Ozone stimulates the production of anthocyanins as a secondary antioxidant defense in *Arabidopsis thaliana*

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INTRODUCTION

Ozone is now the major air pollutant damaging crops, forests, and other vegetation. It enters plants during gas exchange and causes oxidative stress leading to chlorosis and necrosis. In response to such stress, plants increase the synthesis of secondary metabolites for repair processes and antioxidant defenses. Anthocyanins are naturally occurring secondary metabolites that increase in concentration if plants are exposed to unfavorable conditions. Anthocyanins can be antioxidants that fight free radicals and lower the rate of cell death in leaves. *Arabidopsis thaliana* belongs to the Brassicaceae family and has a short life cycle, small size, and prolific seed production, making it an ideal model organism. Three seed-lines varying in anthocyanin production (TT-3, BLH-1, Pap-1) were compared to wild type (Col-1). Three other lines were selected with mutations in other antioxidant defenses (Vtc-1, GPA-1, DES-1).

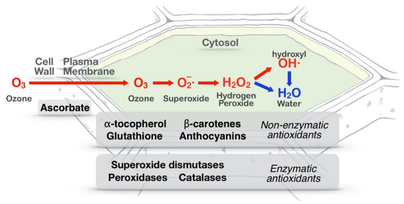
Seed lines:

- Col-1: wildtype, reference line
- Pap-1: T-DNA mutant with highly increased anthocyanins (Borevitz et al., 2000)
- Vtc-1: ozone sensitive mutant, deficient in ascorbate, another antioxidant defense (Conklin et al., 1996)
- Blh-1: natural accession with higher anthocyanins
- TT-3: no anthocyanins, deficient in dihydroflavonol 4-reductase, compromised in transcription regulators affecting flavonoid synthesis (Charles et al., 2009)
- GPA1: less sensitive to ozone, lacking the α subunit of G protein, prevents oxidative burst of reactive oxygen species inside leaf cells (Joo, JH. et al., 2005)
- DES1: deregulated sulfur-responsive genes, enhancing antioxidant defenses (Alvarez, C. et al., 2010)

OBJECTIVES

The objective of this work is to study anthocyanins and their responses to different ozone concentrations as one mechanism of increased ozone resistance in different seed-lines of *Arabidopsis thaliana.* As ozone enters a leaf, the primary antioxidant defense is ascorbate in the cell wall spaces. This prevents some or all of the ozone from reaching the cell membrane and propagating reactive oxygen species into the cell. With high ozone exposure, some ozone does penetrate the cell. Secondary antioxidant defenses include enzymatic defenses, such as superoxide dismutase, and non-enzymatic defenses, such as α-tocopherol. Various signaling processes trigger increases in these secondary defenses once oxidative stress is detected. Anthocyanins are among the secondary antioxidant defenses available to the plant.

Among the seed-lines of *Arabidopsis* tested in this project, some had initially higher anthocyanin concentrations. This could give them greater resistance to ozone stress. Some of the seed-lines tested had mutations in other antioxidant defenses. For mutant lines missing a defense, anthocyanin production could be stimulated more under ozone stress to compensate. For mutant lines with enhanced resistance, enhanced anthocyanin production would not be necessary.



RESULTS

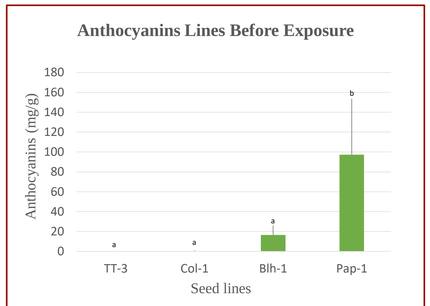


Fig. 1 Pap-1 had significantly higher anthocyanin concentrations than the other lines before ozone exposure. N=25.

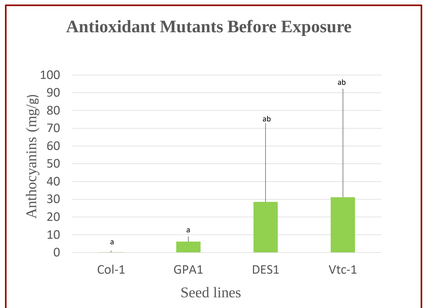


Fig.2 DES1 and Vtc-1 has significantly higher anthocyanin concentration than wildtype col-1 before exposure. GPA1 had no significantly higher anthocyanin concentration than wild type Col-1.

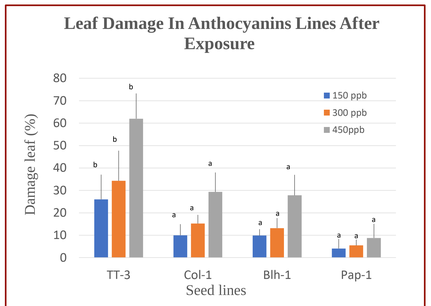


Fig.5 TT-3 showed the highest percentage of leaf damage after ozone stress. Pap-1 showed the least leaf damage. Leaf damage increased with increasing ozone concentration. N=10.

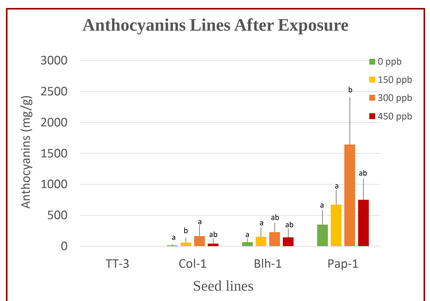


Fig. 3 Anthocyanins increased the most at 300ppb. Pap-1 has the highest increased anthocyanin concentrations. N=25.

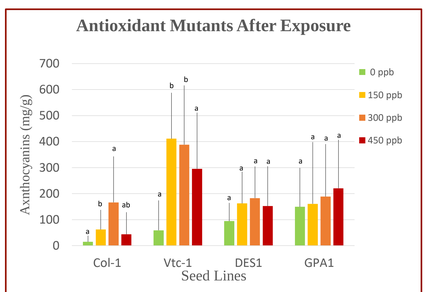


Fig. 4 Vtc-1 has significantly increased anthocyanin concentrations than wild type Col-1 in response to ozone stress. DES1 and GPA1 did not significantly increase anthocyanins in response to ozone stress. N=25.

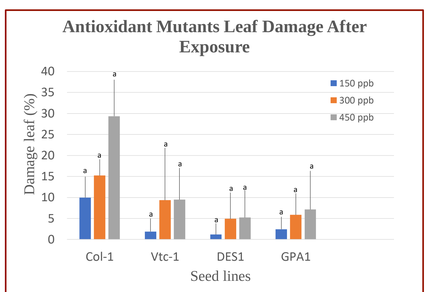


Fig. 6 The three antioxidant mutants showed less leaf damage than the wild type Col-1 under all ozone exposures. Vtc-1 had more leaf damage than DES1 and GPA1 at 300ppb and 450ppb ozone exposure. N=10.

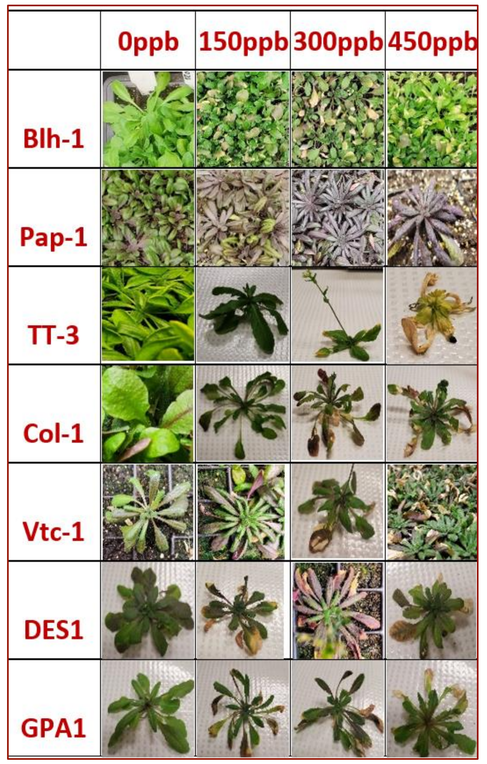


Fig. 7 Pictures showing different seed lines after exposure under 0ppb, 150ppb, 300ppb, and 450ppb ozone respectively.

MATERIALS AND METHODS

Three-to-four week old seedlings were exposed under 0ppb, 150ppb, 300ppb, and 450ppb for eight hours on three consecutive days under 16/9 hours photo period. Anthocyanin concentrations were determined before exposure and one week after exposure for 0ppb, 150 ppb, 300 ppb and 450 ppb. One leaf was soaked overnight in a 1:100 mixture of HCL and methanol and kept in the dark. On the following day 300 ml of distilled water and 750ml of chloroform was added to remove a chlorophyll. The absorbance was determined with a spectrophotometer at 530nm and 657nm. The number of total leaves and damaged leaf under four different ozone concentration was recorded.



CONCLUSIONS

Arabidopsis seed-lines with high anthocyanins before the ozone exposure had less leaf damage after the ozone stress. This confirms that anthocyanins can function as a secondary antioxidant defense. Furthermore, almost all of the seed-lines showed higher anthocyanins after the 3-day ozone stress. These higher ozone concentrations could help give the plants more resistance to a subsequent stress. Pap-1 had the highest anthocyanin concentrations due to enhanced anthocyanin production created by activation tagging and showed the lowest percentage of leaf damage. Blh-1, a natural ecotype with higher anthocyanins, had less than 50% leaf damage. TT-3 without any anthocyanins was the most damaged seed line. One week after the ozone exposures, the anthocyanin lines had increased anthocyanin concentrations up to 5X at 300ppb ozone compared to 0ppb, but not as much at 450ppb. At the highest dose, more severe damage and repair demands may limit resources for secondary metabolites like anthocyanins. Among the antioxidant defense mutants, Vtc-1 had the highest anthocyanin concentration increase from 0 ppb to 150 ppb, probably due to absence of its primary defense, ascorbate. The mutants GPA1 and DES1, less sensitive to ozone, showed less leaf damage and little increase in anthocyanins after ozone exposure. This suggests less need for enhanced secondary antioxidant defenses. Overall, the results suggest that anthocyanins, even though only a less regarded secondary antioxidant, do increase in concentration in response to ozone exposure. This could be a valuable tool in genetic engineering of crops for greater ozone resistance.

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