Increasing water-use efficiency of almond trees through Agrobacterium-mediated transformation

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Problem and Solution

Almonds grown in California consume 10% of our water supply; however, it is an $11 billion industry and supplies 80% of the world's almonds. We propose to reduce water usage by almonds through utilizing Agrobacterium-mediated transformation to overexpress a gene that increases water-use efficiency and therefore supports environmental sustainability and the almond economy.

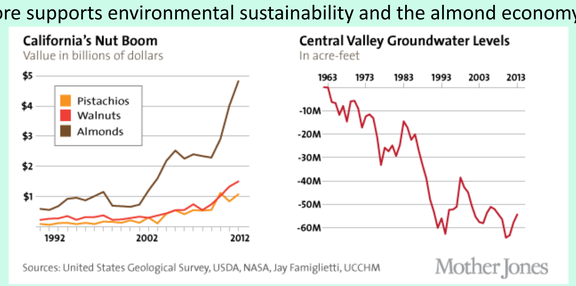

Figure 1: Growth of nut industry (left) , decrease in California ground water levels (right)

History and Current Alternatives

Water usage by California's agricultural industry continues to be a hot button issue. In the past, California has attempted to use drip-irrigation systems to conserve water and nutrients. This type of micro-irrigation system drips water directly onto the roots of a plant, reducing the amount of water lost through evaporation. With the issue of climate change growing exponentially, the likelihood of relapsing into major droughts is high. Although we may be recovering from the recent drought, California's water supply will continue to be of concern if we are unable to drastically reduce our water use, which will lead agricultural businesses to suffer $2.2 billion in part due to high water costs according to UC Davis economists. These costs have forced California farmers to use an extreme amount of groundwater, leading to ground level depreciation and soil salinization. Auto emissions alone have been estimated to have amplified the effects of the drought by 15-20%. There is a dire need to solve this problem in a way that balances economic growth, agricultural progress, and environmental sustainability.

Ethical and Societal Concerns

Public concern stems from the fear of foreign genes expressed in our food. The *PsbS* gene we're modifying will not be expressed in the almond nut; it would only be expressed in plant stomata, which are located along the stems and leaves. However, this does not necessarily mean the *PsbS* gene does not affect the molecular pathways involved in the growth and maturation of the seeds.

In order to precisely select modified crops we will use a marker gene. This marker gene traditionally contains an antibiotic or anti-herbicide, which can cause public concern about the acceleration of "superbugs" and "super plants." To address these concerns, we will be using CRISPR technology to remove the isolated anti-herbicide marker gene.

In terms of safety and contamination concerns, the tests will be confined to a lab or an isolated greenhouse with only plant and bacterial subjects. Also, FDA approved tests will be implemented for food safety as well as nutrition.

Technical Challenges

Because the effect on the tree's mass, growth rate, and yield can only be predicted using results from previously transformed tobacco plants, we cannot completely for see the outcome of our manipulation. We expect to see little consequence on the tree's growth, because *PsbS* expression has shown to have an unsubstantial impact on net CO_2 assimilation. Another challenge arises due to the long maturation period of around 5-12 years, potentially making testing procedures uneconomical. However, government subsidies reserved for agricultural water use can be allocated towards research and development of our crop. Lastly, we will likely find many issues with the process of facilitating the transformation of the plants by A. *tumefaciens*. To optimize efficiency of the process, many additional factors must be controlled (plant-bacteria co-incubation time, periods and intensity of light, pretreatment with phytohormones, different *A. tumefaciens* helper strains).

Detailed Solution and Sketches

We chose to genetically modify almond plants via Agrobacterium-mediated transformation using a binary plasmid system to increase the water-use efficiency of almond trees. Our vector of interest, A. *tumefaciens,* is a bacterium that normally causes cancer in the plants it infects through the integration of oncogenes - Auxin and Cytokinin. We will utilize restriction enzymes to remove these oncogenes from the bacterium's Tumor-inducing plasmid. Then, we will engineer and incorporate binary plasmids containing a T-DNA strand with the Photosystem 2 Subunit S (*PsbS*) gene which, when overexpressed in the almond trees, will make stomata less prone to opening. This method was shown to cause a 25% reduction in water consumption without significant loss of CO_2 assimilation.

This works by blocking electron transfer to Chloroplastic Quinone A (QA), the primary electron acceptor downstream of photosystem 2 whose redox state directly affects stomatal opening in response to light. The *PsbS* protein plays a central role in the activation of non-photochemical quenching (NPQ) through light-regulated interactions with the antenna proteins of Photosystem 2. Therefore the increased expression of this gene decreases the rate at which light energy is used to reduce QA. By keeping QA more oxidized, we will be decreasing the frequency of stomatal opening in response to light. Though the specific mechanisms of *PsbS* proteins and their effect on NPQ are unknown, research suggests they play a role in controlling the macro-organization of the light-harvesting supercomplexes of PS2 and the activation of NPQ. Along with the *PsbS* gene, we will also be incorporating a reporter gene (either GFP or the herbicide resistance gene) for the purpose of identifying the successfully mutated bacteria and plants.

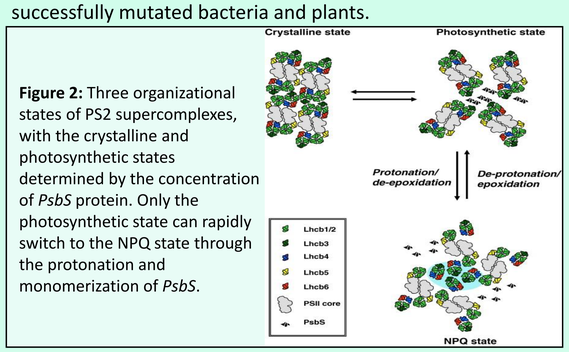

Figure 2: Three organizational states of PS2 supercomplexes, with the crystalline and photosynthetic states determined by the concentration of *PsbS* protein. Only the photosynthetic state can rapidly switch to the NPQ state through the protonation and monomerization of *PsbS*.

To infect the plants, we chose a floral-dip transformation procedure over the alternatives because it is simpler, cheaper, free from somaclonal variation, and does not require a sterile environment or tissue culture regenerative abilities from the plant. This is especially important because almond trees, a type of woody crop, are most likely recalcitrant to regeneration based on studies of similar crops. In the floral-dip method, the female reproductive cells are transformed by dipping the reproductive plant parts into a solution of 5% sucrose, 0.01-0.05% Silwet L-77, and Agrobacterium cells carrying the gene of interest.

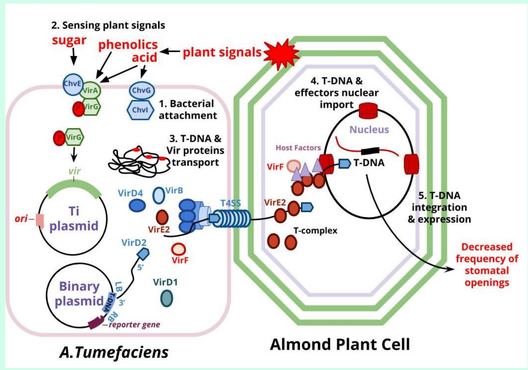

Figure 3: Agrobacterium-mediated Transformation

Upon infection, plant phenolics will bind to the bacterium's *VirA* transmembrane protein, triggering the activation of *VirG*, a transcriptional activator of the Virulence region (*vir*) genes on the Tumor-inducing (*Ti*) plasmid. The *vir* proteins resulting from *vir* gene expression will aid in the processing and delivery of our T-strand into the host cell's nucleus. Both *VirD1* and *VirD2* cut our T-strand at its left and right border, with *VirD2* connecting to the 5' end to pilot the DNA into the host cell. This T-complex will be transferred into the host cell via the *vir*-encoded Type IV secretion system (T4SS), along with the help of *VirE2*, *VirD4*, and various *VirB* proteins to facilitate and protect the package from damage. After infiltrating the cell, *VirD2*, *VirE2*, and the plant's own host factors will integrate the T-complex into the host cell's nucleus, and *VirF* will disassemble the T-complex for efficient T-DNA integration. Upon expression of the newly incorporated T-DNA in the host cell's nuclear DNA, we will isolate the successfully engineered plants by the expression of the reporter gene. If we use herbicide resistance (as opposed to fluorescence) for identification, the ampicillin resistance gene incorporated will be removed using CRISPR/Cas9 technology to mitigate potential public concern.

Table 1: Selection Matrix for **Gene of Interest**

SELECTION CRITERIA 1 = worst 2 = neutral/unknown 3 = best	**Selected Solution:** *PsbS*	**Alternative 1:** CspA and CspB	**Alternative 2:** HARDY	**Alternative 3:** DRO1
% H2O reduction	**2** 25%	**1** <15%	**3** About 55% in WUE	**1** For drought tolerance, no change in H2O consumption.
Size of the gene (bp) - transformation efficiency	**1** 3203	**2** 1748	**3** 554	**2** 2207
mRNA expression one-channel array signal intensity	**3** 3704.118	**1** 648.907	**1** 22.163	**2** n/a
Side effects on yield	**2** Unsure whether the yield will decrease.	**2** Unsure whether the yield will decrease.	**1** Stunts plant's growth if overexpressed	**2** Unsure whether the yield will decrease.
Functional complexity	**3** Found in almonds (WT)	**2** Can be added to almonds	**1** Not found in almonds	**3** Found in almonds (WT)
UV absorption (@ 280 nm) = (W, Y and F residues/aa length)	**3** 22 / 265 = 8.30 %	**2** 19 / 301 = 6.31 %	**3** 15 / 184 = 8.15 %	**1** 14 / 287 = 4.88 %
Sum	14	10	12	11
Rank	1	4	2	3

Table 2: Selection Matrix for **Method of Bacterial Transformation**

SELECTION CRITERIA 1 = worst 2 = neutral/unknown 3 = good 4 = best	Rate of Success for Almonds	Necessity for Regeneration	Cost Time	Complexity	Range of transformable species	Sum	Rank
Suggested Solution: Floral dip Agrobacterium-mediated transfer	3	4	4	3	3	17	1
Alternative 1: Cell culture, Agrobacterium-mediated	4	1	1	1	2	9	7
Alternative 2: Injection of Agrobacterium into tissue	3	3	3	2	3	14	3
Alternative 3: Vacuum infiltration, Agrobacterium-mediated	3	4	3	3	3	16	2
Alternative 4: Pollen tube mediated gene transfer	1	3	3	3	3	13	4
Alternative 5: Electroporation	2	1	3	3	4	13	5
Alternative 6: lipofection	1	1	4	4	2	12	6
Alternative 7: Microinjection	3	3	1	1	4	12	6
Alternative 8: Particle bombardment	3	1	3	2	3	12	6

Testing, Benchmarking, and Comparison to Alternatives

Testing:

Pre-transformation of crop:

DNA sequencing - sequence plasmid to ensure target DNA incorporation in Agrobacterium.

Post transformation of crop:

Microassay - for protein concentration and identification to measure protein product.

DNA sequencing, for successfulness rate of genetic transformation.

Goal: determine the most efficient quantity of copies of PsbS to insert to provide the most water efficiency without compromising the plant.

Benchmarking:

Use crop quality tests such as size and nutrition in addition to water consumption quantity tests such as transpiration rate tests to compare the modified almond crop's water efficiency and seed quality with the those of other crops containing PsbS gene overexpression such as tobacco and Arabidopsis. Product quality and quantity tests are important to determine the projected marketability of the product.

Comparison to Alternative:

In the event that our method of genetic transformation is inadequate in making almond crops more water efficient, we could use the same Agrobacterium-mediated transformation process with the addition of CRISPR technology to accurately change the promoter of the PsbS to be more active. The reason why we are not attempting this initially is because our current plan has been successful historically in many successful GMOs and is less complex.

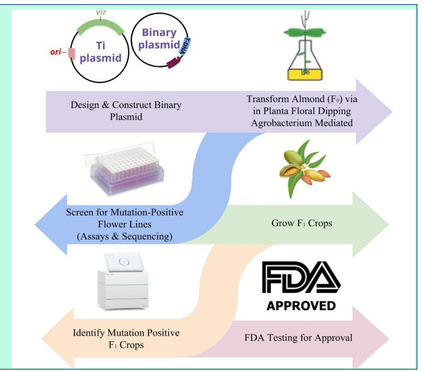

Figure 4: Procedural Flowchart

Sequential procedure for implementing our Agrobacterium-mediated transformation solution of almond plants.

References and Acknowledgements

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Thanks to Anubhav

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