FINAL PROJECT REPORT
WTFRC project number: AH-03-317A
Project Title: Evaluation of apple lines overexpressing the Antioxidant APX

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Objectives:

- Evaluation of three transgenic apple lines overexpressing APX. Evaluations will be conducted in both West Virginia (Kearneysville) and Oregon (Corvallis). Evaluation of material will be as self-rooted plants and as scions grafted on a commercial rootstock.
- Evaluation will consist of monitoring growth parameters, photosynthetic and antioxidant parameters on lines grafted on commercial rootstocks, especially under conditions of environmental stress.
- Continued characterization of the resistance of potted plants to environmental stresses such as drought, and high and low temperatures, and high light.
- Construction of transgenic lines of apple overexpressing other antioxidant enzyme genes such as SOD (superoxide dismutase).
- Characterization of seasonal expression of the APX gene, and enzyme activity in different tissues (leaves, stem, and bud).
- Determining how overexpression of APX affects other parameters of the antioxidant system (SOD, glutathione reductase, catalase, etc.).

Significant Findings and Accomplishments over the Life of the Project:

Ten to Fifteen Lines of Transgenic Apple Were Created and Several of the Lines Were Shown to Have Increased Resistance to Environmental Stress. We were the first to demonstrate that Overexpression of the Antioxidant Enzyme Gene for Ascorbate Peroxidase (APX) Could Enhance the Resistance of Apple to Several Different Types of Environmental Stress (Low and High Temperature, Drought, and UV-B).

- APX levels were higher in transgenic leaves than in wild-type leaves and had significantly higher total antioxidant status.
- The overexpression of APX produced plants that were more resistant to freezing stress, high temperature stress, and drought stress. (See details in previous annual reports and final report).
- Plants were also resistant to UV-B-induced oxidative stress.
- Transgenic APX plants exposed to drought maintained higher levels of photosynthesis.
- Higher levels of APX in transgenic leaves were consistent throughout the growing season.
- Higher levels of APX levels in transgenic leaves resulted in lower levels of hydrogen peroxide compared to wild-type leaves when leaves were exposed to UV stress.
- No significant differences were observed in growth rates between the wild-type and transgenic lines indicating that the overexpression of APX did not have a negative impact on general metabolism. This is an important finding when assessing the cost of increasing resistance to environmental stress.
- Several genetically-enhanced line of apple were established that overexpress a cytosolic SOD (superoxide dismutase) gene. This line is now being propagated to assess its resistance to environmental stress.

Methods Used in Final Year of Study:

Drought Stress and Enzyme Activities

MATERIALS AND METHODS

Plant materials

Wild type and APX transgenic *royal gala* apple plants were cultured and selected with selective medium. Plants were planted in 8 inch pots and grown under controlled environment conditions (PPFD 200 μmol m^{-2} s^{-1} during a 16 h photoperiod at 25 °C) in a greenhouse.

Drought stress

Leaves were freshly detached and weighed. Then leaves were exposed to the air on a bench in the laboratory (25°C) for various periods and the weight loss were recorded. Parallel samples were dried at 80 ^{o}C to determine the water content of the leaves.

Enzyme activity assay

Abbreviations: dehydroascorbate reductase (DHAR); monodehydro-ascorbate reductase (MDAR); glutathione reductase (GR); ascorbate peroxidase (APX);

Four enzymes involved in the ascorbate-glutathione cycle were studied. Antioxidant enzymes were extracted according to Grace and logan (1996). Leaf discs were weighed, ground with a pre-cooled mortar and pestle in 2.5 ml extraction buffer containing 50 mM KH_2PO_4-KOH (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.3% (w/v) Triton X-100, and 4% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The extract was then centrifuged at 13, 000 *g* for 10 min in an Eppendorf microcentrifuge, and the supernatant was used immediately for enzyme activity assay.The ascorbate consumption was monitored by the reduction of absorbance at 290 nm taking 2.8 (mmol/ $L)^{-1}$ cm^{-1} as the absorption coefficient (Nakano and Asada, 1981)

For the MDAR assay, a reaction mixture containing 0.9 ml of 2 mmol/L ASA in phosphate buffer (pH 7.0), 0.04 ml of ASA oxidase (2 units) in phosphate buffer (pH 5.6), 0.03 mL of 2 mmol/L NADPH in buffer (pH 7.6) and 0.03 mL crude enzyme was used. The consumption of NADPH was monitored by the reduction of absorbance at 340 nm taking 6.2 (mmol /$L)^{-1}cm^{-1}$ as the absorbance coefficient (Krivosheeva *et al*, 1996).

For the DHAR assay, a reaction mixture containing 0.7 ml buffer (pH 7.0) , reduced glutathione (GSH) [20 mmol/L 0.1 ml in buffer (pH 7.0)], 2 mmol/L DHA 0.1 mL and crude enzyme 0.1 ml was used. DHA was freshly prepared and kept on ice until addition of the reaction mixture in the cuvette to prevent oxidation at room temperature. The reduction of DHA to ASA was monitored by the increase in absorbance at 265 nm using 14 (mmol/ $L)^{-1}$ cm^{-1} as the absorbance coefficient (Krivosheeva *et al*, 1996).

Glutathione reductase (GR) activity was determined at 340 nm by following the change in absorbance occurring as NADPH is oxidized to NADP (Glutathione reductase assay kit, Calbiochem).

All the enzyme assays were repeated three times.

Results:

In the drought stress experiment the APX enzyme activity of the wildtype (control) leaves increased rapidly after only 10% loss of water and then quickly decreased after 20% and 30% water loss. In contrast the APX activity of the transgenic leaves gradually increased after 10% and 20% loss in water followed by a gradual decrease at 30% water loss.

The rapid increase in APX activity after 10% water loss suggests that wild type were able to acclimate quickly to the level of the unstressed transgenic leaves but further stress caused a rapid loss of APX activity. It is interesting to note that APX enzyme activity of the APX transgenic plants responded slower than the wild type suggesting that perhaps the higher initial APX activity of the transgenic plants was sufficient to withstand the gradual loss of water followed by a gradual increase in activity to acclimate to the continued loss of water.

The higher initial APX enzyme activity in the APX transgenic plant leaves may enable the stressed cells to better scavenge the hydrogen peroxide produced in response to the stress thus protecting the cells from being oxidized. The initial higher APX activity of the transgenic plants may be important for enabling the transgenic plants to have a buffer against rapid and greater exposure to stresses. The higher maintenance of APX activity of the transgenic leaves with increasing water stress up to 30% water loss suggests that the transgenics were able to acclimate to water stress than the wild type plants.

The initial and sustained higher APX activity of transgenic shows better protection from drought stress.

APX activity was sensitive to the rapid changes in water content of leaves. In contrast the MDAR and DHAR activities seemed much lower, and responded slower to drought stress than APX.

The activity of MDAR and DHAR is higher in the APX transgenic than the wild type plants. These enzymes are important for regenerating ascorbate and the results are consistent with the higher APX activities found in the transgenic plants. This implies that the APX transgenic plants have faster ascorbate-glutathione cycle.

The GR activity in the transgenic plants was lower initially and responded slower to 10% water loss than the wild type. At 20% water loss the GR of the transgenic plants increased rapidly and was greater than the GR activity of the wild type. At 30% water loss GR activity of both plant types decreased however, the activity was greater in the transgenic plants.

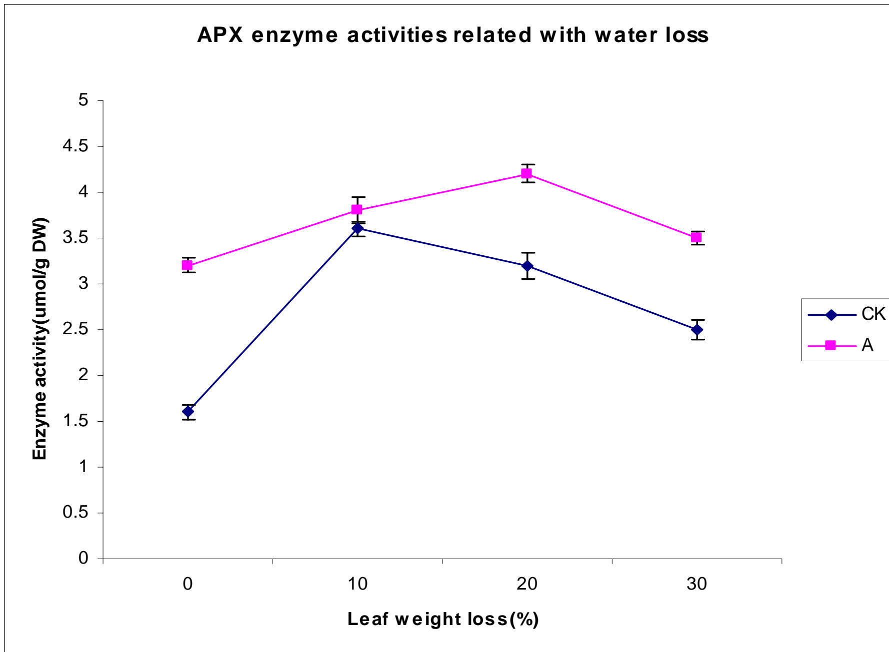

Fig1. APX enzyme activities change with leaf water loss

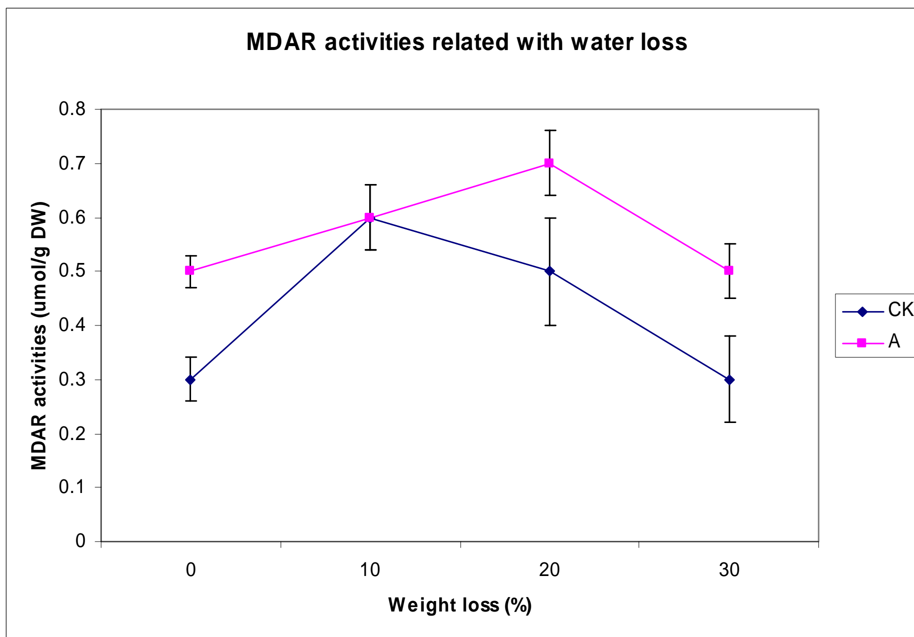

Fig2. MDAR activities with leaf water loss

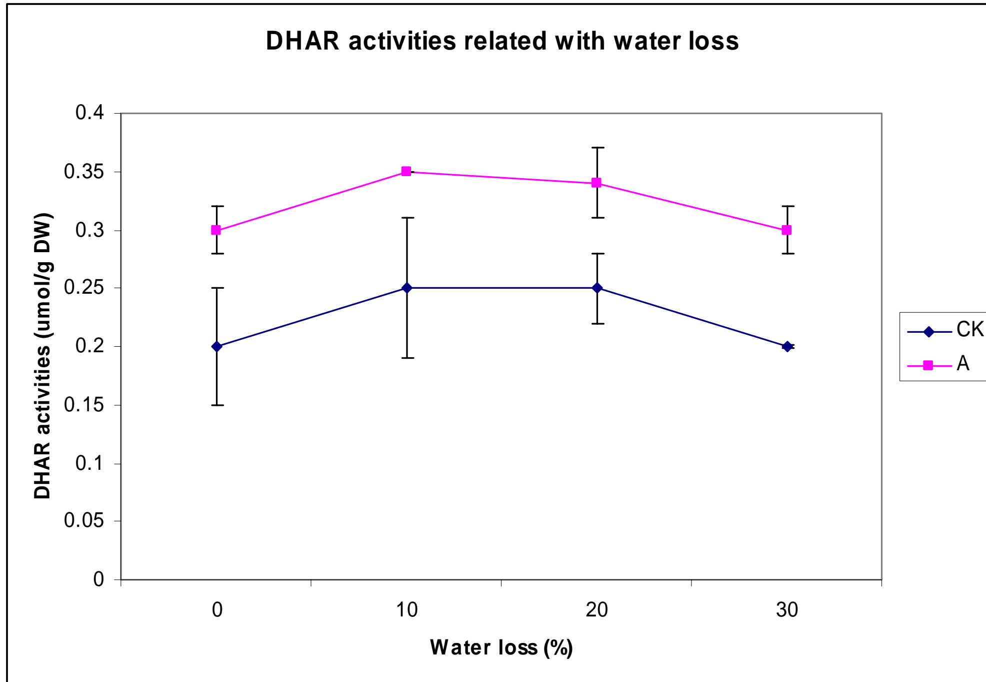

Fig3.DHAR activities with leaf water loss

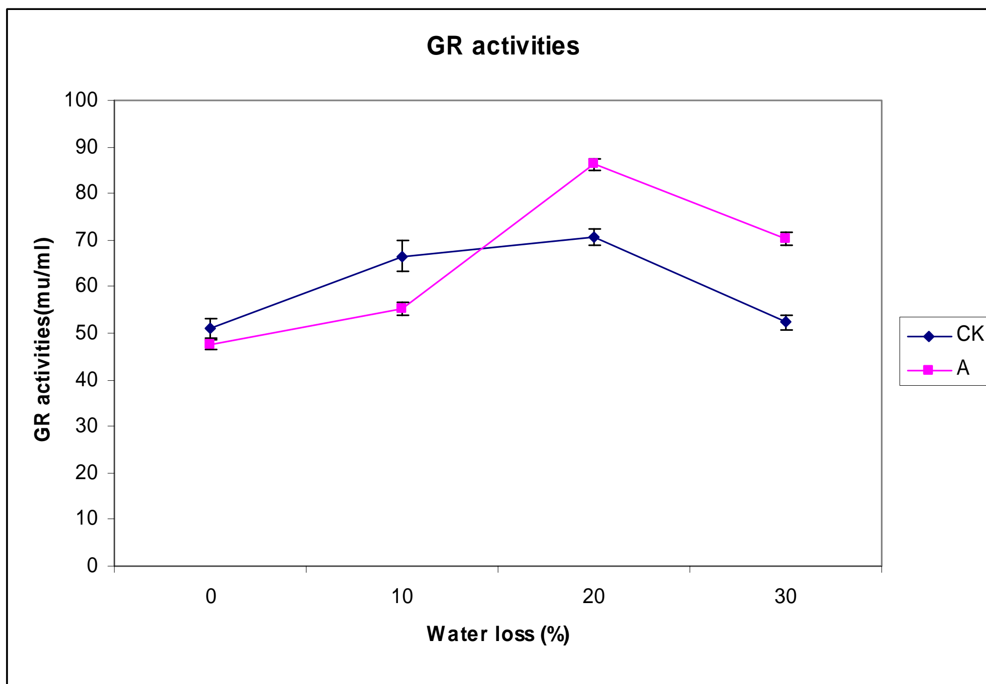

Fig 4. Glutathione Reductase (GR) activity in relations to changes in water loss (%) of leaves from wild-type and APX-trangenic plants

Seasonal change of APX enzyme activity and shoot growth

Material:

Two leaf discs from first fully expanded leaves from wild type and APX transgenic 'Gala' apples growing under natural conditions at Corvallis, OR were collected monthly from April to October. The samples were put into 1.5ml centrifuge tube and placed into liquid nitrogen immediately. All samples were stored in a freezer at -80°C until analysis.

Shoot growth

Shoots from different lines and wild type plants growing in containers at Corvallis, OR as described previously were labeled, and during the beginning of each month, the shoot length was determined. Six replications per each line and wild type were determined.

Extraction and assay of APX

The method for APX assay was the same as described above. One sample from wild type was used as a reference protein. It was divided into 0.5ml centrifuge and 25 µl/ tubes. All tubes were put into the freezer (-80°C) immediately. The enzyme assays by native gels for all the plant types.

Results:

1.
The APX enzyme activity of the wild type and APX transgenic plants showed two peaks during the 2005 season (Fig5). In general the APX activities of the transgenic liens were greater throughout the sampling period than the wild type plants. In both the wild type and transgenic lines the activity increased slowly to a peak at June followed by a decrease in July in the transgenic line and a decrease in July and further decrease in August by the wild type. The activity increased again to a new peak in August for the transgenic line and in September for the wild type plants. This trend was similar as the results of 2004.

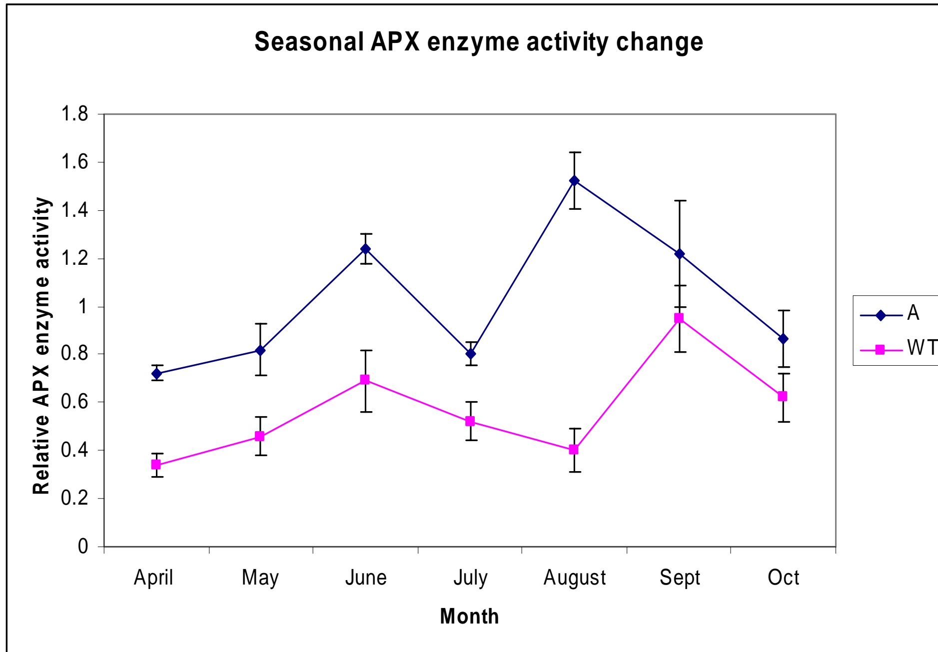

Fig 5. Seasonal APX enzyme activity change

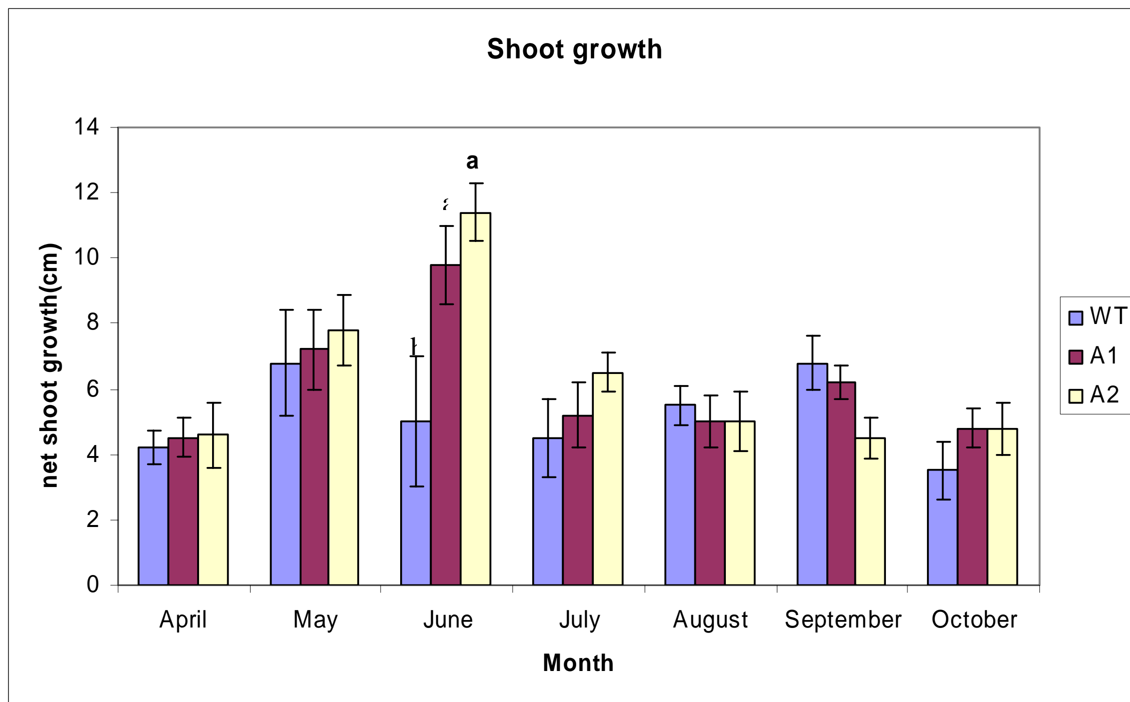

Fig 6. Seasonal shoot growth of APX transgenic plants and wild type

2.
The only significant difference found in the monthly growth between the wild type and two transgenic lines were found in June when both transgenic lines grew significantly more than the wild type plants. At all other sampling periods from April to October no differences in growth were found in general between the plant types. The only other exception was found in September when the growth of the wild type was greater than one of the transgenic line.

The Relationship of Chlorophyll Fluorescence under drought stress

Material
Three year old potted apple trees were used. Six replications of transgenic plants and wild type plants growing in 8 inch pots containers were completely randomized at Oregon State University, Corvallis, OR.
Plants were treated without watering for 3 days. Chlorophyll [variable fluorescence/maximal fluorescence (Fv/Fm)] ratios were taken on the same leaf at day 0,1,2,3 in the morning.

Measurements of chlorophyll fluorescence

Modulated Chlorophyll fluorescence measurements were made in attached leaves of apple plants at midday with a Hansatech portable FMSII fluorometer (Hansatech, UK)

The plant was dark adapted overnight prior to the measurement by put the plants into the dark room at night. Upon the exposure to a saturating flash (8000 mol m^{-2} s^{-1} for 1 s) of light, fluorescence increases from the ground state value (Fo) to its maximum value, Fm. In this condition, QA, the first electron acceptor of PSII, is fully reduced. This enables one to determine the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given by Fv/Fm = (Fm-Fo)/Fm.

Results:

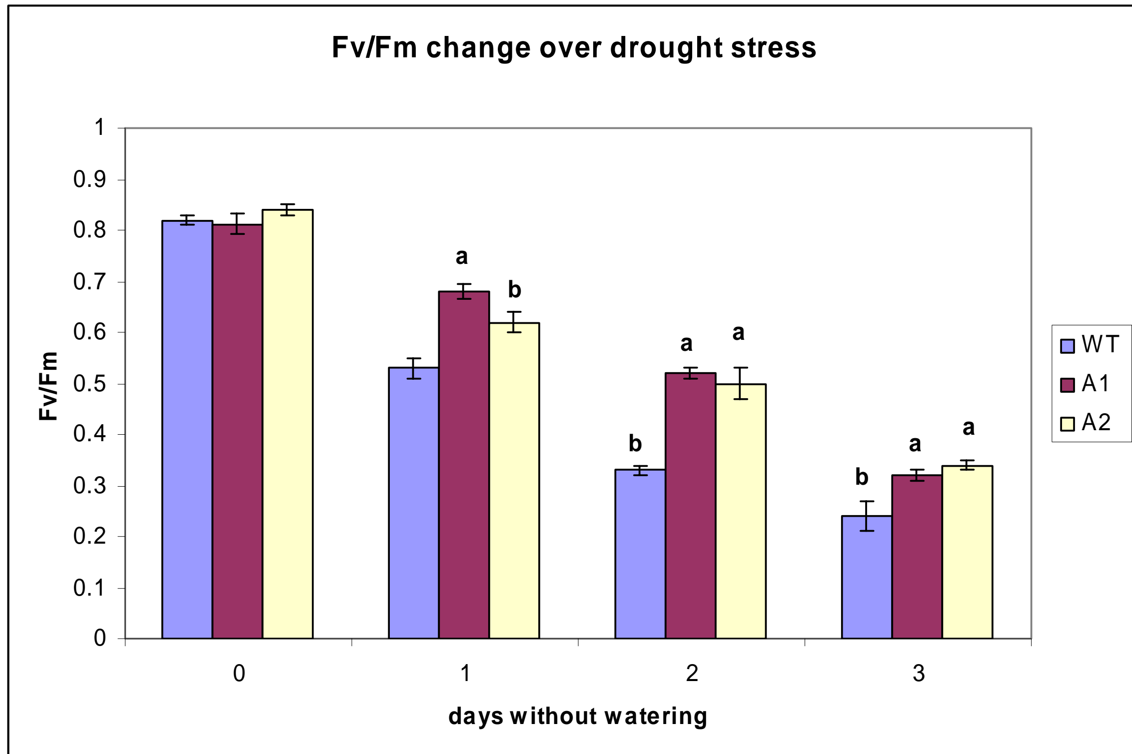

Fig 7: PSII efficiency of wild type and transgenic plants under drought stress

Efficiency of PSII photochemistry (F_v/F_m) is an indicator of photoinhibition.. After one, two and three days of water stress the APX transgenic plants maintained significantly higher PSII efficiency(Fig.7) than the wild type plants, This suggests that a proportion of PSII reaction centers in the wild type plants suffered greater damage due to exposure of plants to water stress conditions. This study suggests that higher APX enzyme activity in the APX transgenic lines can protect apple leaves from photoinhibition.

Budget

Production and Evaluation of Transgenic Apple Overexpressing Enhanced Antioxidant Enzyme Systems

Principal Investigator:
Michael Wisniewski
Co-Principal Investigator:
Les Fuchigami
Project Duration:
2003-2005
Current Year:
2005
Project Total (3 years):
$45,000 – **Represents Awards at Levels Significantly Less than Requested. Although the project was approved, it was funded during the first two years at half the level requested and funding was drastically reduced in the third year.**

Budget: USDA-ARS

Item	Year 1 (2003)	Year 2 (2004)	**Year 3 (2005)**
Supplies	5,000	5,000	**0**
Total	**5,000**	**5,000**	**0**

Budget: Oregon State University

Item	Year 1 (2003)	Year 2 (2004)	**Year 3 (2005)**
Wages	15,000	15,000	**5,000**
Benefits (3%)	0	0	**0**
Supplies	0	0	**0**
Total	**15,000**	**15,000**	**5,000**