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Abiotic stresses induce different localizations of anthocyanins in Arabidopsis

Nik Kovinich[1,2], Gilbert Kayanja[1,2], Alexandra Chanoca[3], Marisa S Otegui[3,4], and Erich Grotewold[1,2,*]

[1]Center for Applied Plant Sciences (CAPS); The Ohio State University; Columbus, OH USA; [2]Department of Molecular Genetics; The Ohio State University; Columbus, OH USA; [3]Department of Botany; University of Wisconsin-Madison; Madison, WI USA; [4]Department of Genetics; University of Wisconsin-Madison; Madison, WI USA

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Abbreviations: A5, cyanidin 3-*O*-[2-xylosyl-6-*O*-(4(glucosyl)-*p*-coumaroyl)]5-[6-*O*-(malonyl)glucoside]; A11, cyanidin 3-*O*-[2-(2-(sinapoyl)xylosyl)-6-*O*-(4(glucosyl)-*p*-coumaroyl)glucoside]5-[6-*O*-(malonyl)glucoside]; AIC, anthocyanin induction condition; C3G, cyanidin 3-*O*-glucoside; Col, Columbia-0; HPLC-PDA, extinction coefficient, ε; high performance liquid chromatography- photodiode array detection; ROS, reactive oxygen species.

Anthocyanins are induced in plants in response to abiotic stresses such as drought, high salinity, excess light, and cold, where they often correlate with enhanced stress tolerance. Numerous roles have been proposed for anthocyanins induced during abiotic stresses including functioning as ROS scavengers, photoprotectants, and stress signals. We have recently found different profiles of anthocyanins in Arabidopsis (*Arabidopsis thaliana*) plants exposed to different abiotic stresses, suggesting that not all anthocyanins have the same function. Here, we discuss these findings in the context of other studies and show that anthocyanins induced in Arabidopsis in response to various abiotic stresses have different localizations at the organ and tissue levels. These studies provide a basis to clarify the role of particular anthocyanin species during abiotic stress.

Anthocyanins are plant pigments of the flavonoid subclass of phenylpropanoids characterized by a 3,5,7-trihydroxylated flavylium backbone.[1] The red-to-purple color imparted by anthocyanins to flowers, fruits, and seeds act as visual deterrents to herbivores, and attractants to pollinators and seed dispersers. Anthocyanins and other flavonoids also contribute to stress tolerance in plants. There is a growing interest in understanding the mechanisms by which anthocyanins help plants cope with abiotic stress, most importantly in the context of crop yield reduction due to global climate change. Anthocyanins are commonly induced in plant vegetative tissues in response to a number of different abiotic stresses including drought, salinity, excess light, sub- or supra-optimal temperatures, and nitrogen and phosphorous deficiency.[2-8] The proposed roles of anthocyanins during abiotic stresses include quenching of ROS,[9,10] photoprotection,[11,12] stress signaling,[13,14] and xenohormesis (i.e., the biological principle that relates bioactive compounds in environmentally stressed plants and the increase in stress resistance and survival in animals that feed from them).[15,16]

Plants as a group produce hundreds of structurally distinct anthocyanin species. Arabidopsis (*Arabidopsis thaliana*) alone produces more than 20 different types of anthocyanins, but whether they have specific functions is unknown. Whereas all anthocyanins could have identical roles, the high metabolic cost of adding numerous decorations (e.g. sugar and acyl groups) to the flavylium backbone in the different anthocyanin species makes this scenario very unlikely.

We recently reported that distinct profiles of anthocyanins are induced in seedlings of Arabidopsis in response to different abiotic stresses.[4] We analyzed seedlings grown in 8 abiotic stress conditions, including high salinity, cold, and an artificial stress medium termed anthocyanin induction condition (AIC), which consists of 3% sucrose and no additional nutrients. The fact that distinct profiles of anthocyanins are induced by different abiotic stresses suggested that different anthocyanins, or profiles of anthocyanins, have different functions *in planta*. Another recent study demonstrated that only a subset of Arabidopsis anthocyanins (A11, A9, A8 and A5) were induced in response to short-term drought stress.[17] The anthocyanin profile induced by drought[17] was most similar to that induced in response to salt ($MgSO_4$) stress in our experiments.[4] Therefore, it is possible that plants exposed to drought and salt stresses take advantage of the same anthocyanin, or set of anthocyanins, whereas plants subjected to other abiotic stress conditions may benefit from different anthocyanin species.

A11 was the major anthocyanin induced in Arabidopsis in response to salt ($MgSO_4$)[4] and drought[17] stresses. The synthesis of A11 is metabolically more costly than C3G, an anthocyanin that has widespread occurrence in plant species, as A11 has 6 additional sugar and acyl decorations, and has 3 times the mass of C3G. A recent study demonstrated that acylated

*Correspondence to: Erich Grotewold; Email: grotewold.1@osu.edu

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Table 1. Light absorbance comparisons between anthocyanins A11 and C3G

Wavelength (nm)	530	300
A11 ε (L cm^{-1} mol)	61,300	61,300
C3G ε (L cm^{-1} mol)	34,700	17,300
A11/C3G	1.8	3.5

(coumaroylated) anthocyanins in the epidermal cells of sweet basil conferred tolerance to excess light.[12] A11 is a coumaroylated anthocyanin and absorbs 1.8 times more visible light (at 530 nm) and almost 3 times more UV-B (at 300 nm) than C3G at equivalent concentrations (**Table 1**). In addition, A11 also contains a sinapoyl group, which has been recently shown to confer major increases in the antioxidant capacity of anthocyanins.[18] This raises the question of whether A11 serves as a photoprotectant, as an antioxidant, both, or perhaps neither *in vivo.* Constitutive anthocyanin accumulation in plants overexpressing the anthocyanin regulatory gene *PAP1,* conferred enhanced tolerance to drought and

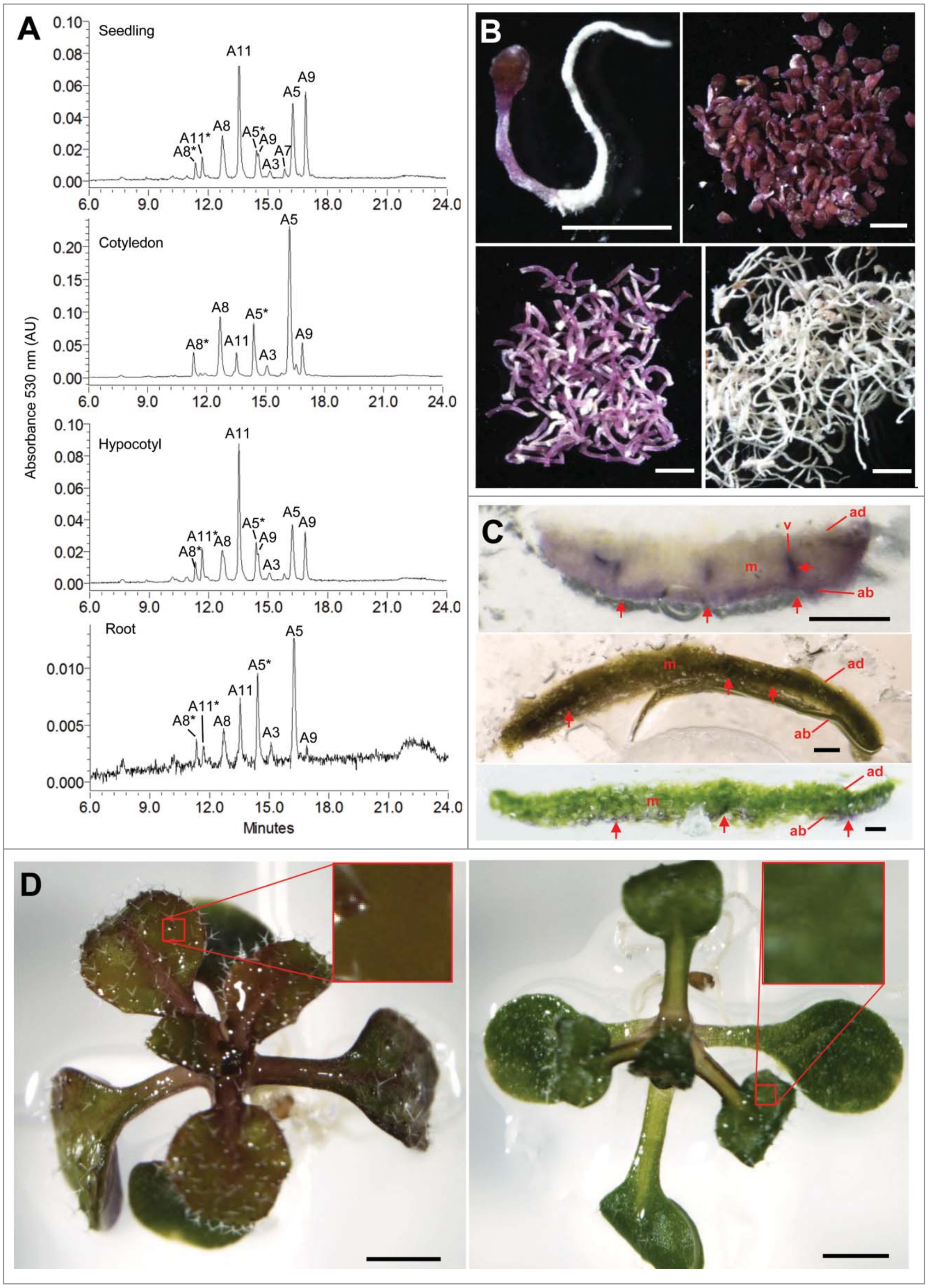

Figure 1. Localization of anthocyanins in Arabidopsis during AIC and $MgSO_4$ stresses. (**A**) HPLC-PDA chromatograms of aqua-methanol extracts from whole seedling (Top), cotyledon (second from top), hypocotyl (second from bottom), and root (bottom). Note scale of chromatograms differs. (**B**) Microscopic analysis of the seedling tissues analyzed in (**A**). Seedlings grown for 5 days in AIC were lyophilized for 3 days and imaged. Whole seedling prior to dissection (top left), dissected cotyledons (top right), hypocotyls (bottom left), and roots (bottom right). Scale bar 1mm. (**C**) Detection of anthocyanin pigmentation in cotyledons of seedlings grown in AIC (top), or on 1/2MS 1% sucrose agar medium containing 100 mM $MgSO_4$ under 150 μmol m^2 s^{-1} light (middle) or 40 μmol m^2 s^{-1} light (bottom). Mesophyl, m; abaxial epidermis, ab; adaxial epidermis ad; vasculature, v. Scale bar 200 μm. (**D**) Leaf color of seedling leaf grown for 10 days on 1/2MS 1% sucrose agar medium containing 100 mM $MgSO_4$ under 150 μmol m^2 s^{-1} light (left) or 40 μmol m^2 s^{-1} light (right). Note: anthocyanins developed a brown color indicative of oxidation under 150 μmol m^2 s^{-1} light. Scale bar 1 mm.

oxidative stresses.[10] A11 is by far the predominant anthocyanin induced by *PAP1* in leaves, but not in petioles, hypocotyls or roots.[10] Whereas these studies cannot rule out that another compound induced by PAP1 enhanced tolerance to the oxidative stresses, the predominant induction of A11 specifically in leaves during oxidative stresses suggest that A11 has a major function in quenching excess ROS generated by photosynthesis.

To determine whether A11 is always the major anthocyanin in Arabidopsis leaves, we measured A11 levels in the cotyledons of seedlings grown in AIC, a stress condition where visible chlorophyll does not develop, and thus anthocyanin would not be required to protect highly photosynthetically active machinery from excess ROS. Our results show that A11 was not a major anthocyanin in the cotyledons in AIC, despite the fact that A11 is among the most abundant anthocyanins in the whole seedling extract (**Fig. 1A**). We visually confirmed that there was no cross-contamination among dissected seedling organs by microscopy (**Fig. 1B**). We found the majority of A11 induced in AIC was in the hypocotyl (**Fig. 1A**, second from bottom). By analyzing cross sections of the hypocotyl, we detected anthocyanin accumulation in all cell layers (**Fig. S1**), suggesting that A11 has a unique function in this organ. By contrast, A5, with no sinapoyl group, was the predominant anthocyanin in cotyledons (**Fig. 1A**, second from top) and roots from seedlings in AIC (**Fig. 1A**, bottom), as well as in roots of seedlings overexpressing *PAP1*.[17] These results show that both cotyledons with low photosynthetic activity and non-photosynthetic tissues under AIC favor the synthesis of A5 and not A11 as the major anthocyanin. In addition, A5 lacks the extra antioxidant capacity afforded by the sinapoyl group in A11, suggesting that A5 does not have a major role in protection against photosynthetically-derived ROS.

Under excess salinity conditions, chloroplast generates ROS (reviewed by[19]) and plants accumulate predominantly A11.[4] To determine whether the localization of anthocyanins in leaves differ between oxidative or non-oxidative stress growth conditions, we analyzed seedlings grown in high salt and high light (100 mM $MgSO_4$, 150 μmol m^2 s^{-1}) and AIC, respectively. In cotyledons grown in AIC, anthocyanins accumulated mainly in the abaxial epidermis and vasculature, but not in mesophyll cells (**Fig. 1C**, top). By contrast, anthocyanins localized primarily to the mesophyll cells of seedlings grown in high salt and high light conditions (**Fig. 1C**, middle). To determine whether the induction of anthocyanins in mesophyll cells correlated with high light that potentially generated excess ROS, we repeated the experiment under low light (40 μmol m^2 s^{-1}). We found that anthocyanins were not present in the mesophyll cells under low light conditions (**Fig. 1C**, bottom). Interestingly, the anthocyanins induced in response to high salt had a brown coloration, (compared to purple color of anthocyanins in AIC; **Fig. 1B–C**), indicative of anthocyanin oxidation only in seedlings grown under high light (**Fig. 1D**, left), supporting their potential roles as antioxidants.

These results, in combination with our recent finding that A11 was the predominant anthocyanin induced in response to $MgSO_4$ stress,[4] suggest that A11 functions as an antioxidant to protect photosynthetic mesophyll cells from oxidative damage caused by the generation of excess ROS. It is possible that A11 in the hypocotyl of seedlings grown under AIC functions as an 'insulator' to prevent ROS signals from traveling between the root and the cotyledon. In addition, A5 in roots and other non-photosynthetic tissues may not act as an antioxidant, and warrants further investigation.

Methods

Plant materials and growth conditions

Wild-type seeds of Arabidopsis thaliana (Col) were surface-sterilized, stratified, and grown as indicated in.[4]

Chemical analysis

Anthocyanins were extracted from equal dry weights of tissue and analyzed by HPLC-PDA as indicated in.[4]

Anthocyanin distribution

Fresh leaf tissues were imbedded in Paraplast X-tra tissue medium (Fisher) and hand-sectioned prior to imaging with an SMZ1500 stereomicroscope equipped with a Digital Sight DS-Fi1 camera (Nikon).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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