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Protective role of anthocyanins in plants under low nitrogen stress

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ABSTRACT

Nitrogen (N) is a major nutrient of plants but often a limiting factor for plant growth and crop yield. To adapt to N deficiency, plants have evolved adaptive responses including accumulation of anthocyanins. However, it is still unclear whether the accumulated anthocyanins are part of the components of plant tolerance under low N stress. Here, we demonstrate that low N-induced anthocyanins contribute substantially to the low N tolerance of *Arabidopsis thaliana*. *pap1-1*, a mutant defective in MYB75 (PAP1), a MYB-type transcription factor that positively regulates anthocyanin biosynthesis in *Arabidopsis*, was found to have significantly decreased survival rate to low N stress compared to its wild-type plants. Similarly, *tt3*, a mutant with severe deficiency in dihydroflavonol 4-reductase (DFR), a key enzyme in anthocyanin biosynthesis, also showed much lower survival rate under low N stress. These results indicate that anthocyanins are substantial contributors of plant tolerance to low N stress. Furthermore, a metabolomics analysis using LC-MS revealed changes in flavonoid profile in the *pap1-1* and *tt3* plants, which established a causal relationship between plant adaptation to low N stress and these compounds including anthocyanins. Our results showed an important role of anthocyanins rather than flavonols in conferring plant tolerance to low N stress.

1. Introduction

Nitrogen (N) is an important nutrient element of plants. Lack of N will affect plant growth, development and even survival. To harvest crops with high production, substantial N fertilizer is widely used [1]. However, due to the low utilization efficiency of N fertilizer by crop plants, the big loss of N fertilizer brought increasingly severe environmental problems such as the groundwater nitrate contamination [2]. Thus, it is urgent to understand the mechanisms of plant tolerance to low N for improving N use efficiency and therefore reducing N fertilizer input. To adapt to low N, plants have evolved a set of adaptive responses, such as retardation of growth, reduction of photosynthesis, remobilization of N from old organs to new ones and accumulation of abundant anthocyanins [3–6].

Anthocyanins, a branch of flavonoids, have many biological functions in higher plants. They provide a variety of colors for plants acting as insect and animal attractants [7]. They also serve as important molecules in defense against UV irradiation, pathogen infection and ROS scavenging [8–10]. Recent studies have found that anthocyanins could confer tolerance to low temperature, drought and high salinity stress in plants [11]. However, whether anthocyanins can improve the tolerance of plants to low N is still uncertain.

In *Arabidopsis thaliana*, the anthocyanin biosynthetic pathway can be mainly divided into three parts. In the upstream, phenylalanine (Phe) was converted to 4-coumaroyl CoA catalyzed by a series of enzymes such as phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl-CoA ligase (4CL). Then three enzymes including chalcone synthase (CHS), chalcone isomerase (CHI) and flavonone 3-hydroxylase (F3H) are involved to convert the 4-coumaroyl CoA into dihydrokaempferol (DHK). In the downstream, DHK can also be converted to dihydroquercetin (DHQ) by flavonoid 3′-hydroxylase (F3′H). Both DHK and DHQ are then converted to leucoanthocyanidins by dihydroflavonol 4-reductase (DFR), and leucoanthocyanidins are eventually converted to anthocyanins by anthocyanidin synthase (ANS) and UDPG-flavonoid glucosyl transferase (UFGT). Alternatively, DHK and DHQ can be converted to the basic flavonol structure kaempferol and quercetin respectively by flavonol synthase (FLS), which can be further modified by UDP-dependent glycosyltransferase (UGT) [7,12]. Therefore, DFR is a key enzyme in anthocyanin biosynthesis that controls the carbon flux direction. Glycosylation, acylation, and methylation of aglycone

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structures are important steps in the formation of anthocyanins (cyanidin and pelargonidin derivatives) and flavonols (quercetin and kaempferol derivatives) [13,14].

The expression of anthocyanin and flavonol biosynthetic genes are regulated by different transcription factor (TF) families including MYB, basic helix-loop-helix (bHLH), WD40, and WRKY families [15–17]. Among these TF families, the MYB family is regarded as a major regulator of the flavonoid pathway [15]. In many plant species, overexpression of the an MYB TF MYB75 (PAP1), leads to anthocyanin hyper-accumulation [13]. The studies using PAP1 loss-of-function mutant (*pap1-1*) and PAP1 over-expressing plant (*pap1-D*) revealed that PAP1 is not only involved in anthocyanin biosynthesis, but also in regulation of the flavonol pathway [13,18]. To regulate the expression of anthocyanin-specific genes, MYB75 forms a ternary complex with bHLH and WD40 (MYB/bHLH/WD40) TFs, which binds directly to the anthocyanin biosynthetic genes [16,19].

In this study, the survival rates of *pap1-1* and a *DFR*-deficient mutant *tt3*, were found to be significantly lower than that in the wild-type plants after N starvation, suggesting a positive role of flavonoids including anthocyanins in improving plant tolerance to low N. In consistence with the survival rate changes, the results of flavonoid profiling by LC-MS showed decrease in anthocyanin accumulation in both mutants. Based on all these results, we conclude that low N-induced anthocyanin accumulation plays a substantial role in plant tolerance to low N stress.

2. Materials and methods

2.1. Plant materials and growth conditions

Two *Arabidopsis thaliana* ecotypes Nossen and Landsberg *erecta* (L*er*) were used in this study. Loss of function mutant of PAP1 (*pap1-1*) in Nossen background was a gift from Prof. Brian E. Ellis (University of British Columbia). *DFR* deficient mutant (*tt3*) in L*er* background [20,21] was gifted by Prof. Hongxuan Lin (Chinese Academy of Sciences). Seeds were sterilized, sown on 1/2 MS or N-free MS agar medium and incubated for 2 d at 4 °C in the dark to break dormancy (stratification), then they were transferred to a growth chamber with a photoperiod of 16 h light and 8 h dark at 22 °C.

2.2. Seed germination assays

180 to 200 seeds of each genotype were plated on 1/2 N-free MS agar medium and incubated for 2 d at 4 °C in the dark before being transferred to a growth chamber (22 °C, with a 16h/8h light/dark cycle) for 7 d. Germination was defined as the first sign of radical tip emergence and scored. The germination results were calculated based on three independent experiments.

2.3. Seedling survival rate assay as a determination of low N stress tolerance

180 to 200 seeds of each genotype were plated on 1/2 N-free MS agar medium and were grown for 50 d for N starvation treatment. The N-deprived seedlings were then transferred to 1/2 MS agar plates for 7 or 14 d to allow the plants to recover from N deprivation. The seedlings recovery rate was recorded from samples with three independent biological replicates.

2.4. Measurement of anthocyanin content

The anthocyanin content was measured as described by Kim et al. [22] with minor modifications. Briefly, the anthocyanins were extracted by incubating 50 mg of shoot tissue in 300 μL of extraction solution (1% HCL in methanol) overnight at 4 °C in darkness. After this, 200 μL of water and 200 μL of chloroform were added and the mixture was centrifuged at 14000 rpm for 2 min to remove the tissue debris. 70 μL of the supernatant solution was used to measure the anthocyanin content, which was detected by a spectrophotometer and calculated as (A530−0.33*A657) per gram of fresh weight.

2.5. Measurement of chlorophyll content

For chlorophyll content assay, 0.1 g shoot tissue was extracted with 10 mL of 80% acetone for 12 h, then the supernatant was measured at wavelengths $\lambda = 645$ and 663 nm by a spectrophotometer. The chlorophyll *a and b* contents were calculated as chlorophyll *a* (mg/g) = (12.7*A663−2.69*A645)/(100*fresh weight) and chlorophyll *b* (mg/g) = (22.9*A645−4.68*A633)/(100*fresh weight) [23]. The total chlorophyll = chlorophyll *a* + *b*.

2.6. Flavonoids collection and preparation

8-d-old seedlings grown up on 1/2 MS medium were transferred to N-free medium for 16 d, then shoots were collected, rapidly frozen with liquid nitrogen and stored at −80 °C until use. Accurately 40 mg of sample was used for metabolites extraction. The extraction was done by adding 600 μL of methanol-water (7:3, v/v) mixed solution and 20 μL of internal standard (2-Chloro-L-phenylalanine, 0.3 mg/mL) dissolved in methanol to each sample. The resulting mixture was ultrasonicated for 30 min in ice water bath, incubated at −20 °C for 20 min and then centrifuged at 14000 rpm (4 °C) for 10 min. A 200 μL aliquot of supernatant in a glass vial was ready for LC-MS analysis subsequently.

2.7. LC-MS analysis of metabolites

3 μL of metabolites sample extracted above was injected into a Waters UPLC system (Waters Corporation, Milford, USA). It was coupled with a Q-TOF Mass Spectrometer equipped with an electrospray interface. Separation was carried out on an Acquity BEH C18 column (100 mm × 2.1 mm × 1.7 μm, Waters) with the temperature maintained at 45.0 °C. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The program of UPLC gradient elution was as follows at a flow rate of 0.40 mL/min: 0–2 min 5–20% B, 2–8 min 20–60% B, 8–12 min 60–100% B, holding at 100% B for 2 min, 14–14.5 min 100%–5% B, and then holding at 5% B for 1 min. A Waters Q-TOF Mass Spectrometer equipped with an electrospray ionization source was used to collect MS data in both positive and negative ion detection mode. The temperature of source and desolvation were maintained at 120 °C and 500 °C, respectively, and the desolvation gas was at a flow rate of 900 L/h. Full-scan mass spectra were collected from 50 to 1000 mass to charge ratio (*m*/*z*) at ten scans per second and the interscan delay was 0.02 s.

2.8. Metabolomic data analysis and flavonoids identification

The software progenesis QI (Waters Corporation, Milford, USA) was used to analyze the acquired MS data from LC-MS by baseline filtering, peak identification, integration, retention time correction, peak alignment and normalization, which finally produced a matrix of features with the retention time, *m*/*z* and peak intensity. The flavonoids were identified by the progenesis QI and public databases such as http://www.hmdb.ca/and https://metlin.scripps.edu/. The data represented the means from six biological replicates. The heatmap analysis was constructed based on the relative amount of each metabolite and implemented using the R pheatmap package.

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