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Nitric oxide mediates brassinosteroid-induced flavonoid biosynthesis in *Camellia sinensis* L.

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ABSTRACT

Flavonoids are one of the key secondary metabolites determining the quality of tea. Although exogenous brassinosteroid (BR), a steroidal plant hormone, can stimulate polyphenol biosynthesis in tea plants (*Camellia sinensis* L.), the relevance of endogenous BR in flavonoid accumulation and the underlying mechanisms remain largely unknown. Here we show that BR enhances flavonoid concentration in tea leaves by inducing an increase in the endogenous concentration of nitric oxide (NO). Notably, exogenous BR increased levels of flavonoids as well as NO in a concentration dependent manner, while suppression of BR levels by an inhibitor of BR biosynthesis, brassinazole (BRz), decreased the concentrations of both flavonoids and NO in tea leaves. Interestingly, combined treatment of BR and BRz reversed the inhibitory effect of BRz alone on the concentrations of flavonoids and NO. Likewise, exogenous NO also increased flavonoids and NO levels dose-dependently. When the NO level in tea leaves was suppressed by using a NO scavenger, 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), flavonoid concentration dramatically decreased. Although individual application of 0.1 μM BR increased the concentrations of flavonoids and NO, combined treatment with exogenous NO scavenger, cPTIO, reversed the effect of BR on flavonoid concentration. Furthermore, BR or sodium nitroprusside (SNP) promoted but cPTIO inhibited the transcription and activity of phenylalanine ammonia-lyase (PAL) in leaves, while combined treatment of BR with SNP or cPTIO had no additive effect. The results of this study suggest that an optimal level of endogenous NO is essential for BR-induced promotion of flavonoid biosynthesis in tea leaves. In conclusion, this study unveiled a crucial mechanism of BR-induced flavonoid biosynthesis, which might have potential implication in improving the quality of tea.

1. Introduction

Flavonoids are a vast group of plant secondary metabolites, predominantly occurring as glucosylated compounds in most plant tissues. Characteristically, they consist of two aromatic rings in a three-carbon aliphatic chain (C6-C3-C6), which forms an oxygen-containing pyran ring through condensation. Over 10,000 structures of flavonoids have been identified, which are divided to several sub-classes such as flavones, flavanones, flavonols, flavanonols, flavan-3-ols, chalcones, dihydrochalcones, anthocyanidins, isoflavones, aurones and biflavonoids (Julkunen-Tiitto et al., 2015). Flavonoids have been suggested to function as key antioxidants under a plethora of abiotic stresses in higher plants (Di Ferdinando et al., 2012). The lower redox potentials (0.23–0.75 V) of flavonoids enable them to reduce highly oxidizing free radicals such as O_2^-, H_2O_2, and OH·, which have redox potentials in the range 2.13–1.0 V. Moreover, flavonoids can inhibit the activities of several enzymes involved in reactive oxygen species (ROS) production (Pietta, 2000; Tossi et al., 2011).

Notably, tea, the most popular non-alcoholic beverage in the world, contains the highest contents of flavonoids among common food and beverages (Tounekti et al., 2013). Green teas that are manufactured from the buds and leaves of *Camellia sinensis* L., contains a special group of flavonoids called catechins belonging to the flavan-3-ols sub-class. Catechins are major bioactive compounds accounting for 12–24% of dry weight of tea leaves. In plants, flavonoids play important roles in growth and development (Julkunen-Tiitto et al., 2015). Some flavo-

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noids such as derivates of quercetin affect auxin transport at intra- and intercellular levels, thereby regulating the development of a specific organ and thus whole plant (Di Ferdinando et al., 2012). Additionally, the antioxidative potential of flavonoids protects plants from oxidative stress. In humans, tea catechins provide protection against cancer, cardiovascular disease, diabetes, obesity and metabolic syndrome (Tounekti et al., 2013). The health benefits of green tea are partly responsible for greatly increased overall consumptions of tea across the world. Now-a-days, green tea and its flavonoids are used as therapeutic agents and functional ingredients in foods (Tounekti et al., 2013). Therefore, manipulation of flavonoids contents in tea leaves is an important topic to tea researchers (Li et al., 2016).

Flavonoids biosynthesis occurs via shikimate pathway, where chorismate is formed as a precursor of phenylalanine (Tounekti et al., 2013). Afterward, phenylalanine ammonia lyase (PAL) deaminates phenylalanine to form cinnamic acid towards transformation into coumarate and 4-coumaroyl-CoA. A polyketide synthase known as chalcone synthase (CHS) catalyzes condensation of 3-malonyl-CoA with 4-coumaroyal-CoA into a chalcone, leading to isomerization of chalcone to various flavanones. These intermediates finally yield different classes of flavonoids (Xi et al., 2013). Flavonoid biosynthesis in tea leaves is influenced by various external factors such as day length, rainfall, sunlight, temperature, horticultural and harvesting practices (Tounekti et al., 2013). In addition, plant growth regulators including signal molecules and phytohormones can also alter flavonoid contents in plants (Tossi et al., 2011; Ahammed et al., 2012). For instance, phytohormones such as gibberellins or abscisic acid (ABA) affect catechin contents and transcript levels of their biosynthetic genes in tea leaves (Rani et al., 2012; Singh et al., 2009). Salicylic acid (SA) can enhance flavonoid concentration and CHS activity in leaves of *Zingiber officinale* (Ghasemzadeh et al., 2012). Likewise, brassinosteroids (BR), a group of plant hormones, stimulate increased flavonoid concentration in tomato leaves under both normal and stressful conditions (Ahammed et al., 2012). Very recently, we reported that exogenous BR could stimulate the biosynthesis of tea polyphenols in tea leaves (Li et al., 2016). However, the relevance of endogenous BR in flavonoid accumulation and the mechanisms by which BR influences flavonoid biosynthesis still remain poorly understood in tea plants.

Nitric oxide (NO) is a freely diffusible small signaling molecule, playing crucial roles in diverse physiological processes relating to plant growth, development and responses to stress (Dixon and Paiva, 1995; Wang and Wu, 2005). This ubiquitous molecule is produced in plants by both enzymatic and nonenzymatic routes (Cohen et al., 2006). Since NO scarcely occurs as polar molecule, it can freely cross membranes and move from one compartment to the other (Simontacchi et al., 2013). NO has also been considered as a broad-spectrum anti-stress molecule particularly for its antioxidant activity similar to that of flavonoids (Tossi et al., 2011). Characteristics such as an unpaired electron and a redox potential of 0.4 V allow NO to attain a high reactivity with O_2, O_2^- and redox active metals. Moreover, NO can attenuate the formation of OH· by scavenging ROS such as superoxide or metal ions such as iron (Lorenzo et al., 2003). NO plays a critical role in the transcriptional regulation of genes involved in the phenylpropanoid biosynthetic pathway in maize and *Arabidopsis*. It induces the expression of transcription factors encoding genes such as *HY5*, *MYB12* and *ZmP,* which results in the upregulation of transcripts of *CHS* and *CHALCONE ISOMERASE* (*CHI*). Eventually, stimulation in this signaling pathway results in an increased flavonoid content (Tossi et al., 2011). Exogenous NO stimulates PAL activity and phenol contents in *Matricariacha momilla* roots during nitrogen deficiency (Kovacik et al., 2009).

On the other hand, NO is considered as an essential component for a wide range of hormone-regulated processes in plants (Simontacchi et al., 2013). It is involved in seed germination, root development, stomatal closure, senescence and responses to stress, the processes that are fine-tuned by the orchestration of various phytohormones (Simontacchi et al., 2013). For instance, BR enhances the production of NO in maize leaves, which in turn activates biosynthesis of ABA, leading to an enhanced tolerance to oxidative stress (Zhang et al., 2011). In cucumber plants, NO is required for BR-induced tolerance to cold and oxidative stress (Cui et al., 2011). These reports lead to an assumption that BR may interact with NO to stimulate flavonoid biosynthesis in tea leaves. To verify this assumption, we used pharmacological approach to suppress levels of BR or NO in tea plants, and examined the effect of exogenous BR and NO on flavonoid concentration in tea leaves. Our results show that BR enhances flavonoid concentration by increasing the accumulation of NO in tea leaves and thus NO mediates the BR-induced biosynthesis of flavonoids.

2. Materials and methods

2.1. Plant material and growth conditions

A field experiment was conducted at the tea garden of the Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, Zhejiang province, China (longitude 120°10′ E and latitude 30°14′ N, 16 m above sea level) using Longjing 43 tea (*Camellia sinensis* L.) cultivar. Tea bushes were sprayed with different concentrations of freshly prepared 24-epibrassinolide (BR, Sigma–Aldrich, St. Louis, USA). Working solutions of BR (0.01, 0.1 and 1.0 μM) were prepared by dissolving the solute in ethanol followed by dilution with MilliQ water (ethanol: water (v/v) = 1:10000). MilliQ water containing the same ratio of ethanol was used to spray control (CK) tea bushes. To examine the effect of NO on flavonoid concentration, tea bushes were sprayed with an NO donor, sodium nitroprusside (SNP) at 20, 200 and 2000 μM concentrations. To suppress BR levels, an inhibitor of BR biosynthesis, brassinazole (BRz) at 40 μM, was sprayed once every 24 h for three days, while NO accumulation was inhibited by foliar application of a NO scavenger, 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), at a concentration of 200 μM. For combined treatment with BR and cPTIO or SNP, cPTIO or SNP were sprayed at 8 h prior to treatment with BR. The samples were harvested for analysis 24 h after the final spraying. Each treatment comprises 4 replicates, while each replicate represents an area of 10 m^2 consisting of 20 tea bushes.

2.2. Determination of flavonoids concentration

Flavonoids were extracted from leaf samples in 70% ethanol (v/v) at 100 °C, and the aqueous extract was used to measure the concentration of total flavonoids, which was determined by using $AlCl_3$ method as described previously (Lin and Tang, 2007). Absorbance at 510 nm for flavonoids was determined, and rutin was used as the standard.

2.3. Determination of NO level

The concentration of NO in leaves was assayed spectrophotometrically based on the principle of the conversion of oxyhaemoglobin (HbO_2) to methaemoglobin (MetHb) at 401 nm and 421 nm as described by Pasqualini et al. (2009).

NO accumulation in leaves was visualized using a NO specific fluorescent probe, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-2DA) (Cui et al., 2011). Leaves were cut into small segments and incubated with 5 μM DAF-2DA for 0.5 h at 37 °C followed by washing with 50 mM sodium phosphate buffer (pH 7.4) for three times to remove excess fluorescent probe. Afterward, leaf segments were observed using a Laser Scanning Confocal Microscope (LSCM-500, Zeiss, Germany) with excitation and emission wavelengths at 488 nm and 515 nm, respectively.

2.4. Assay of enzyme activity

PAL enzyme was extracted from 0.3 g tea leaf sample by homo-