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Salicylic acid-induced flavonoid accumulation in *Ginkgo biloba* leaves is dependent on red and far-red light

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ABSTRACT

Flavonoids play important roles in plant immune responses, but the molecular mechanism and influencing factors on flavonoid accumulation during Ginkgo immune responses are largely unknown. In this research, post-harvest Ginkgo leaves cultured in the dark were treated with salicylic acid (SA). A transcriptome analysis and total flavonoid measurement showed decreased flavonoid biosynthesis and accumulation in leaves. Experiments carried out in seedlings, young and adult trees, showed increased flavonoid content under light conditions but decreased content under dark conditions after treatment with SA. A detailed analysis of individual flavonoids showed similar changes for most of their contents, although some showed different change trends. Single-colored light experiments revealed that red and far-red light, but not blue light, were required in SA-induced flavonoid accumulation. The results clarified the importance of light in SA-induced flavonoid accumulation and provided valuable information to the Ginkgo related pharmaceutical industry.

1. Introduction

Flavonoids are a major class of secondary metabolites that are widely distributed in the plant kingdom (Winkel-Shirley, 2001a,b). Because of their diverse chemical structures and varieties, they have a number of important functions in plants, including resistance against pathogens (Mierziak et al., 2014). Non-specific anti-pathogenic properties are partly the result of the anti-oxidative properties of flavonoids. They quench toxic reactive oxygen species (ROS), which are generated after pathogen infections (Dai et al., 1996). Flavonoids are transported to the infection sites and participate in the hypersensitivity reaction and programmed cell death (Beckman, 2000). Furthermore, flavonoids may inhibit enzyme activities of pathogens by chelating metals required for their activities (Treutter, 2005). The anti-pathogenic effects of flavonoids are largely dependent on their structures, and the modification of subgroups greatly changes their anti-oxidative properties (Christensen et al., 1998; Weidenbörner and Jha, 1994).

Ginkgo biloba, a "living fossil", is a long-lived native Chinese tree species with no living relatives (Zhou, 2009). Ginkgo leaf extract, which contains pharmacologically activated flavonoids, is commonly used as an herbal dietary supplement and in the treatment of many diseases (Rimbach et al., 2001). In addition, Ginkgo trees have a broad spectrum of tolerance or resistance to herbivores and pathogens, and because of this hardiness the trees have been widely planted in different areas (Jacobs and Browner, 2000). Despite the wide range of applications for *G. biloba*, the relationship between flavonoid accumulation and plant immune responses is still not clear.

The phytohormone salicylic acid (SA) is a small phenolic compound that has been extensively investigated for its role in the defense against pathogens during plant immunity responses (Pieterse et al., 2012). SA is produced upon pathogen challenge and triggers a global transcriptional reprogramming to induce a systemic acquired resistance (Yan and Dong, 2014). In previous research, SA mediated the fungal elicitor-induced flavonoid accumulation in Ginkgo cells (Xu et al., 2009). Although the relationship between SA signaling and flavonoid biosynthesis is unequivocal, the molecular mechanism and influencing factors are largely unknown.

Light is an important factor in photosynthesis, and it also acts as a ligand in the light-signaling pathway to regulate a variety of plant growth- and development-related processes (Chen et al., 2004). Plants

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have evolved distinct sets of photoreceptors to sense different parts of the light spectrum, ranging from ultraviolet-B (UVB) to far-red wavelengths (Jenkins, 2014; Moglich et al., 2010). Phytochromes respond to red and far-red light by undergoing conformational changes between active and inactive isoforms that are involved in many light-regulated processes (Wang, 2015). Cryptochromes, phototropins, and the ZEITL-UPE family are three types of blue light receptors. These receptors are involved in many processes in plants, including circadian rhythm, flowering, photomorphogenesis, phototropism, chloroplast movement, and stomatal opening (Chaves et al., 2011; Christie et al., 2015). UVB light is perceived by UV RESISTANCE LOCUS 8 (UVR8) (Rizzini et al., 2011). UVR8-mediated UVB signals interpret temperature information to regulate thermomorphogenesis through multiple mechanisms (Hayes et al., 2017).

Here, the flavonoid contents of post-harvest Ginkgo leaves, seedlings, and young and adult Ginkgo trees were measured after various treatments. It was demonstrated that light is indispensable in the SA-induced flavonoid accumulation in Ginkgo leaves. Furthermore, the data indicated that red and far-red light, but not blue light, were required in this process. The results clarify the importance of light in SA-induced flavonoid accumulation and provide valuable information to the Ginkgo related pharmaceutical industry.

2. Materials and methods

2.1. Plant materials

To reduce the individual variations in the transcriptome analyses of Ginkgo trees, previously described post-harvest Ginkgo leaves were used, with minor modifications (Ni et al., 2017). Briefly, Ginkgo leaves with short shoots were maintained in Murashige and Skoog (MS, Gibco) solution (without sucrose) under a constant temperature (25 °C) and continuous darkness for 12 h. The SA treatment was performed by spraying the leaves with 20 mM SA, and samples were harvested after 12 h. H_2O was used as a control treatment. To determine the flavonoid contents and gene expression levels, all of the leaves were cut in halves, with one half used to analyze the flavonoid content and the other for the transcriptome analysis.

Three-month-old seed-derived seedlings were used in the flavonoid analysis. Ginkgo seedlings were grown in a green room under a 16-h light and 8-h dark cycle at a constant temperature (25 °C). The seedlings were subjected to continuous light or darkness for 12 h before the SA treatment. Leaves were harvested 3, 6 and 12 h after treatment. H_2O was used as the control treatment. For red, far-red, and blue light treatments, Seedlings grown in the green room were transferred to three color incubators (25 °C) (Percival, USA) 12 h before SA treatment. The leaves were harvested 3 h after the SA treatment.

In an experimental field, 5-year-old young and 20-year-old adult trees were grown and used to measure total flavonoid contents in adult trees. The experiments were carried out on a sunny day in June 2017. Opaque bags were used to cover tree branches to eliminate exposure to sunlight. Leaves were harvested 3 h after the SA treatment.

2.2. RNA-Seq and data analyses

The leaves were treated with SA for 12 h and then subjected to RNA-Seq (Mortazavi et al., 2008). After data filtering to remove reads containing the adaptor sequences, high contents of unknown bases and low quality reads, clean reads were mapped to reference sequences using HISAT and Bowtie2 tools (Kim et al., 2015; Langmead et al., 2009; Ni et al., 2018). Gene expression levels were quantified by RSEM (Li and Dewey, 2011). NOISeq was used to analyze differentially expressed genes (DEGs) (Tarazona et al., 2011). Biological repeat samples were first grouped and then two groups (control and SA treatment) were compared to screen for DEGs. The DEGs were defined according to the following default criteria: Foldchange ≥ 2 and diverge probability ≥ 0.8. For DEGs in flavonoid biosynthesis, foldchange ≥ 2, diverge probability ≥ 0.6, and genelength ≥ 500.

The gene ontology (GO) annotation analysis of DEGs was performed. After the GO annotation of DEGs, WEGO was used to perform a GO functional classification to understand the distribution of gene functions (Ye et al., 2006). The numbers of mapped DEGs were calculated for every term, and a hypergeometric test was used to find significantly enriched GO terms to perform a GO enrichment analysis. The pathway enrichment analysis of DEGs was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using the same calculating formula used in the GO analysis (Kanehisa et al., 2008).

2.3. Measurement of flavonoids in Ginkgo leaves

The total flavonoid content was measured using an $Al(NO_3)_3$ colorimetric assay. Briefly, the thoroughly dried leaf powder was extracted using 60% ethanol in a 70 °C water bath. The total flavonoid in supernatant was analyzed by $NaNO_2$ and $Al(NO_3)_3$ additions, and then coloration was induced by the addition of NaOH. The spectrophotometer wavelength was set to 510 nm. Rutin (R106912, Aladdin) was used to define a standard curve. The extraction and HPLC analysis of flavonoids were carried out as previously described with minor modifications (Ni et al., 2017). Rutin standard was used for confirmation of flavonoid in peak 4. For addition of standard, 30 µl of standard solution was added to 1 ml of the Ginkgo leaf extract for HPLC analysis.

2.4. Statistical analysis

Data are reported as the mean ± standard deviation (SD). The data sets are expressed as mean values of three biological replicates, and two technical replicates were run for each biological replicate. Metric bars indicate SD. SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA) was used to perform all the tests.

3. Results

3.1. SA treatments induced necrotic lesions on post-harvest Ginkgo leaves

To investigate the responses to SA signaling in Ginkgo leaves, post-harvest Ginkgo leaves were treated with SA for different periods. For the first 6 h after SA treatment, no significant differences were observed between the SA-treated and the control leaves. A large proportion of SA-treated leaves (21/25) showed necrotic lesions at the edge of leaves 9 h after SA treatment. Thereafter, large areas of necrotic lesions were shown in SA-treated leaves and these leaves wizened (Fig. 1). Thus, post-harvest Ginkgo leaves have normal defense responses to SA treatments with regard to necrotic lesions.

3.2. Basic sequencing data

To further investigate the transcriptome responses to SA signaling in Ginkgo leaves, total RNAs were extracted and isolated from post-harvest Ginkgo leaves cultured in the dark after the SA treatment. The cDNA libraries, which were reverse-transcribed from RNAs, were subjected to high-throughput sequencing using an Illumina HiSeq SE50. As a result, 144.63 M clean reads with 7.23 Gb clean bases were obtained after removing adaptor sequences and low-quality reads. The ratios of clean reads in each replicate were more than 99%, indicating that the quality of the sequencing data was acceptable (Table S1). After filtering, clean reads were mapped to the published reference sequences, and the mapping ratios of each sample were ~90% (Table S2). In addition, the numbers of identified expressed genes and the distributions of gene numbers at different expression levels for each sample were analyzed (Fig. S1).