °C, 20 min), aliquots of the extract were stored at –80 °C. Ascorbate peroxidase (APX) activity was determined by monitoring the initial ascorbate oxidation by hydrogen peroxide (H_2O_2) at 290 nm (Lopes-Oliveira *et al.*, 2019). For APX activity determination, 2 mM ascorbate was added to the extraction buffer to preserve the enzymatic activity (Miyake and Asada, 1996). Superoxide dismutase (SOD) activity was measured using nitroblue tetrazolium (NBT) photoreduction at 560 nm (Beauchamp and Fridovich, 1971). One unit (U) of SOD is defined as the amount of enzyme that can inhibit NBT reduction by 50%. Catalase (CAT) activity was determined by following H_2O_2 consumption at 240 nm (Aebi, 1984).

For the analysis of antioxidant enzyme activity under nitration and *S*-nitrosation conditions, crude extracts were incubated with 2 mM of the NO donors diethylamine nonoate (NONOate) or *S*-nitrosoglutahione (GSNO), or the

peroxynitrite donor 3-morpholino-sydnonimine (SIN-1) at 25 °C for 30 min, before determining APX, SOD and CAT activities (Rodríguez-Ruiz *et al.*, 2019 and references therein). As control of GSNO decomposition residue, samples were incubated with 2 mM reduced glutathione (GSH).

For H_2O_2 content, approximately 300 mg FW of frozen powdered fruit pericarp was extracted with 1 ml of trichloroacetic acid (0.2% in methanol) and centrifuged at 16 000 × *g* for 5 min at 4 °C. After 1 h of reacting with KI in phosphate buffer (0.1 M, pH 7.5) in dark conditions, H_2O_2 content was measured at 390 nm (Lopes-Oliveira *et al.*, 2019).

S-nitrosoglutathione reductase (GSNOR) activity

GSNOR was extracted and analyzed according to (Zuccarelli *et al.*, 2017), with modifications. Briefly, frozen samples were ground with a mortar and pestle in liquid nitrogen and the cellular content was extracted with a solution composed of 20 mM HEPES (pH 8.0) buffer. After centrifugation (20 000 × *g*, 20 min, 4 °C), aliquots of the supernatant were assayed for 30 min at 30 °C in the presence of 200 mM NADH and 400 mM GSNO, and the NADH decomposition was spectrophotometrically monitored at 340 nm.

Fruit color, ethylene emission, and measurement of compounds related to ethylene biosynthesis

Fruit surface color was determined at the pedicellar and stylar fruit regions using a colorimeter (Konica Minolta, CR-400, 8-mm aperture, D65 illuminant), as described in (Cruz *et al.*, 2018). Ethylene emission was determined in whole fruits immediately after harvesting via gas chromatography (Trace GC Ultra, Thermo Electron) with a flame-ionization detector (Cruz *et al.*, 2018). ACC content and *in vitro* ACO activity were determined as described in (Cruz *et al.*, 2018).

Primary and secondary metabolite profiling

Carotenoid, chlorophyll, phenylpropanoid and ascorbate quantification were carried out on a Hewlett-Packard series 1100 high-performance liquid chromatography (HPLC) system coupled with a diode array detector (Agilent Technologies series 1200), as described in (Alves *et al.*, 2020). Tocopherol profiling was performed using a Hewlett-Packard series 1100 HPLC system coupled with a fluorescence detector (Agilent Technologies series 1200), as described in (Lira *et al.*, 2017). The profiling of polar and apolar compounds was performed in a gas chromatograph (Agilent Technologies 7890B) equipped with auto-injector (CombiPAL, CG sampler 80) and a mass selective detector (Agilent Technologies 5977A), as described by (Alves *et al.*, 2020).

Volatile compound profiling

Volatile compound profiling was performed by solid-phase microextraction (SPME) and subsequent analysis in a gas chromatograph (Hewlett-Packard model 6890) coupled with a mass detector (Hewlett-Packard model 5973), as described in Silva Souza *et al.*, 2020.

RNA sequencing and quantitative PCR (qPCR) analysis

RNA sequencing was performed at the Bk stage in triplicates. RNA extraction, library preparation, and sequencing using the Illumina HiSeq2500 system (Illumina, Inc.), as well as RNA-Seq mapping, annotation and differential expression analysis were performed as described in (Alves *et al.*, 2020). Differential expression analysis was performed using the negative binomial function, applying the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) for multiple tests to avoid false positives. Only genes with false discovery rate (FDR) ≤ 0.05 were considered differentially expressed. Gene Set Enrichment Analysis (GSEA) was performed with FDR ≤ 0.05 as

significance threshold, using the online tool PANTHER™ (Mi *et al.*, 2019), with PANTHER™ GO slim ontology (version 15.0, based on GO release 2018-07-03, released 2020-02-14) with default parameters. GO terms obtained from PANTHER were summarized (redundancy removal) using the online tool REVIGO (Supek *et al.*, 2011) with default parameters. For MapMan functional categorization, tomato reference genome SL3.0 with the ITAG3.2 annotation (cDNA sequences) was mapped to MapMan classification using the online tool Mercator4 v2.0 (Schwacke *et al.*, 2019). DEGs with FDR ≤0.05 were analyzed using MapMan software Version 3.6.0RC1 and the mapping file generated by Mercator4. Significant under- and over-represented biochemical pathways categories (bins) were identified by the Wilcoxon rank-sum test with FDR adjustment, according to Benjamini and Hochberg, 1995 and $P \leq 0.05$ as the significance threshold. Custom Python and R scripts used in the bioinformatics analysis are available upon request.

cDNA synthesis and RT–qPCR analysis were performed as described in (Cruz *et al.*, 2018). Transcript abundance was normalized against the geometric mean of two reference genes, *CAC* and *EXPRESSED*. Primer sequences used are presented in Supplementary Table S1.

Statistical analysis

Pairwise comparisons of biochemical and RT–qPCR data were performed by a Student's *t*-test to determine whether samples were normally distributed, and homogeneity of variances were presented. Otherwise, the comparisons were performed by applying a Wilcox test. Multiple comparisons were performed by Analysis of Variance (Anova) followed by Tukey's test. Metabolomic analysis was performed using the web-based platform Metaboanalyst 4.0 (Chong *et al.*, 2018).

1.3. Results

Global transcriptional changes in tomato fruits exposed to nitric oxide-enriched atmosphere

In the presence of oxygen, NO is rapidly converted into nitrogen dioxide (NO_2). Therefore, the delivery of NO to plant tissues has often been conducted under ultra-low oxygen atmospheres (Wills *et al.*, 2000; Liu *et al.*, 2019*a*), exposing the plant cells to hypoxic conditions, which in turn can trigger physiological responses not necessarily associated with the presence of NO itself.

To circumvent the potential interference of either NO_2 formation or hypoxic conditions, here we employed the delivery of high flow rates of NO-enriched air mixture during the first 96 h after fruit harvest, which successfully delayed fruit ripening without causing necrosis or other collateral effects on fruit appearance (Fig. 1A). The progressive acquisition of distinctive red fruit coloration was restricted, and the green shoulder in the pedicellar region was partially retained in response to NO treatment (Fig. 1A), as confirmed by the Hue angle measurements (Fig. 1B).

The transcriptome profile revealed that approximately one-third of the 17 779 transcripts identified at the Bk stage was significantly affected by NO treatment (Supplementary Table S2). Compared to control samples, 3391 and 2570 genes were up- and down-regulated, respectively, in response to NO exposure. Validation of RNA-Seq results performed for 15 genes using the RT–qPCR technique revealed consistency (r^2 correlation =0.79) between both methods ().

Enrichment analysis of MapMan ontogeny groups via Wilcoxon rank-sum test analysis ($P \leq 0.05$ as a threshold) identified photosynthesis, lipid metabolism, ethylene action, cell cycle organization, cutin and suberin metabolism, as well as vesicle

trafficking, as over-represented in NO-treated fruits (Fig. 1C; Supplementary Table S3, S4). Gene Set Enrichment (GSE) analysis confirmed that genes grouping around GO terms related to these categories were significantly affected by NO treatment (Supplementary Fig. S2; Table S5).

Out of the 271 photosynthesis-related genes detected in Bk fruits, 212 and 18 loci were up- and down-regulated, respectively, in response to NO, with a large proportion of these genes related to photophosphorylation, followed by Calvin cycle and photorespiration reactions (Fig. 1D). Genes related to photosynthesis and plastid organization and function were also among the most highly over-represented GO terms identified in NO-treated fruits (Supplementary Fig. S2; Table S5). In agreement, NO treatment delayed chlorophyll degradation (Supplementary Table S6) by inducing and repressing genes related to chlorophyll production (i.e. tetrapyrrole biosynthesis) and degradation, respectively (Fig. 1D,E).

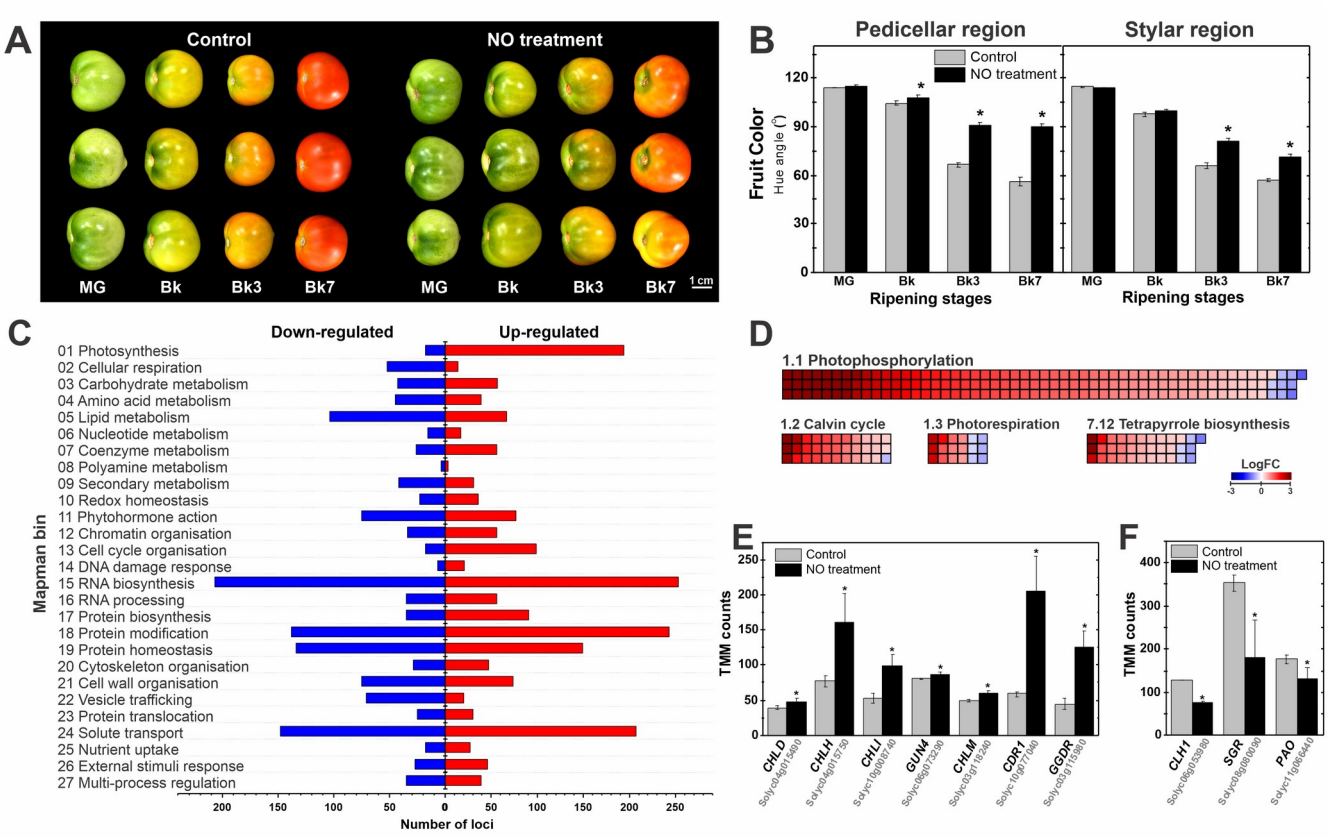


Fig. 1. Changes in tomato fruit transcriptome during NO-induced delay in ripening. Fruits harvested at mature green (MG) stage were left to ripen off-the-vine under control and NO-enriched (100 ppm) atmosphere for 96 h, followed by sampling at MG (immediately after the NO treatment), breaker (Bk), Bk3 and Bk7 (3 d and 7 d after Bk, respectively) stages. (A) Representative fruits at each sampling stage. (B) Fruit color estimated by the Hue angle at the pedicellar (top) and the stylar (bottom) regions. (C) Mapman4 functional categorization of differentially expressed genes (DEGs, FDR ≤0.05) in NO-treated *versus* control fruits at the Bk stage (Supplementary Table S3, S4). (D) Heatmaps indicate $\log_2$ (fold-change) of DEGs in NO-treated Bk fruits for MapMan categories related to photosynthesis and chlorophyll biosynthesis. (E, F) Trimmed Mean of M-values (TMM) counts for chlorophyll biosynthesis (E) and degradation (F) genes at the Bk stage. Data shown are mean ±SE of at least three biological replicates. In B, E and F, $*P < 0.05$ (Student's t-test) compared with control samples. CHLH/CHLD/CHLI/GUN4, magnesium chelatases; CHLM, Mg-protoporphyrin IX methyltransferase; CLH, chlorophyllase; CRD1, Mg-Proto IX monomethyl ester cyclase; GGDP, geranylgeranyl diphosphate; SGR, stay green; PAO, pheophorbide *a* oxygenase.

Nitric oxide acts early in the tomato ripening regulatory network and represses ethylene biosynthesis, perception and signal transduction

Several genes encoding major ripening-related developmental regulators, including *CNR*, *RIN*, *NOR*, *FRUITFULL1* (*FUL1*), *APETALA2a* (*AP2a*) and *TOMATO*

AGAMOUS-LIKE 1 (*TAGL1*), were down-regulated at the Bk stage in response to NO treatment (Fig. 2A). From all hormone-related Mapman sub-categories, only the ethylene group was predominantly down-regulated in NO-treated fruits (Fig. 2B; Supplementary Table S3, S4). In agreement, NO inhibited climacteric ethylene emission (Fig. 2C) and reduced the transcript accumulation of ripening-associated ethylene biosynthetic genes, including *ACS* (*ACS2* and *ACS4*) and *ACO* genes (*ACO1*, *ACO3*, *ACO6*; Fig. 2D, compared with the controls. Among S-adenosyl-L-methionine synthetase (SAM)-encoding genes, *SAM2*, *SAM3* and *SAM4* were also downregulated in response to NO treatment Fig. 2D. Moreover, reduced ACO activity and increased ACC content were detected in NO-treated fruits at MG stage Fig. 2E).

Compared with controls, NO treatment also down-regulated genes encoding ripening-related ethylene receptors, particularly ETR3 and ETR4, and ethylene signaling proteins, such as EIN3-BINDING F-BOX (EBFs), CONSTITUTIVE TRIPLE RESPONSE 1(CTR1) and sub-class-E ERFs, at the Bk stage Fig. 2F; Supplementary Fig. S4). Time-course analysis revealed that the NO-triggered down-regulation of ripening- and ethylene-related genes was transitory, mostly restricted to the Bk stage, followed by the recovery of their expression at more advanced ripening stages (Supplementary Fig. S5; Table S7). The expression of fruit softening-related genes, which are widely accepted targets of RIN/NOR- and ethylene-dependent signaling cascades (Fujisawa *et al.*, 2012; Zhong *et al.*, 2013), including *PECTATE LYASE* (*PL*) and *POLYGALACTURONASE* 2a (*PG2a*), was also transiently repressed at the Bk stage, and subsequently promoted at the Bk3 stage (upplementary Fig. S6; S7).

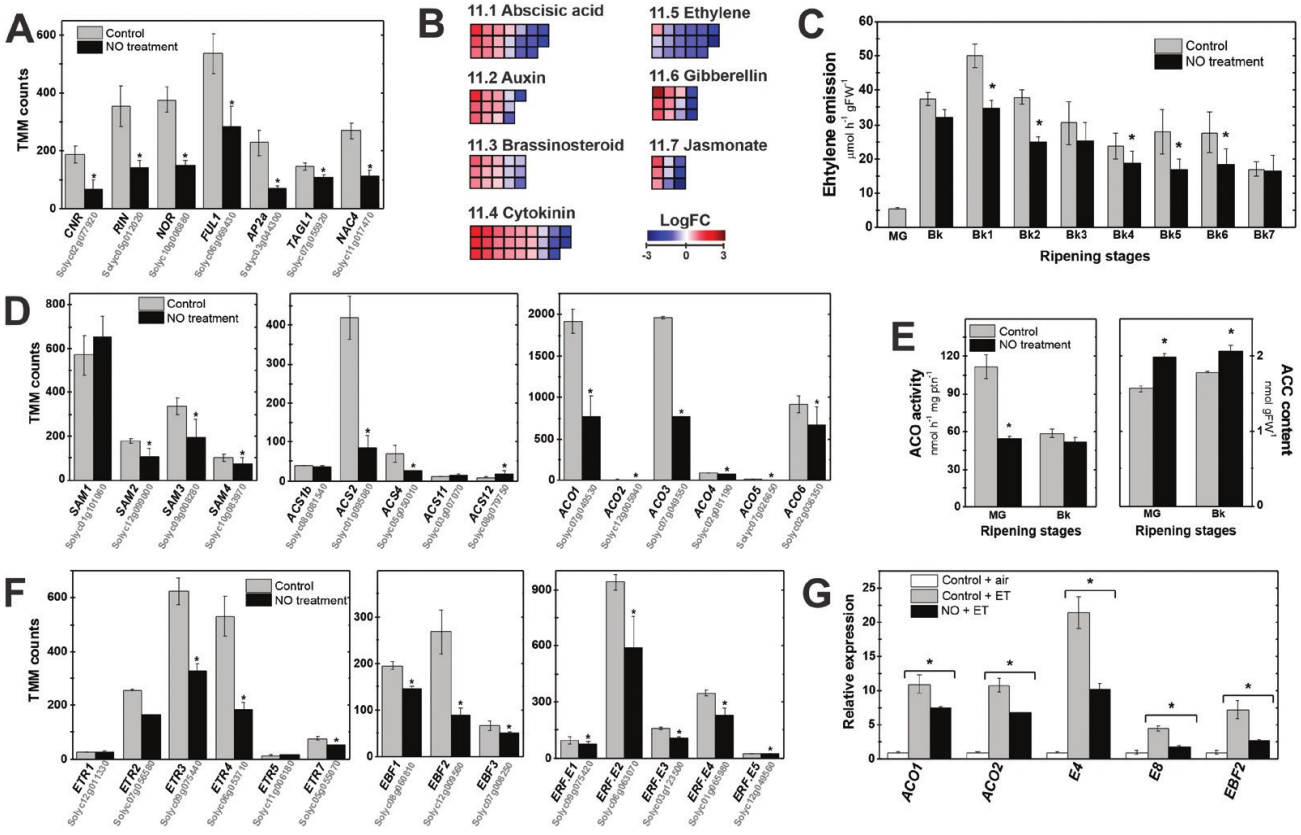


Fig. 2. NO treatment down-regulates early components in tomato fruit ripening regulatory networks and represses ethylene biosynthesis, perception and signal transduction. (A) Transcript abundance of ripening regulators at the breaker (Bk) stage. (B) Heatmap representation of $\log_2$(fold-change) of DEGs between NO-treated and control fruits at the Bk stage for MapMan categories related to phytohormone action. (C) Ethylene emission. (D) Trimmed Mean of M-values (TMM) counts of ethylene biosynthesis genes at the Bk stage. (E) 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) activity and endogenous ACC content. (F) TMM counts of ethylene perception and signaling genes at the Bk stage. (G) Transcript abundance of ethylene-inducible genes in control and NO-treated fruits exposed to 50 ppm ethylene for 6 h. Data shown are mean ±SE of at least three biological replicates. In A and C-F, $*P < 0.05$ (Student's t-test) compared with control samples. Bk3, 3 d after Bk; Bk7, 7 d after Bk; ACS, ACC synthase; AP2a, apetala2a; CNR, colorless non-ripening; ETR, ethylene response; ERF, ethylene response factor; EBF, EIN3-binding F-box; FUL1, fruitfull1; NOR, non-ripening; RIN, ripening inhibitor; SAM, S-adenosyl-L-methionine synthetase; TAGL1, tomato agamous-like 1.

To investigate whether the NO-mediated transcriptional repression of ethylene perception and signaling components affected the fruit tissue responsiveness to this hormone at the early ripening stage, intact fruits were subjected to a short ethylene exposure period (6 h) immediately after the 96 h NO treatment (Fig. 2G). At the end of the ethylene treatment, analysis of the expression of ethylene-inducible genes, such

as *ACO1*, *ACO2*, *E4*, *E8,* and *EBF2*, revealed that the NO exposure significantly (Student's t-test, $P < 0.05$) limited the fruit responsiveness to this hormone compared with fruits not exposed to NO.

Nitric oxide treatment reduces tomato fruit hydrogen peroxide scavenging capacity, promotes ascorbate biosynthesis and intensifies protein S-nitrosation and nitration

In climacteric fruits, an oxidative burst typically coincides with the increase in respiratory rates at early ripening stages (Jimenez *et al.*, 2002; Kumar *et al.*, 2016; Corpas *et al.*, 2018). In agreement, endogenous H_2O_2 concentration increased at the climacteric phase (Bk3 stage) in both control and NO-treated tomato fruits (Fig. 3A). Moreover, higher H_2O_2 concentrations were detected in NO-treated fruits at all ripening stages analyzed (Fig. 3A), which correlated with the NO-induced reduction in activities of the two major H_2O_2-scavenging enzymes, ascorbate peroxidase (APX) and catalase (CAT; Fig. 3B). In contrast, the H_2O_2-generating superoxide dismutase (SOD) activity was only slightly reduced at the MG stage (Fig. 3B).

As the expression of genes encoding the APX, CAT, and SOD isoforms most highly expressed in fruits were either not affected (*APX4*, *CAT4*) or slightly reduced (*SOD1*) by NO treatment (Supplementary Fig. S7A), we next investigated the potential modulation of these antioxidant enzymes via NO-PTMs in tomato fruits (Fig. 3C). APX and CAT activities were inhibited in the presence of either NO donors employed (NONOate or GSNO). Compared with controls, reductions of approximately 80% were observed upon NONOate treatment for both enzymes, and GSNO diminished APX and CAT activities by about 25% and 40%, respectively (Fig. 3C). The treatment with GSH (used as a control for GSNO) did not affect APX and CAT enzymatic activities. Moreover, incubation with the peroxynitrite donor SIN-1

inhibited CAT activity by 45%, and caused a slight reduction of APX activity (5%), compared with controls. In contrast, SOD activity was not significantly affected (Tukey test, $P < 0.05$) by either NO or peroxynitrite donors (Fig. 3C).

Mapman categories related to non-enzymatic ROS scavenging, H_2O_2 removal, and chloroplast redox homeostasis were significantly promoted (Wilcoxon rank-sum test, $P < 0.05$) in NO-treated fruits (Supplementary Fig. S7B). A closer examination revealed that many of the redox-related genes up-regulated in response to NO were involved in the biosynthesis of ascorbate, which correlated well the NO-triggered increment of approximately 25% in total ascorbate (reduced ascorbate + dehydroascorbic acid) content at the Bk7 stage (Fig. 3D). The NO treatment up-regulated multiple genes involved in the *D*-mannose/*L*-galactose pathway of ascorbate biosynthesis (Fig. 3E; Supplementary Fig. S7C), including genes encoding the rate-limiting enzymes GDP-L-galactose phosphorylase (GGP) and GDP-mannose pyrophosphorylase (GMP), responsible for the production of the intermediates *L*-Galactose-1P and GDP-*D*-Mannose, respectively.

In contrast to the decline in *S*-nitrosogluthatione reductase (GSNOR) activity and increment in protein Tyr-nitration observed in ripening sweet pepper fruits (Chaki *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2016), both these parameters were maintained relatively constant throughout the ripening of control tomato fruits Fig. 3F, G). However, despite the intensification in GSNOR-mediated removal of excess GSNO (Fig. 3F) and overaccumulation of hydrophilic antioxidants (i.e. ascorbate, flavonoids), NO-treated tomato fruits were under intense nitro-oxidative stress at climacteric phase, as indicated by the more abundant and diverse profile of nitrated and *S*-nitrosated proteins at all analyzed ripening stages (Fig. 3G, H).

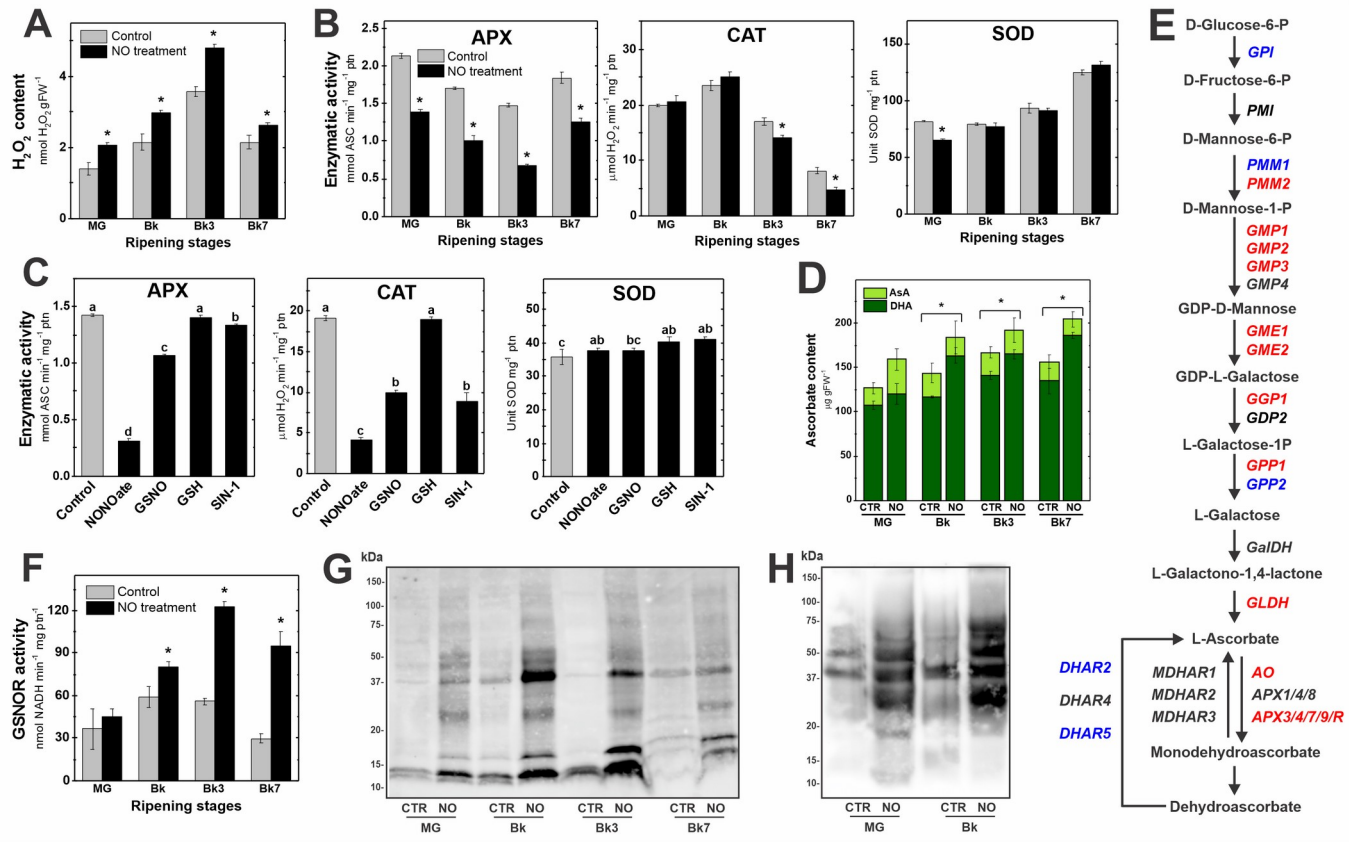


Fig. 3. NO treatment reduces fruit hydrogen peroxide (H_2O_2) scavenging capacity, promotes ascorbate biosynthesis, and intensifies protein *S*-nitrosation and nitration. (A) Hydrogen peroxide content. (B) *In vitro* activities of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD). (C) APX, CAT, and SOD activities from mature green (MG) tomato fruits assayed under nitration and *S*-nitrosation conditions. Activities were measured in the presence of 2 mM diethylamine nonoate (NONOate, a NO donor), GSNO (a NO donor), reduced glutathione (GSH) or 3-morpholino-sydnonimine (SIN-1, a peroxynitrite donor). (D) Reduced ascorbate (Asa) and dehydroascorbic acid

(DHA) content in control and NO-treated fruits. (E) Schematic representation of ascorbate biosynthesis via the *D*-mannose/*L*-galactose pathway. Biosynthetic genes up- and down-regulated in response to NO treatment at the breaker (Bk) stage are highlighted in red and blue, respectively. Transcript abundance and gene abbreviations are detailed in Supplementary Fig. S7. (F) *In vitro* activity of tomato GSNO reductase (GSNOR). (G) Detection of tyrosine-nitrated proteins. Proteins (20 µg) were separated by SDS-PAGE and visualized by immunoblotting using 3-nitrotyrosine antibody (dilution 1:200). (H) Detection of *S*-nitrosated proteins via the biotin switch technique. Proteins (70 µg) were separated by SDS-PAGE and visualized by immunoblotting using anti-biotin antibody (dilution 1:100). Data shown are mean ±SE of at least three biological replicates. In A, B, D, and F, $*P < 0.05$ (Student's t-test)compared with control samples. In C, different letters indicate statistically significant differences ($P < 0.05$). Bk3, 3 d after Bk; Bk7, 7 d after Bk.

NO differentially affects carotenoid, tocopherol, and flavonoid metabolism

NO-treated ripening fruits under-accumulated lycopene, phytoene and phytofluene (Fig. 4A), but exhibited similar amounts of β-carotene and other photosynthesis-related carotenoids, compared with control counterparts (Supplementary Table S6). As a result, total carotenoid content was approximately 70% lower in NO-treated than in control fruits at the Bk7 stage (Fig. 4A). In contrast, total tocopherol content was not affected by the NO treatment, with the exception of a slight reduction in all four tocopherol forms (α-, β-, γ- and δ-tocopherol) at the Bk3 stage (Fig. 4A; Supplementary Table S6).

The methylerythritol 4-phosphate (MEP) intermediate geranylgeranyl diphosphate (GGDP) is the common metabolic precursor for carotenogenesis and the production of phytyl-diphosphate (PDP), which in turn drives chlorophyll and tocopherol biosynthesis (Fig. 4B); (Almeida *et al.*, 2011). At the Bk stage, the expression of genes encoding MEP pathway enzymes involved in GGDP synthesis, such as *1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE 1* (*DXS1*) and *GGDP SYNTHASE1* (*GGPS1*) remained unchanged, or were only slightly reduced (*GGPS2*) in response to NO (Fig. 4B, C). In contrast, all major carotenogenesis genes,

including those encoding enzymes that catalyze rate-limiting steps in tomato fruit carotenoid pathway, such as PHYTOENE SYNTHASE 1 (PSY1) and PHYTOENE DESATURASE (PDS)(McQuinn *et al.*, 2018), were down-regulated by NO at the Bk stage (Fig. 4B, C).

Transcripts encoding GGDP reductase (GGDR) over-accumulated in NO-treated fruits at the Bk stage, suggesting an intensification of the GGDP-to-PDP conversion in response to NO treatment (Fig. 4B, C). Other key genes involved in tocopherol biosynthesis, including *TOCOPHEROL C-METHYL TRANSFERASE* (*VTE4*) and *PHYTOL KINASE* (*VTE5*), were also up-regulated by NO at the Bk stage. RT–qPCR-based time-course analysis revealed that the NO-triggered down-regulation of carotenogenesis genes at the Bk stage was either sustained (*PDS*) or reversed (*PSY1*) at more advanced ripening stages (Fig. 4D;Supplementary Table S7). In contrast, PDP- and tocopherol-related genes, such as *GGDR* and *VTE4*, were consistently induced by NO, mainly at the initial phase of ripening (Fig. 4D; Supplementary Table S7).

All major flavonoids typically found in tomato fruits, except rutin and rutin pentoside, over-accumulated in ripe NO-treated fruits compared with the control treatment (Fig. 5A). Total flavonoids were approximately 60% higher in NO-treated fruits than in control counterparts, reflecting the NO-triggered increment of up to 3.2-fold in naringenin chalcone, naringenin glucoside, kaempferol rutinoside, quercetin diglycoside, and apigenin derivative. Contrary to the expected, transcriptome data revealed an overall down-regulation of most flavonoid biosynthesis genes in NO-treated fruits at the Bk stage (Fig. 5B, C). However, time-course analysis revealed that the expression of key genes such as *PHENYLALANINE AMMONIA LYASE 5a* (*PAL5a*), *CHALCONE SYNTHASE* (*CHS1*), and *FLAVONOL SYNTHASE* (*FLS*) were

several-fold higher in NO-treated than in control fruits after the Bk stage (Fig. 5D; Supplementary Table S7).

Therefore, rather than homogenously affecting carotenoid, tocopherol and flavonoid metabolism, the NO treatment differentially affected the biosynthetic routes of each of these antioxidant classes.

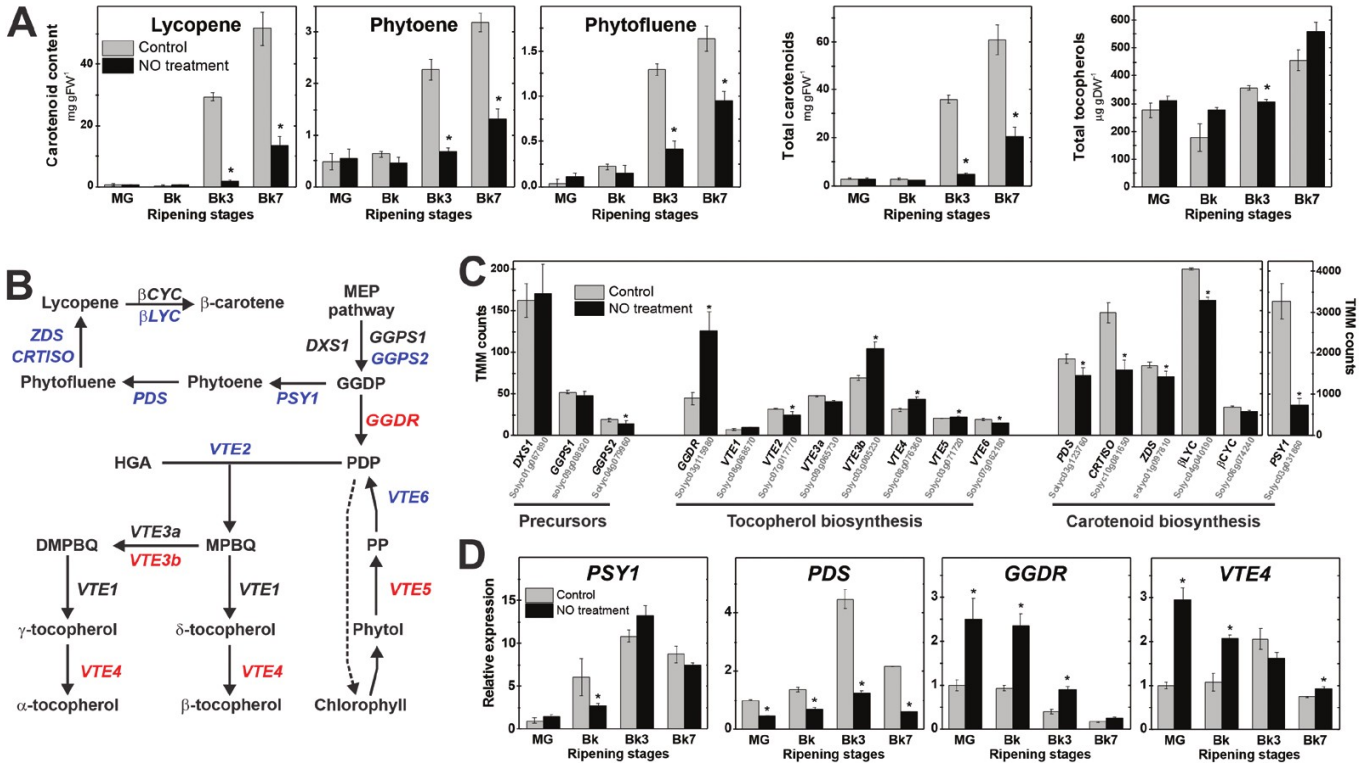


Fig. 4. NO differentially affects tocopherol and carotenoid biosynthesis in tomato fruits. (A) Lycopene, phytoene, phytofluene, total carotenoids and tocopherol content in control and NO-treated fruits. Individual carotenoid and tocopherol concentrations are presented in Supplementary Table S6. (B) Schematic representation of isoprenoid metabolism interconnecting carotenoid, tocopherol and chlorophyll pathways. Intermediate reactions are omitted. Biosynthetic genes up- and down-regulated in response to NO treatment at the breaker (Bk) stage are highlighted in red and blue, respectively. (C) Transcript abundance of isoprenoid biosynthetic genes at the Bk stage expressed as Trimmed Mean of M-values (TMM) counts. (D) Transcript abundance of key carotenoid and tocopherol biosynthetic genes profiled via RT–qPCR and normalized against the control samples at the mature green (MG) stage. Gene abbreviations and data from RT–qPCR analysis for other isoprenoid biosynthesis genes are detailed in Supplementary Table S7. Data shown are mean ±SE of at least three biological replicates. *$P < 0.05$ (Student's t-test) compared with control samples. Bk3, 3 d after Bk; Bk7, 7 d after Bk, DMPBQ, 2,3-dimethyl-6-phytyl benzoquinone; GGDP, geranylgeranyl diphosphate; HGA,

homogentisic acid; MEP, methylerythritol 4-phosphate; MPBQ, 2-methyl-6-geranylgeranylbenzoquinol; PDP, phytyl diphosphate; PP, phytyl phosphate.

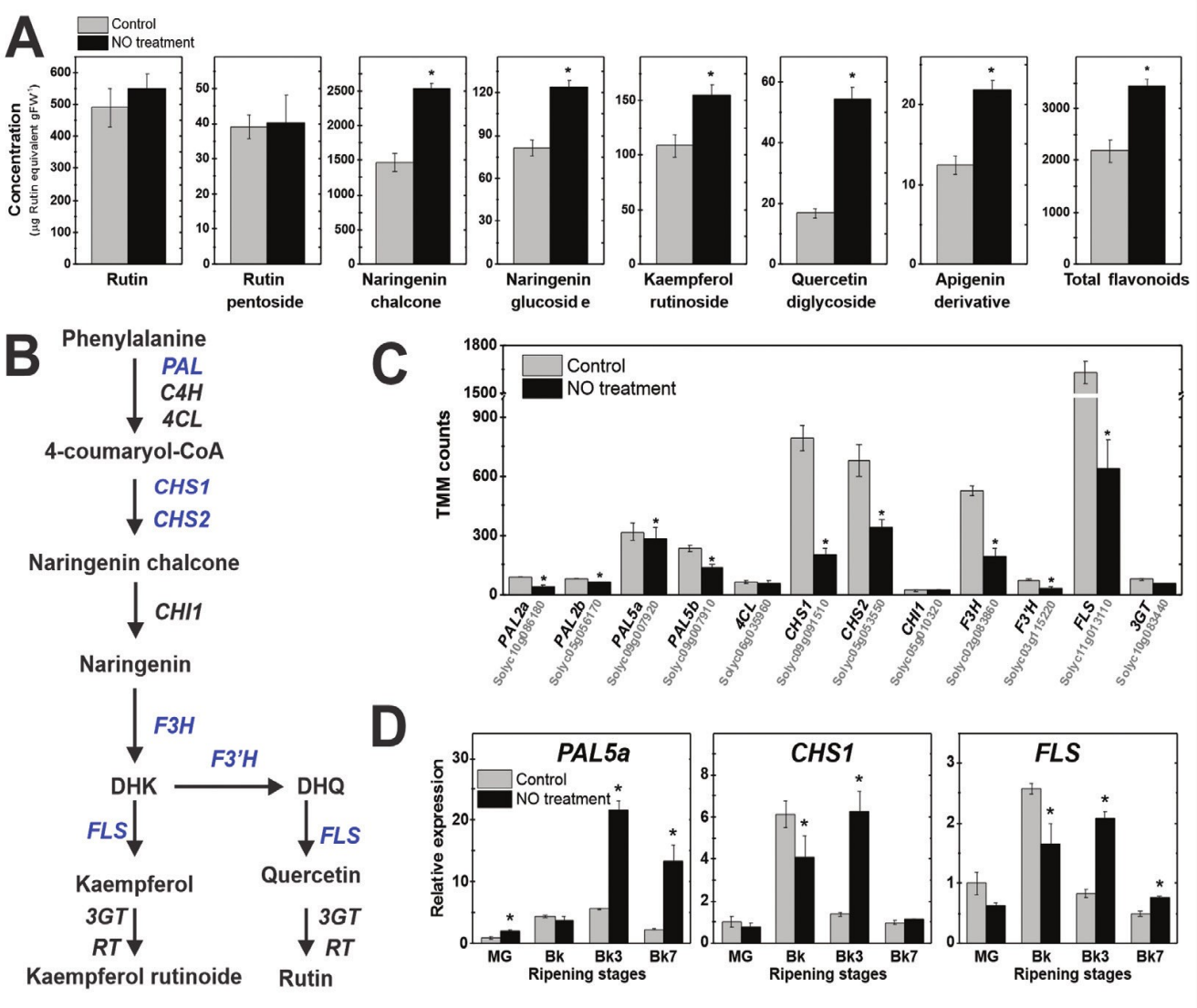


Fig. 5. NO modifies flavonoid production dynamics in ripening tomato fruits. (A) Flavonoid composition at the Bk7 (breaker + 7 d) stage. (B) Schematic representation of flavonoid metabolism, in which biosynthetic genes up- and down-regulated in response to NO treatment at the Bk stage are highlighted in red and blue, respectively. (C) Transcript abundance of flavonoid biosynthetic genes at the Bk stage expressed as Trimmed Mean of M-values (TMM) counts. (D) Transcript abundance of key flavonoid biosynthetic genes profiled via RT–qPCR and normalized against the control samples at the mature green (MG) stage. Data shown are mean ±SE of at least three biological replicates. **P* < 0.05 (Student's t-test) compared with control samples. Bk3, 3 d after Bk; 3GT, flavonoid 3-O-glucosyltransferase; 4CL, 4-coumaroyl CoA ligase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DHK, dihydrokaempferol; DHQ, dihydroquercetin; F3'H, flavone

3′-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine ammonia lyase; RT, rhamnosyl transferase.

NO has limited influence on fruit primary metabolites and volatiles

Tomato flavor depends on the balance between the accumulation of sugars, organic acids, amino acids and volatile compounds, which can be significantly disturbed depending on fruit ripening conditions (Vogel *et al.*, 2010; Tieman *et al.*, 2017). Large-scale mass spectrometry (MS)-based polar and apolar profiling revealed that 26 out of the 93 metabolites identified were significantly altered by NO treatment at some stages of ripening (Fig. 6; Supplementary Table).

In tomato, starch is converted into soluble sugars at the early ripening stage (Schaffer and Petreikov, 1997; Bianchetti *et al.*, 2017). At the Bk stage, NO promoted fructose and glucose accumulation (Fig. 6A), a response preceded by the NO-induced up-regulation at the Bk stage of multiple genes classified into Mapman categories related to starch and sucrose metabolisms (Fig. 6B; Supplementary Table S3, S4). At the ripe stage, NO treatment had no major impact on the sugars most predominantly accumulated in ripe tomato fruits (i.e. fructose, glucose, and sucrose), and only galactose and mannose, which are closely linked to ascorbate biosynthesis, were at lower levels in ripe NO-treated fruits than in control counterparts (Fig. 6A).

Glutamate and aspartate, which are important contributors to tomato taste (Zgola-Grześkowiak and Grześkowiak, 2012), as well as the majority of other amino acids, were not affected by NO treatment (Fig. 6). Proline and cysteine were found at higher amounts in NO-treated fruits, probably linked to their roles in stress responses and redox signaling-related processes in plants, respectively (Szabados and Savouré, 2010). Multiple DEGs classified into MapMan categories related to amino acid biosynthesis were identified in NO-treated Bk fruits, from which a large proportion of

them associated with the biosynthesis of aspartate- and shikimate-family of amino acids (Fig. 6B). Overall, organic acids were detected at similar concentrations in both control and NO-treated fruits (Fig. 6A).

Within fatty acids, hexadecenoic acid and octadecenoic acid derivatives represent the most abundant fraction in cutin monomer composition (Saladie *et al.*, 2007), and in this study, octadecenoic acid was significantly (Student's t-test, $P < 0.05$) over-accumulated in NO-treated fruits (Fig. 6). Alkanes, which are also important constituents of cuticular wax in tomato fruits (Vogg *et al.*, 2004; Lara *et al.*, 2014), were found at similar concentrations in control and NO-treated fruits (Supplementary Table S8). Numerous genes within MapMan categories related to fatty acid metabolism were differentially expressed in NO-treated fruits, including cutin and suberin biosynthetic genes (Fig. 6B).

SPME headspace extraction followed by GC-MS profiling revealed that concentrations of 1-penten-3-one and 2-isobutylthiazole, two volatile compounds responsible for the characteristic ripe tomato fruit (Buttery and Ling, 1993; Rambla *et al.*, 2014), were unaltered and increased, respectively, in NO-treated fruits at the Bk3 stage (A; Table S9). At the Bk7 stage, the volatile compound profile of NO-treated and control fruits was indistinguishable (A). Citral (geranial), which is one of the most abundant aldehydes contributing to tomato aroma, derives from lycopene in tomato fruits via the activity of the carotenoid cleavage dioxygenases CCD1a and CCD1b (Baldwin *et al.*, 2000). Here, both *CCD1a* and *CCD1b* genes were significantly (Student's t-test, $P < 0.05$) up-regulated by NO treatment at the Bk3 stage (B). Genes encoding lipoxygenase (*TomloxC*), hydroperoxide lyase (*HPL*) and alcohol dehydrogenase (*ADH2*) enzymes associated with the biosynthesis of fatty acid volatiles (e.g. 1-penten-3-one) in tomato fruits (Rambla *et al.*, 2014), were also

up-regulated in NO-treated compared with control fruits from Bk3 stage onwards (C). Altogether, these findings indicate an overall promotive effect of NO on the expression of genes encoding volatile biosynthetic enzymes during tomato ripening, resulting in a volatile composition in ripe NO-treated fruits similar to control counterparts.

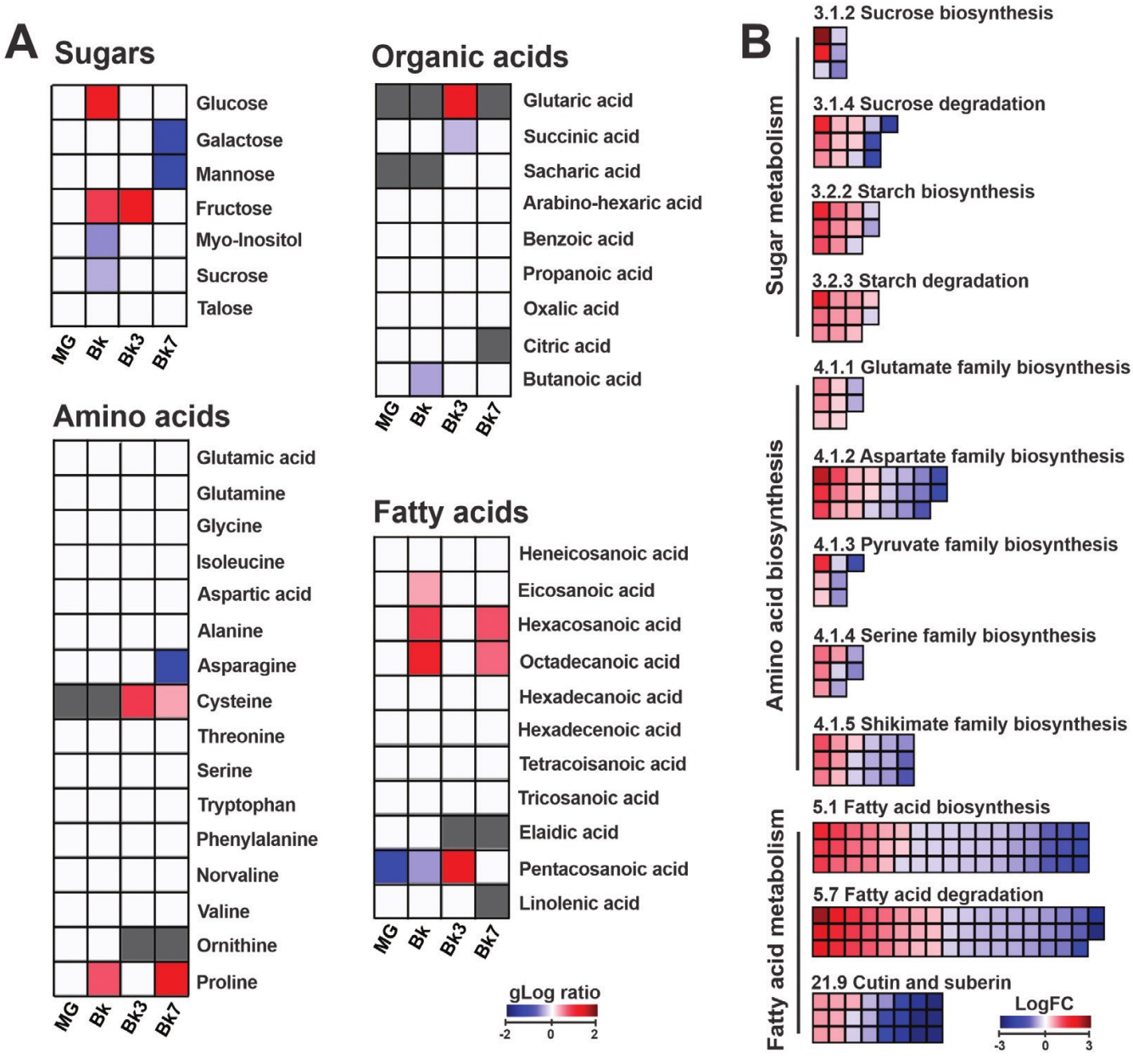


Fig. 6. Influence of NO treatment on fruit polar and apolar metabolome. (A) Heatmaps indicate the difference in metabolite abundance between control and NO-treated samples at each ripening stage, expressed in a generalized logarithm (gLog) of pareto rescaled data. Statistically significant differences in comparison with control samples are represented as colored squares (Student's t-test, $P<0.05$). Dark gray color indicates metabolites not detected.

The relative metabolite abundance values are presented in Supplementary Table S8. (B) Heatmap representation of log_2(fold-change) of DEGs between NO-treated and control fruits at the breaker (Bk) stage for MapMan categories related to sugar, amino acid, and fatty acid metabolism. DEGs in each group are listed in Supplementary Table S3. MG, mature green; Bk3, 3 d after Bk; Bk7, 7 d after Bk.

1.4. Discussion

Multiple biotechnological strategies have been adopted over the years to minimize the post-harvest losses of fleshy fruits, often at the expense of nutritional value, texture, flavor, and aroma (Baldwin *et al.*, 2000; Zhang *et al.*, 2013*b*, 2016). More recently, plant signaling molecules such NO, melatonin and hydrogen sulfide have been demonstrated to modulate fruit ripening when exogenously applied (Corpas and Palma, 2018, 2020; Yao *et al.*, 2018, 2020; Aghdam *et al.*, 2019). In this context, NO donors and NO-enriched atmosphere have emerged as innovative alternatives to extend fleshy fruit shelf life with potentially less extensive detrimental consequences on fruit quality (Manjunatha *et al.*, 2010; Palma *et al.*, 2018, 2019). However, since most studies on NO and fruit ripening have focused on specific metabolic routes, our knowledge on the simultaneous influence of RNS on the intricate and highly connected network of biochemical reactions associated with each fruit trait remains fragmentary. Using tomato as a model, here we describe the multifaceted action of NO during climacteric ripening. We reveal that this molecule can lead to concomitant, multilevel impacts on fruit processes as diverse as climacteric ethylene production and signaling, redox balance, chlorophyll degradation, cell wall softening, and accumulation of nutritionally-relevant compounds (e.g. carotenes, flavonoids, ascorbate, tocopherol), flavor-related substances (e.g. soluble sugar, organic acids, amino acids), and other molecules (e.g. lipid, sterols, and alkanes).

Multilevel interactions between nitric oxide and ethylene during climacteric ripening

Much of the NO action in climacteric ripening has been attributed to its antagonistic relationship with ethylene, with NO mostly acting as a repressor of *ACS* and *ACO* transcript accumulation and enzymatic activity (Palma *et al.*, 2019). In tomato, however, the inhibitory role of NO on both *ACS* and *ACO* gene expression has remained controversial (Eum *et al.*, 2009; Lai *et al.*, 2011). Treatment of pre-ripe tomato fruits with NO (200 ppm, 5 h) under anoxic conditions reduced ethylene emission by down-regulating the transcription accumulation of *ACO* rather than *ACS* genes (Eum *et al.*, 2009). On the other hand, treatment with the NO donor sodium nitroprusside (SNP) resulted in reduced expression of *ACS2*, *ACS4*, and *ACO1* (Lai *et al.*, 2011).

Our findings revealed that NO gas triggers a transitory down-regulation (at Bk stage) of the vast majority of ethylene biosynthesis genes in tomato fruits, including all ripening-associated *ACS* and *ACO* genes. Similar transcriptional changes were observed for genes encoding ripening-associated developmental regulators such as CNR, RIN, NOR, FUL1, AP2a and TAGL1, which form a network of partially redundant components controlling the expression of genes involved in a wide array of ripening-associated processes, including climacteric ethylene metabolism and signaling (Wang *et al.*, 2020). For example, the RIN protein can directly bind to the promoter of genes encoding ripening-related ACS and ACO (Lin *et al.*, 2008; Shima *et al.*, 2013), the ethylene receptors ETR3 and ETR4 (Fujisawa *et al.*, 2012; Shima *et al.*, 2013) and ethylene signaling components, including sub-class-E ERFs (Liu *et al.*, 2016), amongst others. Moreover, RIN and NOR can influence ethylene-independent

ripening events (Osorio *et al.*, 2011; Martel *et al.*, 2011), which makes their gene expression pattern a decisive factor for ripening initiation.

Either as a consequence of the lower abundance of ripening-associated developmental regulators, or via alternative pathways, NO significantly down-regulated key ripening-associated ethylene perception (*ETR3* and *ETR4*) and signaling (*CTR1*, *EBF*s and *ERF*s) genes, which was accompanied by the reduction of fruit sensitivity to exogenously applied ethylene (Fig. 2F, G). Therefore, our findings reveal that the NO-ethylene interplay in climacteric ripening is not limited to the repeatedly described antagonistic action of NO on ethylene production (Eum *et al.*, 2009; Lai *et al.*, 2011), but also relies on an extensive interplay with ethylene perception and signal transduction pathway. In this context, it is of particular notice that out of the nine sub-classes comprising the 77 members of the tomato ERF family (Liu *et al.*, 2016), only sub-class-E *ERF* genes were consistently down-regulated in response to NO treatment. Interestingly, sub-class E members, particularly ERF.E1, ERF.E2, and ERF.E4, have been identified as the most active ERFs in RIN/NOR- and ethylene-dependent ripening, and all sub-class-E ERFs share the presence of the N-terminal MCGGAII/L motif as a distinctive feature (Liu *et al.*, 2016). This motif renders sub-class-E ERFs as targets of post-translational degradation through the N-end rule pathway (NERP) in the presence of NO or oxygen (Licausi *et al.*, 2011; Gibbs *et al.*, 2014, 2015; Weits *et al.*, 2014; Gasch *et al.*, 2016). As a consequence, ERFs containing the N-terminal MCGGAII/L motif have been proposed to act as NO sensors during seed germination, stomatal closure and hypocotyl elongation in Arabidopsis (Gibbs *et al.*, 2014). Whether a similar role as NO sensor can also be attributed to sub-class-E ERFs in fruit ripening remains an important question for future investigation.

Many fruit attributes developed during climacteric ripening strictly depend on the presence and perception of ethylene. Consequently, approaches based on the direct inhibition of ethylene production and perception often leads to irreversible negative impacts on the development of aroma, color and nutritional content in climacteric fruits (Kovács *et al.*, 2009; Cliff *et al.*, 2009; Sivakumar *et al.*, 2010; Xu *et al.*, 2016; Cai *et al.*, 2019). Of particular relevance from the fruit post-harvest perspective, the repressor role of NO on ethylene-related gene expression was restricted to the start of the ripening phase (Bk stage), and this transcriptional pattern was reflected in ethylene- and RIN/NOR-dependent downstream processes, including the expression of genes involved in fruit softening (*PG2a*, *PL*), phenylpropanoid metabolism (*PAL*, *CHS*, *FLS*), and fruit aroma (*TomLOX*, *HPL*, *ADH2*, *CCD*s).

Nitric oxide differentially regulates central players of tomato fruit nitro-oxidative metabolism

Carotenoids, tocopherols, flavonoids and ascorbate are significant contributors of tomato fruit antioxidant capacity (Shi *et al.*, 2004; Moco *et al.*, 2006; Liu *et al.*, 2015*b*; Saini *et al.*, 2015; Cruz *et al.*, 2018), and their metabolic pathways have been extensively studied in tomato fruits under various circumstances (Almeida *et al.*, 2011; Quadrana *et al.*, 2013; Gramegna *et al.*, 2019; Alves *et al.*, 2020), except under NO treatment conditions (Eum *et al.*, 2009; Palma *et al.*, 2019).

Amongst all ethylene-dependent downstream processes analyzed, only the accumulation of lycopene was persistently repressed in NO-treated fruits, which might be linked to three metabolic differences detected in these fruits. Firstly, the enzyme PDS has been shown to become limiting in tomato fruit carotenogenesis, once PSY1 is elevated during ripening (McQuinn *et al.*, 2018). In NO-treated fruits, *PSY1* expression was completely recovered from Bk3 onwards, mirroring the mRNA

abundance profile of ripening-related TFs extensively associated with tomato fruit carotenogenesis, such as RIN, NOR and ERF.E4 (Liu *et al.*, 2015*b*). In contrast, *PDS* was down-regulated in NO-treated fruits at all ripening stages, possibly limiting the poly-*cis*-transformation of phytoene to lycopene, which is one of the bottlenecks in tomato fruit carotenogenesis (McQuinn *et al.*, 2018). Secondly, chromoplasts are intrinsically associated with both the synthesis and accumulation of carotenoids in tomato fruits (Fitter *et al.*, 2002; Nguyen *et al.*, 2014), and the delayed conversion of chloroplasts into chromoplasts, as indicated by the retention of the green shoulder at the pedicellar region and retarded chlorophyll degradation (Fig. 1), may also be linked to the impaired accumulation of lycopene in NO-treated fruits. As a third possibility, *GGDR* was persistently up-regulated in response to NO, suggesting that GGDP was permanently diverted to sustain chlorophyll and tocopherol production rather than carotenogenesis. Tocopherols are known to limit the propagation of lipid peroxidation (Munné-Bosch and Alegre, 2002), which may offer additional protection against the oxidative burst typically associated with climacteric ripening (Corpas *et al.*, 2018).

Amongst water-soluble antioxidants, phenylpropanoid biosynthesis is promoted by NO in different fruit species (Wang *et al.*, 2013; Li *et al.*, 2017), and in some cases, leads to reduced susceptibility to pathogen attack during post-harvest (Li *et al.*, 2017; Hu *et al.*, 2019). In this study in tomato, NO treatment caused an overall down-regulation of phenylpropanoid biosynthetic genes at the beginning of the ripening phase, which was followed by the up-regulation of these same genes at later ripening stages, and higher accumulation of flavonoids at the ripe stage (Fig. 5). As fruits from anthocyanin-overaccumulating tomato lines are known to display suppressed overripening and increase the fruit resistance towards pathogens (Zhang *et*

al., 2013*a*; Bassolino *et al.*, 2013), the NO-triggered increase in phenolics accumulation described here may be associated with the altered resistance to fungal pathogens reported for tomato fruits treated with chemical inhibitors of NO synthesis (Zheng *et al.*, 2011; Li *et al.*, 2020).

Ubiquitously distributed within cell compartments, ascorbate counteracts excessive ROS generation during fruit ripening, thereby contributing to maintaining fruit integrity as ripening progresses (Palma *et al.*, 2015). In sweet pepper, a NO-enriched atmosphere enhanced ascorbate content in about 40% within the fruit tissues, a response associated with higher gene expression and enzyme activity of L-galactono-1,4-lactone dehydrogenase (GalLDH; Rodríguez-Ruiz *et al.*, 2017). Here, we also report the overaccumulation of ascorbate in NO-treated fruits, which was linked to the up-regulation of other key biosynthetic genes rather than *GalLDH*. The oxidation of reduced ascorbate to dehydroascorbic acid via APX activity is a central reaction in the ascorbate–glutathione cycle. As fruits ripen, APX, CAT, and SOD activities fluctuate, thereby influencing the fruit tissue capacity to breakdown H_2O_2 into water (Jimenez *et al.*, 2002; Kumar *et al.*, 2016). As the NO-induced H_2O_2 overaccumulation in ripening tomato fruits was accompanied by a reduction of APX, and to a lesser extent CAT, activities and higher abundance and diversity of *S*-nitrosated and nitrated proteins, our findings suggest that these two enzymes are at the crossroad between nitrosative and oxidative metabolism during tomato fruit ripening. In agreement, both APX and CAT have been reported as central targets of RNS-mediated PTMs in plants (Clark *et al.*, 2000; Begara-Morales *et al.*, 2014; Chaki *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2019), and our findings derived from *in-vitro* assays revealed that nitration and *S*-nitrosation events repress, to distinct degrees, the activities of both these enzymes (Fig. 3F).

GSNOR is a key enzyme to control the concentration of *S*-nitrosothiols under physiological and stress conditions (Leterrier *et al.*, 2011; Xu *et al.*, 2013; Hussain *et al.*, 2019). Evidence from an *in vitro* study using an Arabidopsis recombinant GSNOR indicated that this enzyme undergoes *S*-nitrosation in the presence of NO donors, leading to inhibition of enzyme activity (Guerra *et al.*, 2016). In contrast, in our experimental design, exogenous NO exerted a positive influence on GSNOR activity at all ripening stages. However, it is important to highlight that *S*-nitrosation affects thiol groups of cysteine residues, which are also targets of other molecules in a cellular environment, including hydrogen sulfide, glutathione and H_2O_2, leading to persulfidation, glutathionylation and sulfenation, respectively (Corpas *et al.*, 2019). Therefore, our findings suggest that the post-translational regulation of GSNOR activity *in planta* is more complex than previously anticipated from data obtained under *in vitro*, cell-free conditions (recombinant purified proteins, Guerra *et al.*, 2016; Tichá *et al.*, 2017). A similar suggestion could be made for APX, whose activity was down-regulated by NO in tomato fruits (Fig. 3B), whereas in *vitro* assays with purified recombinant APX in the presence of NO donors exerted a positive effect (Begara-Morales *et al.*, 2013).

Nitric oxide supplementation preserves biochemical pathways related to tomato flavor

Both volatiles and non-volatile metabolites determine tomato flavor (Tieman *et al.*, 2017). Among the non-volatiles, the balance between sugars and acids, as well as the abundance of glutamate and aspartate, are strongly associated with tomato organoleptic quality (Carli *et al.*, 2009; Sorrequieta *et al.*, 2010). As observed in apple and peach (Deng *et al.*, 2013; Han *et al.*, 2018), NO-treated tomato fruits displayed extensive transcriptional changes in sugar-related genes, including key components of

sucrose biosynthesis and degradation. However, in the case of tomato, no significant changes in sucrose, glucose, and fructose were triggered by NO treatment (Fig. 6). In line with our findings, metabolomic analysis performed in ripening fruits of tomato *short root* (*shr*) mutant, which hyperaccumulates NO, revealed similar amounts of these major carbohydrates, compared with the wild type (Bodanapu *et al.*, 2016). Ripening *shr* fruits exhibited increased accumulation of citrate and *cis*-aconitate, which are intermediates of the tricarboxylic acid (TCA) cycle, and amino acids such as tyrosine, asparagine, and glutamine (Bodanapu *et al.*, 2016), none of which were detected here as being altered in NO-treated fruits. In *shr* plants, NO concentrations are presumed to be constitutively high (Negi *et al.*, 2010, 2011), which differs from the short-term (96 h) exposure of pre-ripe fruits to the NO-enriched atmosphere employed in the present study.

Fatty acids, sterols, and alkanes are precursors of cuticular wax and volatile compounds (Valle *et al.*, 2006; Ties and Barringer, 2012; Fernández *et al.*, 2016). Regarded as the most important fatty acid in tomato peels for the formation of volatiles (Ties and Barringer, 2012), linolenic acid concentrations were not affected by NO treatment, which correlates well with the similar concentrations of lipid-derived volatile compounds detected in NO-treated and control fruits. In fact, volatile compound profiling suggested that aroma losses may be minimal, if any, in tomato fruits exposed to a NO-enriched atmosphere, which might have been facilitated by the NO-induced up-regulation of key biosynthetic enzymes at critical ripening stages (Bk3 and Bk7).

1.5. Conclusions

In contrast to the overall reduction in fruit nutritional value, taste, and particularly aroma, typically observed under certain post-harvest practices, such as cold storage

and the use of ethylene perception inhibitors (Mir *et al.*, 2004; Rugkong *et al.*, 2011; Farneti *et al.*, 2015), impaired lycopene accumulation was identified as the main negative effect of post-harvest NO treatment, as other metabolic pathways were either unaffected (e.g. tocopherols, sugars, amino acids, volatiles) or even improved (e.g. flavonoids, ascorbate). Besides providing additional nutraceutical value to the fruit, the NO-induced increases in the content of total ascorbate (25%) and flavonoids (60%) may impact the fruit resistance towards pathogens, which remains one exciting target for future research.

Multiple parallels between the effects of NO on tomato (climacteric fruit) and pepper (non-climacteric fruit, Rodríguez-Ruiz *et al.*, 2017; Corpas *et al.*, 2018); have been observed (e.g. modulation of ascorbate metabolism and ROS/RNS-related enzymes). However, as a model climacteric species, tomato allowed us to expand the landscape of the NO-ethylene interactions during fruit ripening, by revealing that NO represses not only the biosynthesis, but also the perception and signaling of this hormone. By exerting a dynamic control on ethylene and ripening-associated developmental regulators (e.g. RIN, NOR, CNR), NO triggers extensive alterations in the fruit transcriptome, protein activities and consequently metabolomic profile. This reinforces the biotechnological potential of this multifaceted RNS as a promising alternative to adjust post-harvest longevity, while preserving the nutritional quality, taste and aroma of fleshy fruits.

Acknowledgements

This work was supported by the São Paulo Research Foundation (FAPESP) (grants 2018/16389-8, 2016/04924-0, 2017/17935-3 and 2016/01128-9), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grants 422287/2018-0,

305012/2018-5, 303332/2019-0 and 300986/2018-1), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. The research work of FJC and JMP is supported by a European Regional Development Fund-cofinanced grant from the Ministry of Economy and Competitiveness (AGL2015-65104-P and PID2019-103924GB-I00), Spain.

Author contributions

LF and MR conceived the project and supervised the experiments; RZ and MRR conducted most of the experiments; PJLO and GBP conducted part of the experiments; CMF, EP, and GBP provided technical assistance and contributed to data analysis; JMP and FJC contributed to the data analysis and discussion; SCSA conducted most bioinformatics analysis; LF, RZ, MRR, and MR wrote the article with contributions from other authors.

References

Aebi H. 1984. [13] Catalase in vitro. Oxygen Radicals in Biological Systems. Methods in Enzymology. Academic Press, 121–126.

Aghdam MS, Luo Z, Jannatizadeh A, Sheikh-Assadi M, Sharafi Y, Farmani B, Fard JR, Razavi F. 2019. Employing exogenous melatonin applying confers chilling tolerance in tomato fruits by upregulating ZAT2/6/12 giving rise to promoting endogenous polyamines, proline, and nitric oxide accumulation by triggering arginine pathway activity. Food Chemistry **275**, 549–556.

Almeida J, Quadrana L, Asís R, *et al.* 2011. Genetic dissection of vitamin E biosynthesis in tomato. Journal of Experimental Botany **62**, 3781–3798.

Alves FRR, Lira BS, Pikart FC, *et al.* 2020. Beyond the limits of photoperception: constitutively active PHYTOCHROME B2 overexpression as a means of improving fruit nutritional quality in tomato. Plant Biotechnology Journal **18**, 2027–2041.

Baldwin EA, Scott JW, Shewmaker CK, Schuch W. 2000. Flavor Trivia and Tomato Aroma: Biochemistry and Possible Mechanisms for Control of Important Aroma Components. HortScience **35**, 1013–1022.

Bassolino L, Zhang Y, Schoonbeek H, Kiferle C, Perata P, Martin C. 2013. Accumulation of anthocyanins in tomato skin extends shelf life. New Phytologist **200**, 650–655.

Beauchamp C, Fridovich I. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry **44**, 276–287.

Begara-Morales JC, Sánchez-Calvo B, Chaki M, Valderrama R, Mata-Pérez C, López-Jaramillo J, Padilla MN, Carreras A, Corpas FJ, Barroso JB. 2013. Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and *S* -nitrosylation. Journal of Experimental Botany **65**, 527–538.

Begara-Morales JC, Sánchez-Calvo B, Chaki M, Valderrama R, Mata-Pérez C, López-Jaramillo J, Padilla MN, Carreras A, Corpas FJ, Barroso JB. 2014. Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and *S* -nitrosylation. Journal of Experimental Botany **65**, 527–538.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) **57**, 289–300.

Bianchetti RE, Cruz AB, Oliveira BS, Demarco D, Purgatto E, Peres LEP, Rossi M, Freschi L. 2017. Phytochromobilin deficiency impairs sugar metabolism through the regulation of cytokinin and auxin signaling in tomato fruits. Scientific Reports **7**.

Bodanapu R, Gupta SK, Basha PO, Sakthivel K, Sadhana, Sreelakshmi Y, Sharma R. 2016. Nitric Oxide Overproduction in Tomato shr Mutant Shifts Metabolic Profiles and Suppresses Fruit Growth and Ripening. Frontiers in Plant Science **7**.

Boscari A, del Giudice J, Ferrarini A, Venturini L, Zaffini A-L, Delledonne M, Puppo A. 2012. Expression Dynamics of the *Medicago truncatula* Transcriptome during the Symbiotic Interaction with *Sinorhizobium meliloti*: Which Role for Nitric Oxide? Plant Physiology **161**, 425–439.

Buttery RG, Ling LC. 1993. Volatile Components of Tomato Fruit and Plant Parts: Relationship and Biogenesis. In: Teranishi R,, In: Buttery RG,, In: Sugisawa H, eds. Bioactive Volatile Compounds from Plants. Washington, DC: American Chemical Society, 23–34.

Cai H, Han S, Jiang L, Yu M, Ma R, Yu Z. 2019. 1-MCP treatment affects peach fruit aroma metabolism as revealed by transcriptomics and metabolite analyses. Food Research International **122**, 573–584.

Carli P, Arima S, Fogliano V, Tardella L, Frusciante L, Ercolano MR. 2009. Use of network analysis to capture key traits affecting tomato organoleptic quality. Journal of Experimental Botany **60**, 3379–3386.

Chaki M, Álvarez de Morales P, Ruiz C, Begara-Morales JC, Barroso JB, Corpas FJ, Palma JM. 2015. Ripening of pepper (Capsicum annuum) fruit is characterized by an enhancement of protein tyrosine nitration. Annals of Botany **116**, 637–647.

Cheng G, Yang E, Lu W, Jia Y, Jiang Y, Duan X. 2009. Effect of Nitric Oxide on Ethylene Synthesis and Softening of Banana Fruit Slice during Ripening. Journal of Agricultural and Food Chemistry **57**, 5799–5804.

Cherian S, Figueroa CR, Nair H. 2014. 'Movers and shakers' in the regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric fruit. Journal of Experimental Botany **65**, 4705–4722.

Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, Wishart DS, Xia J. 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Research **46**, W486–W494.

Clark D, Durner J, Navarre DA, Klessig DF. 2000. Nitric Oxide Inhibition of Tobacco Catalase and Ascorbate Peroxidase. Molecular Plant-Microbe Interactions **13**, 1380–1384.

Cliff M, Lok S, Lu C, Toivonen PMA. 2009. Effect of 1-methylcyclopropene on the sensory, visual, and analytical quality of greenhouse tomatoes. Postharvest Biology and Technology **53**, 11–15.

Corpas FJ, Barroso JB. 2013. Nitro-oxidative stress vs oxidative or nitrosative stress in higher plants. New Phytologist **199**, 633–635.

Corpas FJ, Freschi L, Rodríguez-Ruiz M, Mioto PT, González-Gordo S, Palma JM. 2018. Nitro-oxidative metabolism during fruit ripening. Journal of Experimental Botany, 1–15.

Corpas FJ, González-Gordo S, Cañas A, Palma JM. 2019. Nitric oxide and hydrogen sulfide in plants: which comes first? (R Brouquisse, Ed.). Journal of Experimental Botany **70**, 4391–4404.

Corpas FJ, Palma JM. 2018. Nitric oxide on/off in fruit ripening (JTM Elzenga, Ed.). Plant Biology **20**, 805–807.

Corpas FJ, Palma JM. 2020. H2S signaling in plants and applications in agriculture. Journal of Advanced Research **24**, 131–137.

Cruz AB, Bianchetti RE, Alves FRR, Purgatto E, Peres LEP, Rossi M, Freschi L. 2018. Light, Ethylene and Auxin Signaling Interaction Regulates Carotenoid Biosynthesis During Tomato Fruit Ripening. Frontiers in Plant Science **9**.

Deng L, Pan X, Chen L, Shen L, Sheng J. 2013. Effects of preharvest nitric oxide treatment on ethylene biosynthesis and soluble sugars metabolism in 'Golden Delicious' apples. Postharvest Biology and Technology **84**, 9–15.

Eum HL, Kim HB, Choi SB, Lee SK. 2009. Regulation of ethylene biosynthesis by nitric oxide in tomato (Solanum lycopersicum L.) fruit harvested at different ripening stages. European Food Research and Technology **228**, 331–338.

Farneti B, Alarcón AA, Papasotiriou FG, Samudrala D, Cristescu SM, Costa G, Harren FJM, Woltering EJ. 2015. Chilling-Induced Changes in Aroma Volatile Profiles in Tomato. Food and Bioprocess Technology **8**, 1442–1454.

Fernández V, Guzmán-Delgado P, Graça J, Santos S, Gil L. 2016. Cuticle Structure in Relation to Chemical Composition: Re-assessing the Prevailing Model. Frontiers in Plant Science **7**.

Fitter DW, Martin DJ, Copley MJ, Scotland RW, Langdale JA. 2002. GLK gene pairs regulate chloroplast development in diverse plant species. The Plant Journal **31**, 713–727.

Fujisawa M, Shima Y, Higuchi N, Nakano T, Koyama Y, Kasumi T, Ito Y. 2012. Direct targets of the tomato-ripening regulator RIN identified by transcriptome and chromatin immunoprecipitation analyses. Planta **235**, 1107–1122.

Gapper NE, McQuinn RP, Giovannoni JJ. 2013. Molecular and genetic regulation of fruit ripening. Plant Molecular Biology **82**, 575–591.

Gasch P, Fundinger M, Müller JT, Lee T, Bailey-Serres J, Mustroph A. 2016. Redundant ERF-VII Transcription Factors Bind to an Evolutionarily Conserved *cis* -Motif to Regulate Hypoxia-Responsive Gene Expression in Arabidopsis. The Plant Cell **28**, 160–180.

Gibbs DJ, Conde JV, Berckhan S, Prasad G, Mendiondo GM, Holdsworth MJ. 2015. Group VII Ethylene Response Factors Coordinate Oxygen and Nitric Oxide Signal Transduction and Stress Responses in Plants. Plant Physiology **169**, 23–31.

Gibbs DJ, Md Isa N, Movahedi M, *et al.* 2014. Nitric Oxide Sensing in Plants Is Mediated by Proteolytic Control of Group VII ERF Transcription Factors. Molecular Cell **53**, 369–379.

González-Gordo S, Bautista R, Claros MG, Cañas A, Palma JM, Corpas FJ. 2019. Nitric oxide-dependent regulation of sweet pepper fruit ripening. Journal of Experimental Botany.

Gramegna G, Rosado D, Sánchez Carranza AP, Cruz AB, Simon-Moya M, Llorente B, Rodríguez-Concepcíon M, Freschi L, Rossi M. 2019. PHYTOCHROME-INTERACTING FACTOR 3 mediates light-dependent induction of tocopherol biosynthesis during tomato fruit ripening. Plant, Cell & Environment **42**, 1328–1339.

Groß F, Durner J, Gaupels F. 2013. Nitric oxide, antioxidants and prooxidants in plant defence responses. Frontiers in Plant Science **4**.

Guerra D, Ballard K, Truebridge I, Vierling E. 2016. S-Nitrosation of Conserved Cysteines Modulates Activity and Stability of S-Nitrosoglutathione Reductase (GSNOR). Biochemistry **55**, 2452–2464.

Han S, Cai H, An X, Huan C, Wu X, Jiang L, Yu M, Ma R, Yu Z. 2018. Effect of nitric oxide on sugar metabolism in peach fruit (cv. Xiahui 6) during cold storage. Postharvest Biology and Technology **142**, 72–80.

Hu M, Zhu Y, Liu G, Gao Z, Li M, Su Z, Zhang Z. 2019. Inhibition on anthracnose and induction of defense response by nitric oxide in pitaya fruit. Scientia Horticulturae **245**, 224–230.

Huang J, Wei H, Li L, Yu S. 2018. Transcriptome analysis of nitric oxide-responsive genes in upland cotton (Gossypium hirsutum). PLOS ONE **13**, e0192367.

Hussain A, Yun B-W, Kim JH, Gupta KJ, Hyung N-I, Loake GJ. 2019. Novel and conserved functions of S-nitrosoglutathione reductase in tomato. Journal of Experimental Botany **70**, 4877–4886.

Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. 2001. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nature cell biology **3**.

Jimenez A, Creissen G, Kular B, Firmin J, Robinson S, Verhoeyen M, Mullineaux P. 2002. Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. Planta **214**, 751–758.

Kang R, Zhang L, Jiang L, Yu M, Ma R, Yu Z. 2016. Effect of postharvest nitric oxide treatment on the proteome of peach fruit during ripening. Postharvest Biology and Technology **112**, 277–289.

Kolbert Z, Barroso JB, Brouquisse R, *et al.* 2019. A forty year journey: The generation and roles of NO in plants. Nitric Oxide **93**, 53–70.

Kovács K, Fray RG, Tikunov Y, Graham N, Bradley G, Seymour GB, Bovy AG, Grierson D. 2009. Effect of tomato pleiotropic ripening mutations on flavour volatile biosynthesis. Phytochemistry **70**, 1003–1008.

Kumar V, Irfan M, Ghosh S, Chakraborty N, Chakraborty S, Datta A. 2016. Fruit ripening mutants reveal cell metabolism and redox state during ripening. Protoplasma **253**, 581–594.

Lai T, Wang Y, Li B, Qin G, Tian S. 2011. Defense responses of tomato fruit to exogenous nitric oxide during postharvest storage. Postharvest Biology and Technology **62**, 127–132.

Lara I, Belge B, Goulao LF. 2014. The fruit cuticle as a modulator of postharvest quality. Postharvest Biology and Technology **87**, 103–112.

Leshem YY, Pinchasov Y. 2000. Non-invasive photoacoustic spectroscopic determination of relative endogenous nitric oxide and ethylene content stoichiometry during the ripening of strawberries Fragaria anannasa (Duch.) and avocados Persea americana (Mill.). Journal of Experimental Botany **51**, 1471–1473.

Leshem YY, Wills RBH. 1998. Harnessing Senescence Delaying Gases Nitric Oxide and Nitrous Oxide: A Novel Approach to Postharvest Control of Fresh Horticultural Produce. Biologia Plantarum **41**, 1–10.

Leterrier M, Chaki M, Airaki M, Valderrama R, Palma JM, Barroso JB, Corpas FJ. 2011. Function of S-nitrosoglutathione reductase (GSNOR) in plant development and under biotic/abiotic stress. Plant Signaling & Behavior **6**, 789–793.

Li S, Chen K, Grierson D. 2019. A critical evaluation of the role of ethylene and MADS transcription factors in the network controlling fleshy fruit ripening. New Phytologist **221**, 1724–1741.

Li R, Sheng J, Shen L. 2020. Nitric Oxide Plays an Important Role in β-Aminobutyric Acid-Induced Resistance to Botrytis cinerea in Tomato Plants. The Plant Pathology Journal **36**, 121–132.

Li G, Zhu S, Wu W, Zhang C, Peng Y, Wang Q, Shi J. 2017. Exogenous nitric oxide induces disease resistance against Monilinia fructicola through activating the phenylpropanoid pathway in peach fruit. Journal of the Science of Food and Agriculture **97**, 3030–3038.

Licausi F, Kosmacz M, Weits DA, Giuntoli B, Giorgi FM, Voesenek LACJ, Perata P, van Dongen JT. 2011. Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilization. Nature **479**, 419–422.

Lin Z, Hong Y, Yin M, Li C, Zhang K, Grierson D. 2008. A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. The Plant Journal **55**, 301–310.

Lira BS, Gramegna G, Trench BA, *et al.* 2017. Manipulation of a Senescence-Associated Gene Improves Fleshy Fruit Yield1[OPEN]. Plant Physiology **175**, 77–91.

Liu M, Lima Gomes B, Mila I, *et al.* 2016. Comprehensive profiling of Ethylene Response Factors expression identifies ripening-associated ERF genes and their link to key regulators of fruit ripening in tomato (Solanum lycopersicum). Plant Physiology, pp.01859.2015.

Liu Y-B, Oh S, Jurick WM. 2019*a*. Response of Aspergillus flavus spores to nitric oxide fumigations in atmospheres with different oxygen concentrations. Journal of Stored Products Research **83**, 78–83.

Liu M, Pirrello J, Chervin C, Roustan J-P, Bouzayen M. 2015*a*. Ethylene Control of Fruit Ripening: Revisiting the Complex Network of Transcriptional Regulation1. Plant Physiology **169**, 2380–2390.

Liu L, Shao Z, Zhang M, Wang Q. 2015*b*. Regulation of Carotenoid Metabolism in Tomato. Molecular Plant **8**, 28–39.

Liu J, Yang J, Zhang H, Cong L, Zhai R, Yang C, Wang Z, Ma F, Xu L. 2019*b*. Melatonin Inhibits Ethylene Synthesis via Nitric Oxide Regulation To Delay Postharvest Senescence in Pears. Journal of Agricultural and Food Chemistry **67**, 2279–2288.

Lopes-Oliveira PJ, Gomes DG, Pelegrino MT, Bianchini E, Pimenta JA, Stolf-Moreira R, Seabra AB, Oliveira HC. 2019. Effects of nitric oxide-releasing nanoparticles on neotropical tree seedlings submitted to acclimation under full sun in the nursery. Scientific Reports **9**, 17371.

Manjunatha G, Gupta KJ, Lokesh V, Mur LA, Neelwarne B. 2012. Nitric oxide counters ethylene effects on ripening fruits. Plant Signaling & Behavior **7**, 476–483.

Manjunatha G, Lokesh V, Neelwarne B. 2010. Nitric oxide in fruit ripening: Trends and opportunities. Biotechnology Advances **28**, 489–499.

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ. 2011. The Tomato MADS-Box Transcription Factor RIPENING INHIBITOR Interacts with Promoters Involved in Numerous Ripening Processes in a COLORLESS NONRIPENING-Dependent Manner. PLANT PHYSIOLOGY **157**, 1568–1579.

McQuinn RP, Wong B, Giovannoni JJ. 2018. AtPDS overexpression in tomato: exposing unique patterns of carotenoid self-regulation and an alternative strategy for the enhancement of fruit carotenoid content. Plant Biotechnology Journal **16**, 482–494.

Melo NKG, Bianchetti RE, Lira BS, Oliveira PMR, Zuccarelli R, Dias DLO, Demarco D, Peres LEP, Rossi M, Freschi L. 2016. Nitric Oxide, Ethylene, and Auxin Cross Talk Mediates Greening and Plastid Development in Deetiolating Tomato Seedlings. Plant Physiology **170**, 2278–2294.

Mi H, Muruganujan A, Huang X, Ebert D, Mills C, Guo X, Thomas PD. 2019. Protocol Update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). Nature Protocols **14**, 703–721.

Mir N, Canoles M, Beaudry R, Baldwin E, Mehla CP. 2004. Inhibiting Tomato Ripening with 1-Methylcyclopropene. Journal of the American Society for Horticultural Science **129**, 112–120.

Miyake C, Asada K. 1996. Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of Ascorbate; Hydrogen Peroxide Decomposes Compound I of Ascorbate Peroxidase. Plant and Cell Physiology **37**, 423–430.

Moco S, Bino RJ, Vorst O, Verhoeven HA, de Groot J, van Beek TA, Vervoort J, de Vos CHR. 2006. A Liquid Chromatography-Mass Spectrometry-Based Metabolome Database for Tomato. Plant Physiology **141**, 1205–1218.

Mukherjee S. 2019. Recent advancements in the mechanism of nitric oxide signaling associated with hydrogen sulfide and melatonin crosstalk during ethylene-induced fruit ripening in plants. Nitric Oxide **82**, 25–34.

Munné-Bosch S, Alegre L. 2002. The Function of Tocopherols and Tocotrienols in Plants. Critical Reviews in Plant Sciences **21**, 31–57.

Negi S, Kharshiing EV, Sharma R. 2011. NO way! Is nitric oxide level in tomato regulated by a mammalian IKK/NF-κB like signaling pathway?: Is nitric oxide level in tomato regulated by a mammalian IKK/NFκB-like signaling pathway? Plant Signaling & Behavior **6**, 1049–1052.

Negi S, Santisree P, Kharshiing EV, Sharma R. 2010. Inhibition of the Ubiquitin—Proteasome Pathway Alters Cellular Levels of Nitric Oxide in Tomato Seedlings. Molecular Plant **3**, 854–869.

Nguyen CV, Vrebalov JT, Gapper NE, Zheng Y, Zhong S, Fei Z, Giovannoni JJ. 2014. Tomato GOLDEN2-LIKE Transcription Factors Reveal Molecular Gradients That Function during Fruit Development and Ripening[W][OPEN]. The Plant Cell **26**, 585–601.

Osorio S, Alba R, Damasceno CMB, *et al.* 2011. Systems Biology of Tomato Fruit Development: Combined Transcript, Protein, and Metabolite Analysis of Tomato Transcription Factor (*nor, rin*) and Ethylene Receptor (*Nr*) Mutants Reveals Novel Regulatory Interactions. Plant Physiology **157**, 405–425.

Palma JM, Freschi L, Rodríguez-Ruiz M, González-Gordo S, Corpas FJ. 2019. Nitric oxide in the physiology and quality of fleshy fruits (R Brouquisse, Ed.). Journal of Experimental Botany **70**, 4405–4417.

Palma JM, Ruiz C, Corpas FJ. 2018. A Simple and Useful Method to Apply Exogenous NO Gas to Plant Systems: Bell Pepper Fruits as a Model. In: Mengel A,, In: Lindermayr C, eds. Methods in Molecular Biology. Nitric Oxide. New York, NY: Springer New York, 3–11.

Palma JM, Sevilla F, Jiménez A, del Río LA, Corpas FJ, Álvarez de Morales P, Camejo DM. 2015. Physiology of pepper fruit and the metabolism of antioxidants: chloroplasts, mitochondria and peroxisomes. Annals of Botany **116**, 627–636.

Quadrana L, Almeida J, Otaiza SN, *et al.* 2013. Transcriptional regulation of tocopherol biosynthesis in tomato. Plant Molecular Biology **81**, 309–325.

Rambla JL, Tikunov YM, Monforte AJ, Bovy AG, Granell A. 2014. The expanded tomato fruit volatile landscape. Journal of Experimental Botany **65**, 4613–4623.

Rodríguez-Ruiz M, González-Gordo S, Cañas A, Campos MJ, Paradela A, Corpas FJ, Palma JM. 2019. Sweet Pepper (Capsicum annuum L.) Fruits Contain an Atypical Peroxisomal Catalase That Is Modulated by Reactive Oxygen and Nitrogen Species. Antioxidants **8**, 374.

Rodríguez-Ruiz M, Mateos RM, Codesido V, Corpas FJ, Palma JM. 2017. Characterization of the galactono-1,4-lactone dehydrogenase from pepper fruits and its modulation in the ascorbate biosynthesis. Role of nitric oxide. Redox Biology **12**, 171–181.

Rodríguez-Ruiz M, Mioto P, Palma JM, Corpas FJ. 2016. S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (Capsicum annuum L.) fruit ripening. Nitric Oxide **68**, 51–55.

Rudell DR, Mattheis JP. 2006. Nitric Oxide and Nitrite Treatments Reduce Ethylene Evolution from Apple Fruit Disks. HortScience **41**, 1462–1465.

Rugkong A, McQuinn R, Giovannoni JJ, Rose JKC, Watkins CB. 2011. Expression of ripening-related genes in cold-stored tomato fruit. Postharvest Biology and Technology **61**, 1–14.

Saini RK, Nile SH, Park SW. 2015. Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. Food Research International **76**, 735–750.

Saladie M, Matas AJ, Isaacson T, *et al.* 2007. A Reevaluation of the Key Factors That Influence Tomato Fruit Softening and Integrity. PLANT PHYSIOLOGY **144**, 1012–1028.

Schaffer AA, Petreikov M. 1997. Sucrose-to-Starch Metabolism in Tomato Fruit Undergoing Transient Starch Accumulation. Plant Physiology **113**, 739–746.

Schwacke R, Ponce-Soto GY, Krause K, Bolger AM, Arsova B, Hallab A, Gruden K, Stitt M, Bolger ME, Usadel B. 2019. MapMan4: A Refined Protein Classification and Annotation Framework Applicable to Multi-Omics Data Analysis. Molecular Plant **12**, 879–892.

Shi J, Kakuda Y, Yeung D. 2004. Antioxidative properties of lycopene and other carotenoids from tomatoes: Synergistic effects. BioFactors **21**, 203–210.

Shima Y, Kitagawa M, Fujisawa M, Nakano T, Kato H, Kimbara J, Kasumi T, Ito Y. 2013. Tomato FRUITFULL homologues act in fruit ripening via forming MADS-box transcription factor complexes with RIN. Plant Molecular Biology **82**, 427–438.

Silva Souza MA, Peres LE, Freschi JR, Purgatto E, Lajolo FM, Hassimotto NM. 2020. Changes in flavonoid and carotenoid profiles alter volatile organic compounds in purple and orange cherry tomatoes obtained by allele introgression. Journal of the Science of Food and Agriculture **100**, 1662–1670.

Sivakumar D, Terry LA, Korsten L. 2010. An Overview on Litchi Fruit Quality and Alternative Postharvest Treatments to Replace Sulfur Dioxide Fumigation. Food Reviews International **26**, 162–188.

Sorrequieta A, Ferraro G, Boggio SB, Valle EM. 2010. Free amino acid production during tomato fruit ripening: a focus on l-glutamate. Amino Acids **38**, 1523–1532.

Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. PLOS ONE **6**, e21800.

Szabados L, Savouré A. 2010. Proline: a multifunctional amino acid. Trends in Plant Science **15**, 89–97.

Tanou G, Minas IS, Karagiannis E, Tsikou D, Audebert S, Papadopoulou KK, Molassiotis A. 2015. The impact of sodium nitroprusside and ozone in kiwifruit ripening physiology: a combined gene and protein expression profiling approach. Annals of Botany **116**, 649–662.

Tichá T, Lochman J, Činčalová L, Luhová L, Petřivalský M. 2017. Redox regulation of plant S-nitrosoglutathione reductase activity through post-translational modifications of cysteine residues. Biochemical and Biophysical Research Communications **494**, 27–33.

Tieman DM, Loucas HM, Kim JY, Clark DG, Klee HJ. 2007. Tomato phenylacetaldehyde reductases catalyze the last step in the synthesis of the aroma volatile 2-phenylethanol. Phytochemistry **68**, 2660–2669.

Tieman D, Zhu G, Resende MFR, *et al.* 2017. A chemical genetic roadmap to improved tomato flavor. Science **355**, 391–394.

Ties P, Barringer S. 2012. Influence of Lipid Content and Lipoxygenase on Flavor Volatiles in the Tomato Peel and Flesh. Journal of Food Science **77**, C830–C837.

Valle MD, Cámara M, Torija M-E. 2006. Chemical characterization of tomato pomace. Journal of the Science of Food and Agriculture **86**, 1232–1236.

Vogel JT, Tieman DM, Sims CA, Odabasi AZ, Clark DG, Klee HJ. 2010. Carotenoid content impacts flavor acceptability in tomato (Solanum lycopersicum). Journal of the Science of Food and Agriculture **90**, 2233–2240.

Vogg G, Fischer S, Leide J, Emmanuel E, Jetter R, Levy AA, Riederer M. 2004. Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid -ketoacyl-CoA synthase. Journal of Experimental Botany **55**, 1401–1410.

Wang R, Angenent GC, Seymour G, de Maagd RA. 2020. Revisiting the Role of Master Regulators in Tomato Ripening. Trends in Plant Science **25**, 291–301.

Wang Y, Loake GJ, Chu C. 2013. Cross-talk of nitric oxide and reactive oxygen species in plant programed cell death. Frontiers in Plant Science **4**.

Weits DA, Giuntoli B, Kosmacz M, Parlanti S, Hubberten H-M, Riegler H, Hoefgen R, Perata P, van Dongen JT, Licausi F. 2014. Plant cysteine oxidases control the oxygen-dependent branch of the N-end-rule pathway. Nature Communications **5**, 3425.

Wills RBH, Ku VVV, Leshem YY. 2000. Fumigation with nitric oxide to extend the postharvest life of strawberries. Postharvest Biology and Technology **18**, 75–79.

Xu S, Guerra D, Lee U, Vierling E. 2013. S-nitrosoglutathione reductases are low-copy number, cysteine-rich proteins in plants that control multiple developmental and defense responses in Arabidopsis. Frontiers in Plant Science **4**.

Xu F, Liu S, Feng X. 2016. Effect of 1-octylcyclopropene on physiological responses and expression of ethylene receptors gene in harvested tomato fruit. Postharvest Biology and Technology **117**, 30–37.

Yao G-F, Li C, Sun K-K, *et al.* 2020. Hydrogen Sulfide Maintained the Good Appearance and Nutrition in Post-harvest Tomato Fruits by Antagonizing the Effect of Ethylene. Frontiers in Plant Science **11**, 584.

Yao G-F, Wei Z-Z, Li T-T, *et al.* 2018. Modulation of Enhanced Antioxidant Activity by Hydrogen Sulfide Antagonization of Ethylene in Tomato Fruit Ripening. Journal of Agricultural and Food Chemistry **66**, 10380–10387.

Zgola-Grześkowiak A, Grześkowiak T. 2012. Determination of Glutamic Acid and Aspartic Acid in Tomato Juice by Capillary Isotachophoresis. International Journal of Food Properties **15**, 628–637.

Zhang Y, Butelli E, De Stefano R, *et al.* 2013*a*. Anthocyanins Double the Shelf Life of Tomatoes by Delaying Overripening and Reducing Susceptibility to Gray Mold. Current Biology **23**, 1094–1100.

Zhang X, Shen L, Li F, Meng D, Sheng J. 2013*b*. Hot air treatment-induced arginine catabolism is associated with elevated polyamines and proline levels and alleviates chilling injury in postharvest tomato fruit: Heat-induced arginine catabolism related to chilling tolerance in tomato fruit. Journal of the Science of Food and Agriculture **93**, 3245–3251.

Zhang B, Tieman DM, Jiao C, Xu Y, Chen K, Fei Z, Giovannoni JJ, Klee HJ. 2016. Chilling-induced tomato flavor loss is associated with altered volatile synthesis and transient changes in DNA methylation. Proceedings of the National Academy of Sciences **113**, 12580–12585.

Zheng Y, Shen L, Yu M, Fan B, Zhao D, Liu L, Sheng J. 2011. Nitric oxide synthase as a postharvest response in pathogen resistance of tomato fruit. Postharvest Biology and Technology **60**, 38–46.

Zhong S, Fei Z, Chen Y-R, *et al.* 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. Nature Biotechnology **31**, 154–159.

Zhou Y, Li S, Zeng K. 2016. Exogenous nitric oxide-induced postharvest disease resistance in citrus fruit to *Colletotrichum gloeosporioides*: Effect of NO on disease resistance in citrus fruit. Journal of the Science of Food and Agriculture **96**, 505–512.

Zhu S, Zhou J. 2006. Effects of Nitric Oxide on Fatty Acid Composition in Peach Fruits during Storage. Journal of Agricultural and Food Chemistry **54**, 9447–9452.

Zhu S, Zhou J. 2007. Effect of nitric oxide on ethylene production in strawberry fruit during storage. Food Chemistry **100**, 1517–1522.

Zuccarelli R, Coelho ACP, Peres LEP, Freschi L. 2017. Shedding light on NO homeostasis: Light as a key regulator of glutathione and nitric oxide metabolisms during seedling deetiolation. Nitric Oxide **68**, 77–90.

Discussão Geral e Conclusões

O esforço no desenvolvimento de novas tecnologias capazes de estender a durabilidade de frutos esbarra no ainda limitado entendimento da complexa rede de sinalização, bioquímica e biologia molecular envolvida nos processos do amadurecimento. Nesse sentido, o nosso trabalho oferece um importante avanço, com a implementação de um protocolo de aplicação de NO em normóxia, em tomateiro, importante espécie modelo, com oferecimento de dosagens fisiologicamente relevantes e com mínima exposição ao NO_2, preservando os frutos sem a indução de necrose ou danos aparentes.

Além disso, demonstramos que os efeitos repressores do NO sobre o etileno (ET) se estendem para além dos mecanismos propostos em outros trabalhos, com a repressão da atividade das enzimas das vias de biossíntese de ET (ACS e ACO). Além desse efeito ter sido confirmado, o NO também demonstrou ser capaz de reprimir a transcrição dos genes codificantes para as mesmas enzimas, assim como outros genes também envolvidos com o amadurecimento, tais como o *CNR*, *RIN*, *NOR*, *FUL1*, *AP2a* e o *TAGL1*. Em adição a isso, o NO também foi capaz de diminuir a sensibilidade do fruto ao ET, pois este também reprimiu a expressão dos genes para os receptores de ET, ETR3 e ETR4, além das proteínas da cascata de sinalização de ET, como EBFs, CTR1 e a sub-classe E de ERFs.

Tomados em conjunto, esses resultados demonstram a capacidade do NO reprimir o amadurecimento de forma global, atuando em diferentes níveis. No entanto, os efeitos demonstrados pelo tratamento com NO possuem caráter transitório e reversível, uma vez que, em

estágios posteriores, o fruto retomou os processos reprimidos anteriormente. Ainda que essa retomada tenha sido apenas parcial para alguns processos, como no caso do acúmulo de carotenoides, resultando na diminuição da sua concentração nos estágios finais do amadurecimento.

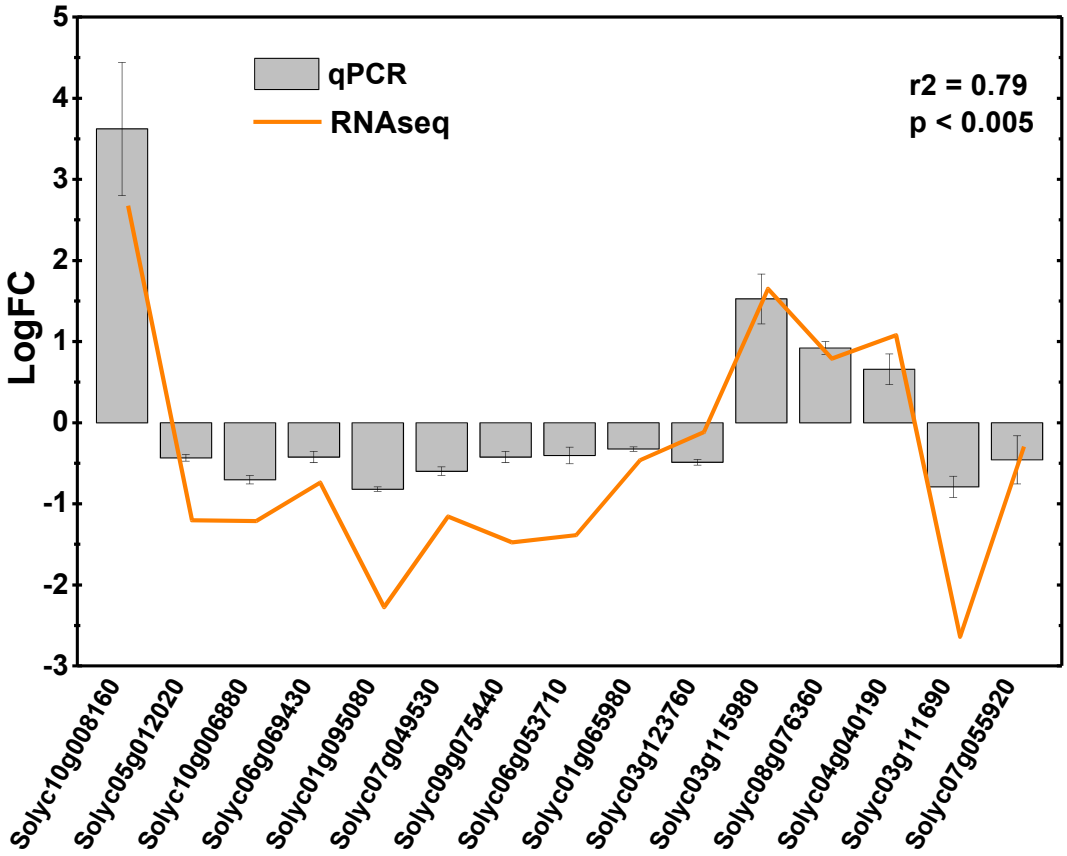
O NO foi também capaz de reprimir a atividade de enzimas do metabolismo antioxidante, com o consequente aumento dos conteúdos de peróxido de hidrogênio e o aumento do estresse nitroxidativo. A extensão e intensidade desse efeito, parece estar dentro de níveis toleráveis, sugerida pelo incremento e manutenção da atividade de sistemas capazes de remover compostos S-nitrosados (GSNOR). Esse efeito também impactou a produção de compostos com atividade antioxidante, como ascorbato e flavonoides, promovendo o acúmulo de tais substâncias, por meio do aumento dos níveis de transcritos de enzimas das vias de biossíntese desses compostos em estágios mais avançados do amadurecimento. Resta ainda, no entanto, estabelecer se o acúmulo de tais substâncias se deu como resposta ao aumento do estresse nitroxidativo ou por outro mecanismo no qual o NO possa atuar diretamente.

Apesar das extensas mudanças em nível transcricional, sinalização de etileno e metabolismo antioxidante promovidas pelo tratamento com NO, outros atributos relacionados à qualidade do fruto, em particular o sabor e aroma foram preservados, mantendo níveis indistinguíveis de açucares, ácidos orgânicos, aminoácidos e compostos voláteis.

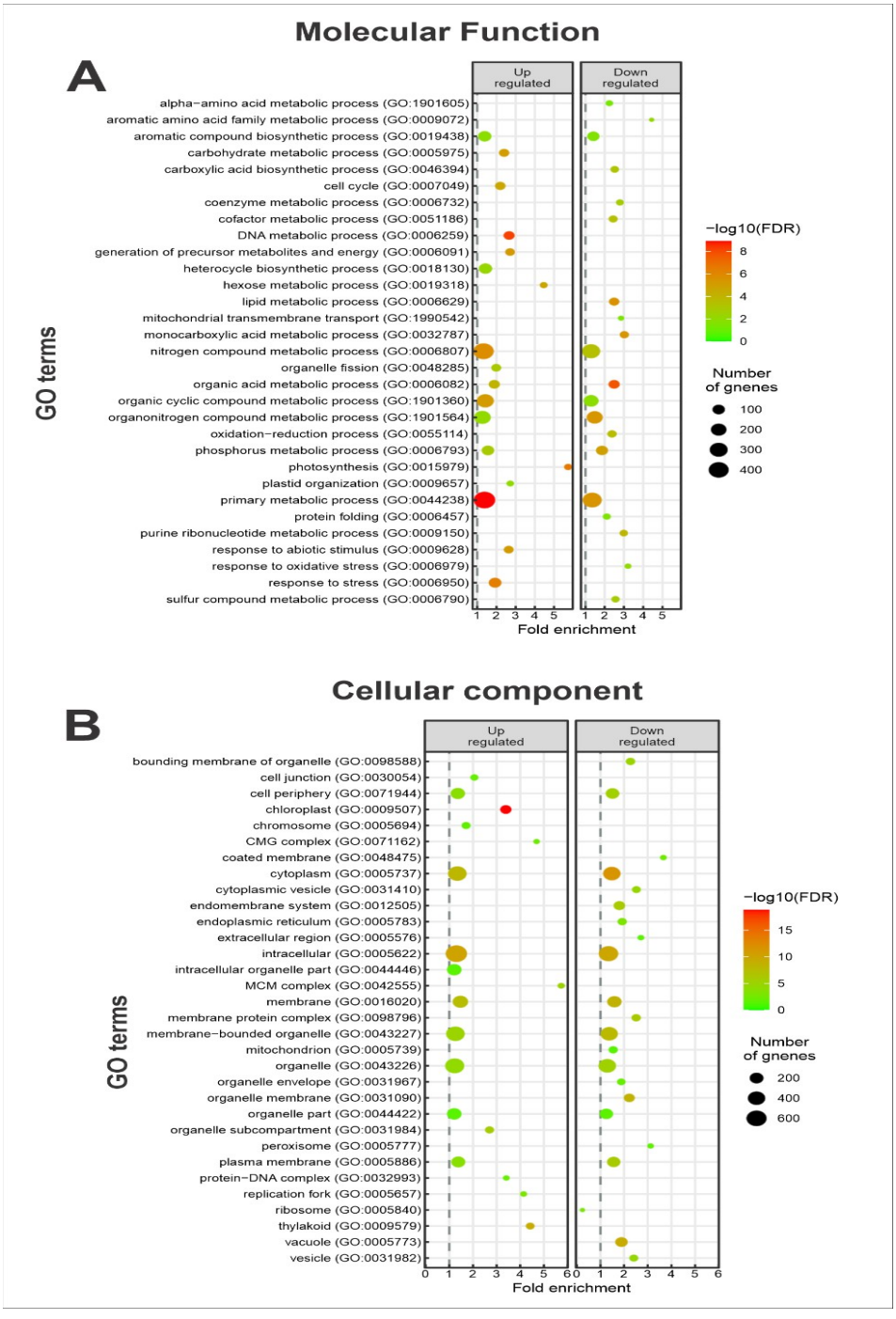
Esses dados proporcionam novas oportunidades de investigação para preservação e melhoria da qualidade dos frutos, pois o incremento do potencial antioxidante pode ser uma oportunidade relevante para o

aumento da resistência a agentes estressores e condições deletérias durante o transporte e armazenamento de frutos.

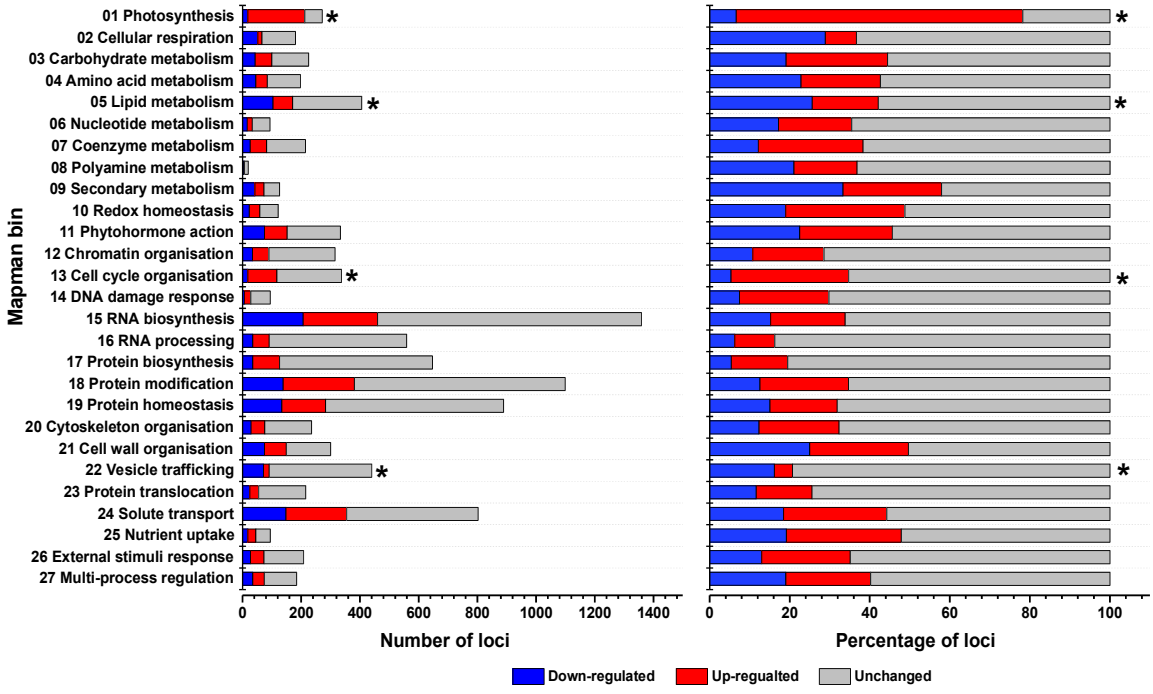
Anexos e Apêndices



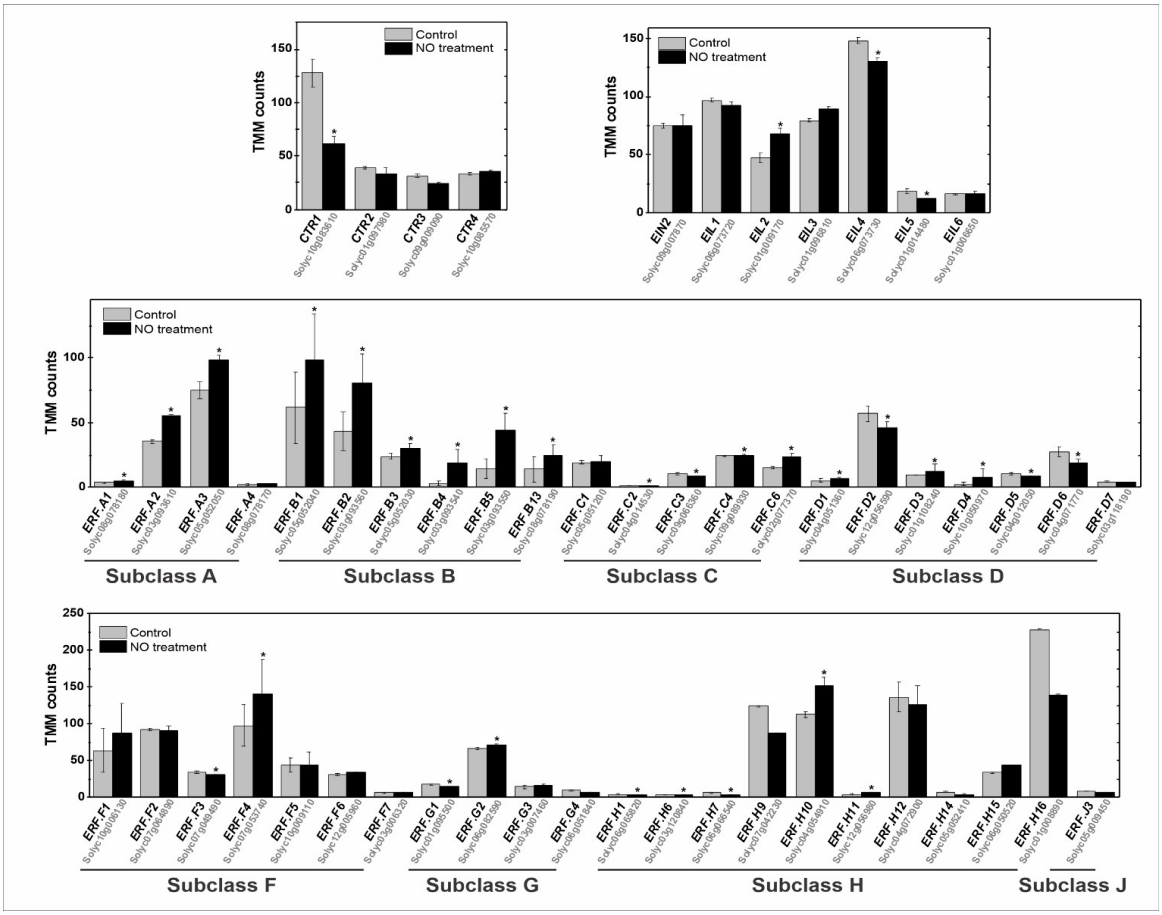
Supplementary Fig. S1. Validation of RNA-Seq analysis via RT-qPCR. Validation was performed for 15 genes comparing control and NO-treated fruits at the breaker stage. Bars represent mean log 2 relative transcript abundances calculated using the 2 - ΔΔCT method. Lines represent mean log 2 expression ratios calculated by pairwise differential expression analysis using edgeR. Primers used for the analyzed genes are included in Table S1. Pearson correlation was calculated, and R 2 and p-values are shown in the figure.



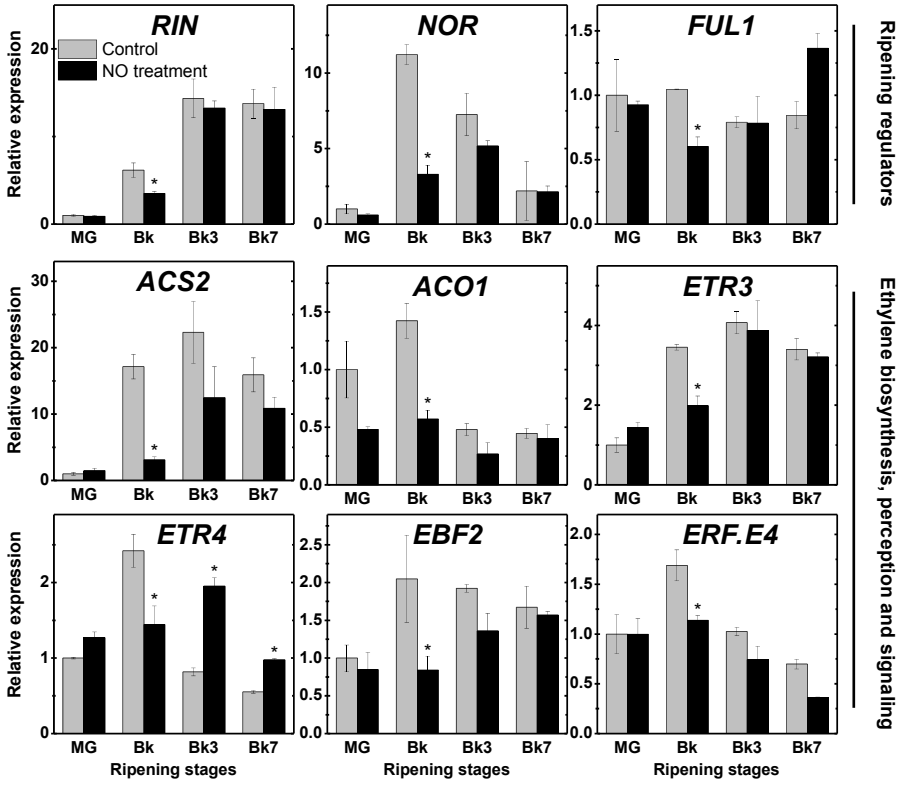
Supplementary Fig. S2. Most significant over-represented GO terms in NO-treated versus control fruits. Biological processes (A) and cellular components (B) are sorted by fold enrichment. The number of DEGs and significance of the enrichment are indicated by the dot size dot color, respectively. The vertical grey dashed line represents a fold enrichment of 1.



Supplementary Fig. S3. Number and percentage of DEGs in each Mapman category. Mapman4 functional categorization of differentially expressed genes (DEGs, FDR ≤ 0.05) in NO-treated versus control fruits at the breaker (Bk) stage. Functional categories that are significantly affected (Wilcoxon rank-sum test with Benjamini Hochberg correction) between the NO-treated and control fruits are indicated by asterisks. The identification of DEGs in each group and statistical data are listed in Supplementary Tables S3 and S4, respectively.

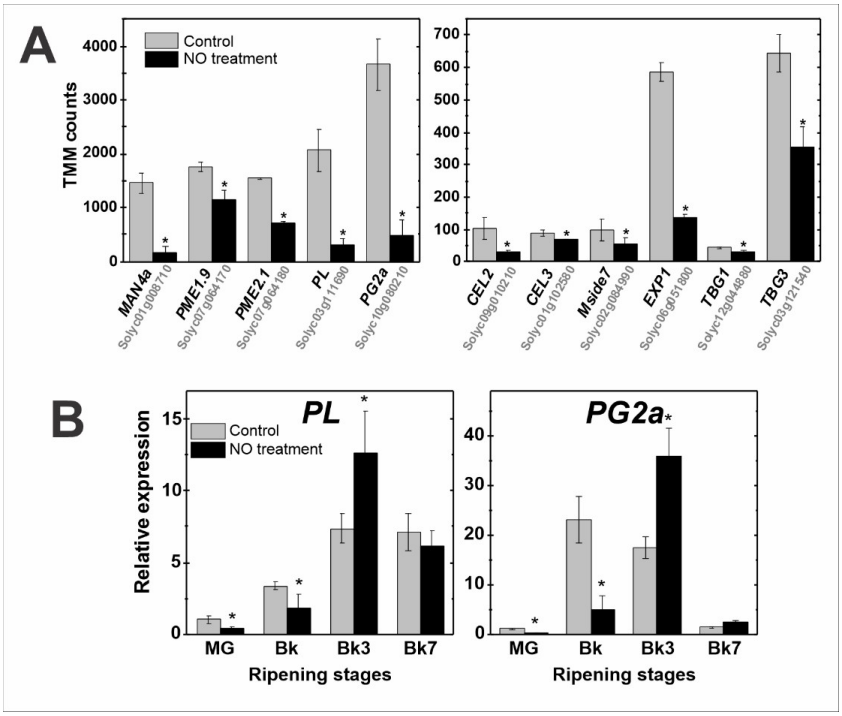


Supplementary Fig. S4. Impact of NO treatment on ethylene signaling genes. Trimmed Mean of M-values (TMM) counts for different classes of ethylene signaling genes at the breaker (Bk) stage. Data shown are mean ± SE of at least three biological replicates. *P < 0.05 (Student's t-test) compared with control samples . CTR, constitutive triple response; EIN3, ethylene insensitive 3; EIL, EIN3- like; ERF, ethylene response factor.

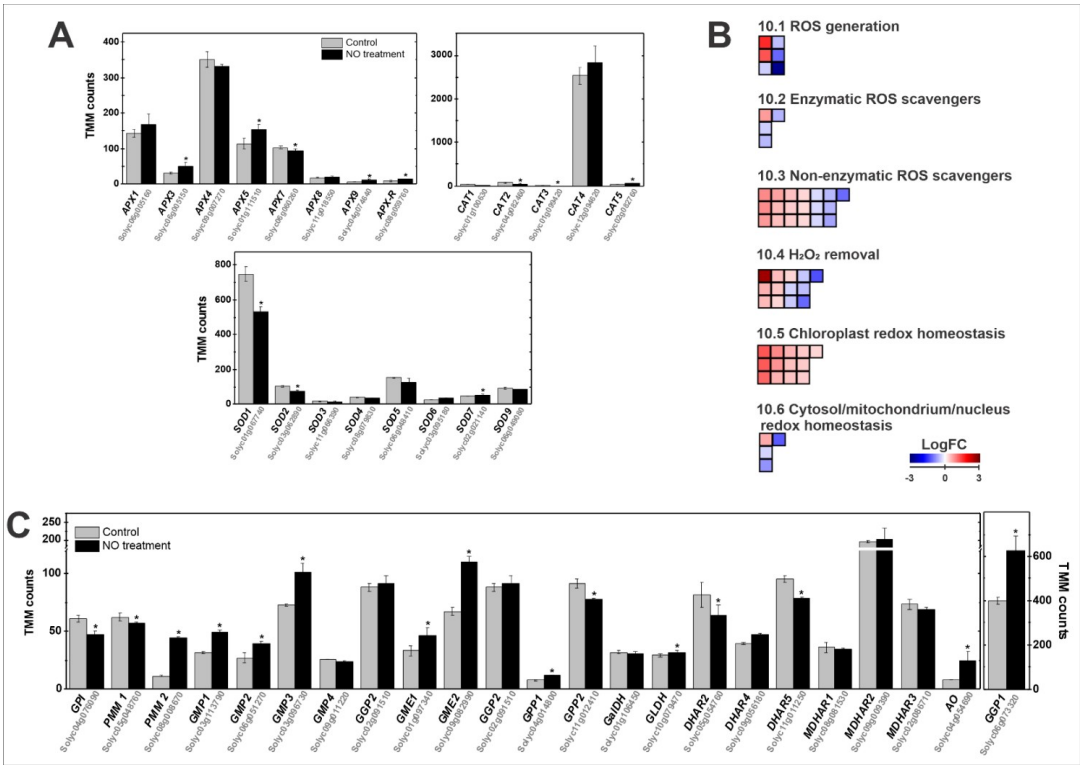


Supplementary Fig. S5. Time-course analysis of ripening- and ethylene-related genes. Transcript

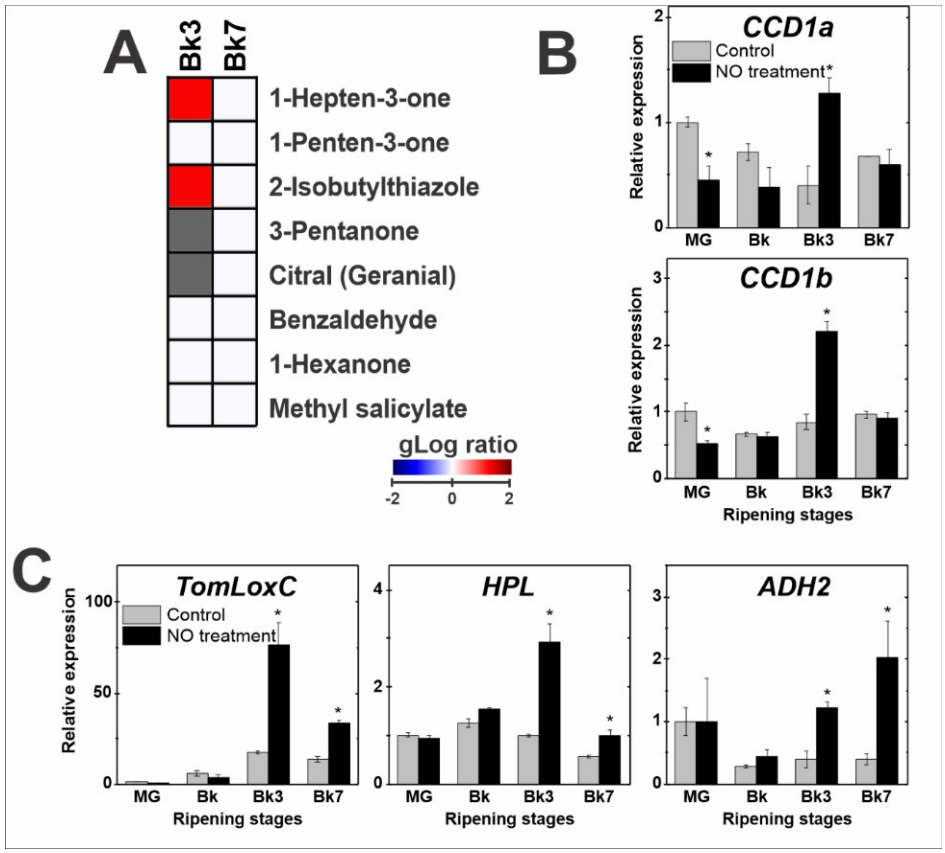
abundance was profiled via RT-qPCR and normalized against the control samples at the mature green (MG) stage. Gene abbreviations and relative transcript values are detailed in Supplementary Table S7. Data shown are mean ± SE of at least three biological replicates. *P < 0.05 (Student's t-test) compared with control samples. Bk, breaker; Bk3, 3 days after Bk; Bk7, 7 days after Bk.



Supplementary Fig. S6. Impact of NO treatment on fruit softening -related genes. (A) Trimmed Mean of M-values (TMM) counts for major cell wall genes at the breaker (Bk) stage. (B) Transcript abundance of PECTATE LYASE (PL) and POLYGALACTURONASE 2a (PG2a), measured via RT-qPCR and normalized against the control samples at the mature green (MG) stage . Data shown are mean ± SE of at least three biological replicates . *P < 0.05 (Student 's t-test) compared with control samples . Bk3, 3 days after Bk; Bk7, 7 days after Bk; CEL, endo-1,4-β-glucanase; EXP, expansin; MAN, endo-1,4-β-mannanase; Mside 7, endo-1,4-beta-mannosidase; PME, pectinesterase;TBG, β-galactosidase.



Supplementary Fig. S7. Impact of NO treatment on redox-related gene expression. (A) Trimmed Mean of M-values (TMM) counts of genes encoding ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) at the breaker (Bk) stage. (B) Heatmaps indicate log 2 (fold-change) of DEGs between NO-treated and control fruits at the Bk stage for redox-related MapMan categories. (C) TMM counts of ascorbate biosynthesis, recycling, and oxidation-related genes at the Bk stage. Data shown are mean ± SE of at least three biological replicates . In A and C, *P < 0.05 (Student's t-test) compared with control samples. Bk3, 3 days after Bk; Bk7, 7 days after Bk; OA, L-ascorbate oxidase; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GLDH , L-galactono-lactone dehydrogenase; GME, GDP-D- mannose-3',5'-epimerase; GMP, GDP -D-mannose pyrophosphorylase; GPI, glucose -6-phosphate isomerase; GPP, L-galactose-1-phosphate phosphatase; MDHAR, monodehydroascorbate; MIOX, myo-Inositol oxygenase; PMM, phosphomannose mutase.



Supplementary Fig. S8. Volatile compound profile and metabolism in NO-treated fruits. (A) Heatmap indicates the difference in volatile compound levels between control and NO-treated samples at Breaker (Bk) plus 3 and 7 days (Bk3 and Bk7, respectively). Data are expressed in a generalized logarithm (gLog) of Pareto rescaled data, and only statistically significant differences in comparison with control samples are represented as colored squares (P<0.05). Gray color indicates not detected metabolites. The relative metabolite abundance values are presented in Supplementary Table S9. (B-C) Transcript abundance of genes associated with the biosynthesis of carotenoid-derived (B) and fatty acid-derived (C) volatiles profiled via RT- qPCR and normalized against the control samples at the mature green (MG) stage. Data shown are mean ± SE of at least three biological replicates. In B-C, *P < 0.05 (Student's t-test) compared with control samples. ADH alcohol dehydrogenase; CCD, carotenoid cleavage dioxygenases; HPL, hydroperoxide lyase; TomLox, lipoxygenase.

Tabelas suplementares disponíveis em:
https://academic.oup.com/jxb/article/72/3/941/5963948#supplementary-data