RESEARCH PLAN

1. Specific Aims

We propose to transform houseplants with genes normally expressed in mammalian liver, genes involved in the detoxification of the most potent gaseous air pollutants. Such transgenic plants can help remove or degrade suspected carcinogens such as chloroform and benzene from indoor air.

When compared to wild-type control plants transgenic tobacco plants expressing the human cytochrome P450 2E1 gene exhibit greatly improved removal rates for benzene, chloroform, and trichloroethylene (up to 10 times higher rates). The proposed study will therefore address the following objectives: transform the common household plant, pothos ivy (*Epipremnum aureum*) with the mammalian cytochrome P450 2E1 gene, and confirm increased degradation of benzene and chloroform by the transformed plants compared to wild type plants. This research should provide the basis for a cost-effective, plant-based technology that enhances indoor air quality.

2. Significance and Related R&D

Health Effects and Sources of Indoor Volatile Organic Compounds. According to standard EPA risk models applied to recent measurements of indoor air pollution exposure [1-3], volatile organic carcinogens (VOCs) in household air account for 50-180 excess cancer cases per million That means that each year as many as 12,000-45,000 excess cancer cases in the US can be attributed to indoor air pollution by VOCs. These cases do not include the effects of second-hand smoke, which greatly increases levels of several VOCs known to be carcinogenic.

Even in cities, indoor air is more polluted with VOCs compared to outdoor air, by a factor of 2-20 fold. There are a variety of VOCs in household air; but benzene, chloroform, carbon tetrachloride, and formaldehyde account for most of the cancer risk [1-3]. Household air is more polluted than office air and school air, and those who spend much of their time at home, such as children and home workers [4]; receive a proportionately higher dose of carcinogens [3]. Infants are particularly susceptible to indoor air pollution due to their low body weight and continuous exposure to indoor air. Importantly, household air is by far the most polluted air that most people breathe in the course of a day. Due to these startling levels of exposure, governments are beginning to recognize the public health importance of indoor air pollution.

Our proposal is focused on decreasing a continuing threat to human health.
How can we reduce exposures to VOCs in indoor air? Some sources of these chemicals can be eliminated or reduced. For example, formaldehyde in household air can be reduced by changing construction and upholstery material compositions. Other carcinogens with multiple sources are more difficult to eliminate; such as benzene, which originates from environmental tobacco smoke (ETS) (i.e., second-hand smoke), attached garages, and from ambient (i.e., outside) air. ETS could be eliminated by prohibition of tobacco use in the home, but that is not likely to be acceptable or effective. Benzene losses from fuel in automobiles and small gas engines in attached garages could be significantly controlled at the source only with major expensive and disruptive regulatory reform of the gasoline refining industry.

Another example of a VOC that cannot be controlled at its source without major societal expense is chloroform. Disinfection byproducts (DBPs), such as chloroform, are produced during chlorination of drinking water. Disinfection of water is essential to eliminate pathogens,

and chlorination is the least expensive and most reliable of disinfection methods. Unfortunately, volatile DBPs (primarily chloroform) are released into home air during showering and washing with chlorinated municipal water. By some estimates, chloroform is the most important gaseous carcinogen in household air [2]. Chlorination is an integral part of drinking water treatment in the United States, so, while it can be reduced by water treatment improvements or avoided by alternative disinfectants such as chloramines, chloroform is likely to continue to be a significant component of municipal water in most of the US. Ingestion of water containing chloroform and other DBPs can be avoided by drinking bottled or purified water, but chloroform and other volatile DBPs released during showering and washing cannot be avoided without the high expense of activated carbon treatment of the entire household water.

Ambient air pollutants from outside the home also contribute to indoor air exposures, especially for carbon tetrachloride. The use of carbon tetrachloride in consumer products has been generally banned in the developed world, but carbon tetrachloride persists in urban air due to its resistance to degradation. Among the top four indoor air pollutants, only carbon tetrachloride is thought to originate from outdoor air infiltration into houses, since the concentrations in indoor and outdoor air are approximately equal [1, 2].

A recent meta-analysis of 77 published studies of indoor air pollutant concentrations found that several P450 2E1 substrates ranked among the highest risk at the levels reported in residences: benzene, mono- and dichlorobenzene, carbon tetrachloride, vinyl chloride, chloroform, ethylbenzene, trichloroethylene, toluene, xylenes and bromomethanes [5].

In summary, volatile gaseous chemicals in indoor household air likely account for thousands of excess cancer cases each year in the US. Eliminating sources of these chemicals in household air is difficult and expensive. An inexpensive method to remove VOCs from household air is needed.

Conventional methods for removal of VOCs from indoor air. Conventional air filters based on activated carbon are too expensive for general application. A typical commercially available air filter for a large room recommends replacement of the carbon filter on a 3-6 month interval at a cost of $15 each[1], so annual costs would be about $45 for each room, perhaps $200 per year for a four-room house. The effectiveness of commercial carbon filters has not been demonstrated, and their performance declines significantly in moderate to high humidity. It is questionable that adequate maintenance of household filters could be sustained. Another high-energy method for removing VOCs from household air is photocatalysis. This approach works for more reduced VOCs such as toluene and benzene [6], but is of doubtful effectiveness for chloroform or carbon tetrachloride, which are highly oxidized. Additionally, the cost of photocatalytic methods is too high [7]. A low-cost, passive method that destroys the pollutants would be preferable to carbon absorbent filters.

Removal of VOCs from indoor air by plants. Household plants have been widely touted as having the ability to remove air pollutants from indoor air. This approach is known as the “green liver” concept and is a central idea of the field of phytoremediation, the use of plants to remove xenobiotic pollutants from the environment [8]. The “green liver” concept is based on the analogy between pollutant metabolism in plants and that in animal livers where toxic compounds are metabolized in the human body. Based on this analogy the hypothesis is drawn that plants can detoxify pollutants in the environment before they can be ingested or inhaled by humans.

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[1] http://www.holmesproducts.com/support_files/pdfs/HAP500_SERIES.pdf

Early studies of air detoxification by household plants found that that formaldehyde was removed from the air of chambers containing spider plants [9, 10]. However, other researchers reported that soil or water alone could explin the removal [11]. Nonetheless, plants can assimilate and metabolize formaldehyde from the air as shown by controlled pure culture plant experiments [12-14]. However, the formaldehyde uptake rate through the leaf surface of typical house plants appears to be insufficient to remove formaldehyde from a typical room without an excessive number of plants (e.g., more than 20 plants per room)[13, 15].

Plants have been genetically modified to overexpress native plant formaldehyde dehydrogenase activity, but the rate of formaldehyde removal was increased by only 25% over unmodified plants [14].

Several studies of plant/soil microcosms have shown that if gaseous benzene or toluene is delivered at sufficiently high concentrations, a benzene-degrading or toluene-degrading microbial population can be accumulated in aerated soil [16-18] or on leaf surfaces [19]. Importantly, levels demonstrated to be effective for increasing benzene degrading populations are 10,000 to 400,000 times more concentrated than typical indoor levels. Household air levels are too low to support significant enhanced benzene-degrading microbial populations, since benzene-degrading microbes must maintain their benzene degrading activities from energy released from benzene degradation. At low benzene concentrations typical of indoor air, that energy is insufficient to sustain the microorganisms. Additionally it appears unlikely to us that household air levels of benzene would be sufficient to induce the expression of the multi-enzyme pathway to metabolize benzene.

Several studies show that common plants can remove VOCs such as formaldehyde and benzene from air, but these studies produce very variable estimates of the rate that a particular plant species removes a given pollutant from air. For example, the rates that seven plant species were found remove benzene from the air by five different labs are shown in Table 1. The pollutant concentration and the removal rate normalized to the leaf area are shown. All of these studies were conducted as batch tests with potted plants in closed vessels dosed with benzene injected into the headspace of the vessels and subsequent air samples analyzed by gas chromatography to provide a time course of benzene removal. The removal rates were corrected for leak rates and for losses with the potting media only. The concentrations used in these tests were several orders of magnitude greater that those typical of home air (e.g., 1-7 ug/m^3 [20]).

To correct for variations in the pollutant concentration, the first order rate constant was calculated by dividing the removal rate by the pollutant concentration, since the removal kinetics in the test vessels was likely controlled by diffusion limitations and by first order kinetics at the enzyme. Note that the air concentrations used in the tests were equivalent to less than 0.2 mg/L in an equilibrated aqueous solution, well below likely Michaelis-Menton half saturation constants for benzene oxidizing enzymes [21]

The rates of benzene removal determined by these experimenters for the plants they tested in common are shown graphically in Figure 1, for seven indoor plant species or genera that the studies had in common. Based on these results it is clear that no indoor plant genus has been shown to be superior to any other.

What accounts for these huge variations in plant efficacy for pollutant removal? Variations in apparatus, materials, procedures, and soil adsorption corrections may have caused the wide

variation in removals, but without experimental controls in common between studies, it is impossible to compare or understand the results of current disparate studies. We propose that future comparative studies of the abilities of indoor plants to remove pollutants from the air be conducted under the following conditions:

1. Hydroponic conditions with plants treated to remove epiphytic bacteria. Such conditions help to minimize the activities of bacteria, which are likely to be induced by the unrealistically high pollutant concentrations used in small scale lab tests.
2. Include in every study a common plant such as tobacco, *Nicotiana tabacum*, so that studies from various labs can be normalized to a common standard. *N. tabacum* has several advantages as a standard plant: large leaf area per plant, complete sequenced genome, extensive botanical and physiological literature, general availability, and clonal propagation.
3. All controls for leaks and for soil only experiments should be completely discussed and data shown.

Table 1. Rates of benzene removal from the air by indoor plants as measured by five labs varied by seven orders of magnitude

Plant	Pollutant concentration, ug/m^3	Removal rate, ug/m^2 leaf/h	First order rate constant, Rate/Concentration, m/h	Ref
Dracaena deremensis cv. Variegata	523	2460	4.70E+00	[22]
Dracaena fragrans	31900	0.006	1.81E-07	[23]
Dracaena Janet Craig	80000	27	3.41E-04	[24]
Dracaena marginata	80000	49	6.12E-04	[16]
Epipremnum aureum	31900	0.005	1.45E-07	[23]
" "	80000	13	1.61E-04	[16]
Hedera helix	2057	166	8.09E-02	[25]
" "	4113	385	9.35E-02	[25]
" "	31900	0.038	1.19E-06	[23]
Howea belmoreana	31900	0.008	2.63E-07	[23]
Howea forsteriana	80000	24	3.03E-04	[16]
Schefflera Amate	80000	17.871	2.23E-04	[16]
Schefflera arboricola	31900	0.005	1.45E-07	[23]
Shefflera elegantissima	31900	0.007	2.17E-07	[23]
Spathiphyllum Petite	80000	31	3.85E-04	[16]
Spathiphyllum Sensation	80000	8.6	1.07E-04	[16]
Spathiphyllum Supreme	523	4440	8.49E+00	[22]
Spathiphyllum wallisii	1744	86	4.94E-02	[25]
" "	3487	499	1.43E-01	[25]
" "	31900	0.008	2.47E-07	[23]
Syngonium podophyllum	1744	55	3.13E-02	[25]
" "	3487	296	8.48E-02	[25]
" "	31900	0.00032	9.87E-09	[23]

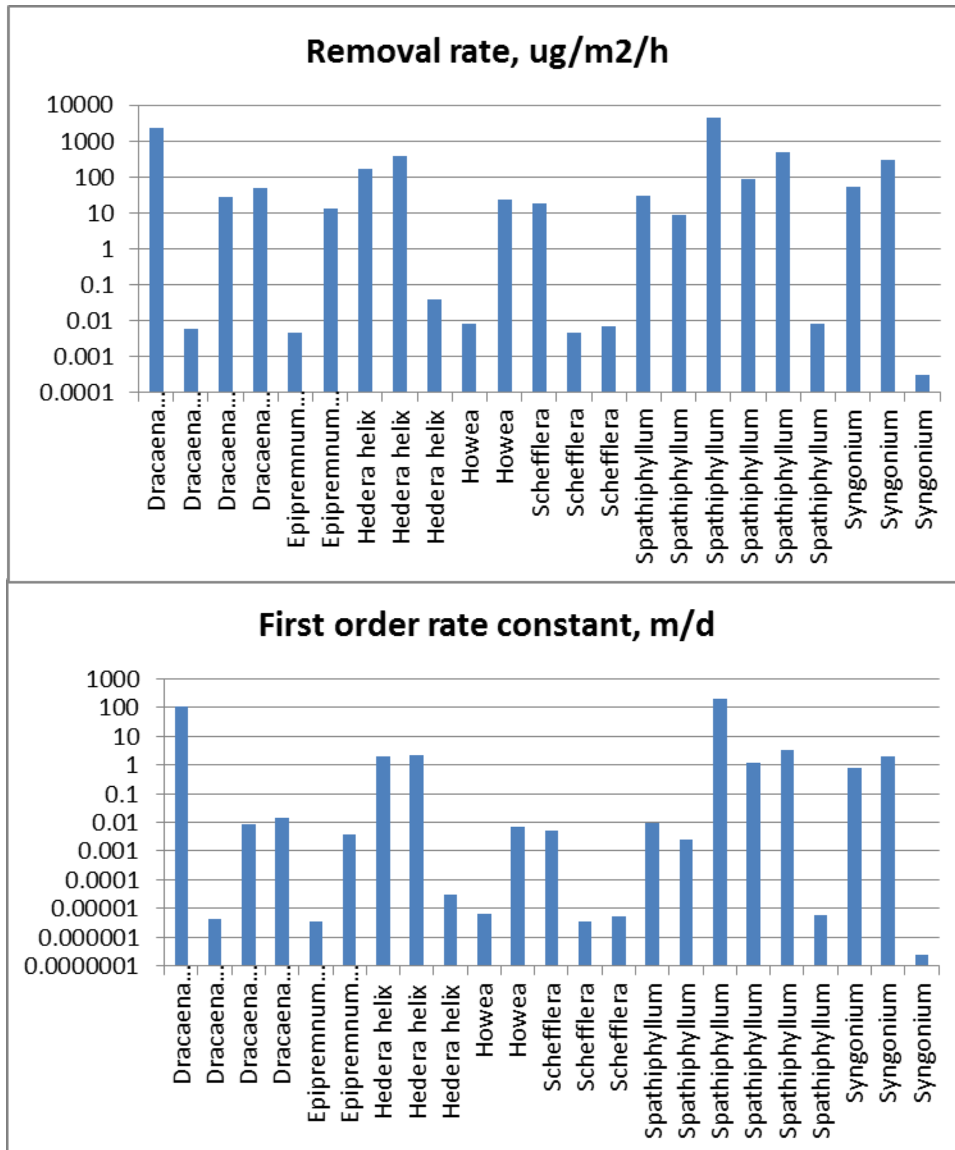


Figure 1 -- Comparison of benzene removal by seven indoor plant species as determined by five studies (see above, Table 1).

These conflicting data notwithstanding, plants do have many attractive features as a platform for metabolism of organic pollutants. Unlike most bacteria, cultivated plants have excess energy available to support catalysis. Plants have high surface areas that facilitate mass transfer of trace gases from the air. Plants do not require the high maintenance typical of bacterial systems. There is certainty of the biological composition of the cultivated plant compared to a soil bacterial community, the population of which cannot be observed directly with present technology. What is needed to increase the detoxification of indoor air is to add to household plants what they lack: a strong ability to metabolize VOCs.

In this proposal, we propose a new approach: we will greatly improve the ability of common houseplants to remove carcinogens from air using biotechnology. Our long-

term goal is to engineer houseplants to break down all of the common gaseous air pollutants found in American homes. Using methods we have developed for reducing water and soil pollution with transgenic plants, we will engineer houseplants to degrade and/or remove the common air pollutants found in American homes. Our first approach is to introduce genes expressed in mammalian liver that are involved in the detoxification of the most potent gaseous air pollutants. We propose to insert the genes for mammalian cytochrome P450s into common household plants. P450s are a group of enzymes that are the main detoxification mechanism in mammals. Our initial focus will be on the specific P450, CYP2E1, which is responsible for the detoxification of the most potent gaseous indoor air pollutants, including chloroform, benzene, and carbon tetrachloride. Later, as time permits, we propose to introduce genes involved in the detoxification of other chemicals, such as mammalian cytochrome P450 2A1 for perchloroethylene degradation and formaldehyde dehydrogenase from yeast, bacteria or mammals [26-28].

Regulatory Considerations. We anticipate that the central concerns surrounding of this technology will be the risks associated with the uncontrolled and unintentional spread of the P450 2E1 gene into a population of sexually compatible local plants. It is our intention to commercialize these transgenic plants for indoor use only. Three government agencies having oversight of GM plants, FDA, EPA, and USDA APHIS, were contacted by Dr. Strand concerning the permitting process for indoor GM plants. With no food or pesticide aspects to the technology, both FDA and EPA deferred to APHIS. APHIS informed Dr. Strand that APHIS does not regulate plants intended for indoor release and provided information for requesting non-regulated status. In addition to molecular genetic characterization of the transgenic Pothos, information on its invasiveness potential, flowering habit, herbicide tolerance, and hardiness zones will be required. Because Jade Pothos is not generally considered invasive in states other than Hawaii, does not flower indoors and only rarely outdoors (in tropical environments outside the US), is capable of being controlled by common herbicides (the 2E1 gene does not confer detoxification to glyphosate or 2,4-D), and has hardiness limited to zones 9-11, it is highly unlikely that the detoxifying gene could spread into a wild population of pothos.

Furthermore, It is important to emphasize that it its very unlikely that P450 2E1 would have any adverse impact in the wild environment of the US. Plants contain hundreds of different P450 sequence varieties compared to mammals, so it is very unlikely that the introduction of another P450 sequence that differs slightly from native plant varieties would have any impact on the fitness of wild plants. Finally the only known activities of the P450 2E1 enzymes are to degrade pollutants, such as ethanol and the VOCs. The slight and speculative risk of transgenic houseplants to degrade pollutants should be compared to the potential benefits of prevention of a portion of the thousands of excess cancers per year thought to be due to indoor VOCs in the US alone.

We will undertake experiments to address questions of ecosafety of the 2E1 transformed pothos. We will determine the susceptibility of the transformed pothos to herbicides, its growth rate relative to the wild type, and its temperature hardiness compared to wild type.

Commercial Opportunities and Societal Benefits. Due to the pervasiveness and carcinogenicity of benzene, chloroform, and other pollutants in indoor air, a real need exists to find low-cost ways to reduce these hazards. The proposed research is focused on increasing the efficiency of VOC removal from indoor air using plants transformed with genes that rapidly degrade these compounds. We will characterize several parameters regulating plant uptake of these dangerous chemicals from the air (e.g., VOC concentration, air velocity, stomatal opening), demonstrate that VOC removal from air is due to these transgenic plants, and

produce useful models of the effects of genetically enhanced plant VOC degradation on indoor air quality, thus facilitating the use of enhanced plants to clean indoor air. Eventually, systems could be built ("phytofiltration systems") that incorporate plants in specialized containers designed to combine the aesthetic advantages of living plants with the functionality of indoor air remediation. Such phytofiltration systems should be particularly attractive in small-scale applications, as in single apartments in buildings for which centralized air filtering is uneconomic. There would seem to be a promising potential market for attractive products that offer both health benefits as well as higher quality of life.

4. Preliminary Studies

Research at the UW on plant remediation of trichloroethylene and carbon tetrachloride, supported by NSF, Dept of Energy and NIEHS has demonstrated that wild type poplar plants have the ability to remove and degrade trichloroethylene and carbon tetrachloride from ground water [29-32]. Our recent studies have demonstrated greatly improved intrinsic removal rates of volatile organic compounds by transgenic tobacco plants expressing human cytochrome P450 2E1 gene [33]. Small intact tobacco plants were completely enclosed in small glass vials with a hydroponic solution. VOCs were injected into the vials and air samples were taken through septum valves attached to the vials. Due to analytical constraints, the initial concentrations of the VOCs in these preliminary experiments ranged from 2 to 2000 mg m^{-3}, which is higher than typical household levels. The plants in the vials were incubated for approximately 7 days with air samples taken daily and analyzed by gas chromatography. The transgenic plants, modified with the rabbit cytochrome P450 2E1 gene, rapidly removed chloroform, trichloroethylene, and benzene from the air of the vials; while the plants without the 2E1 gene ("vector controls") had weak removal of chloroform and trichloroethylene, and no discernible removal of benzene (Figure 1).

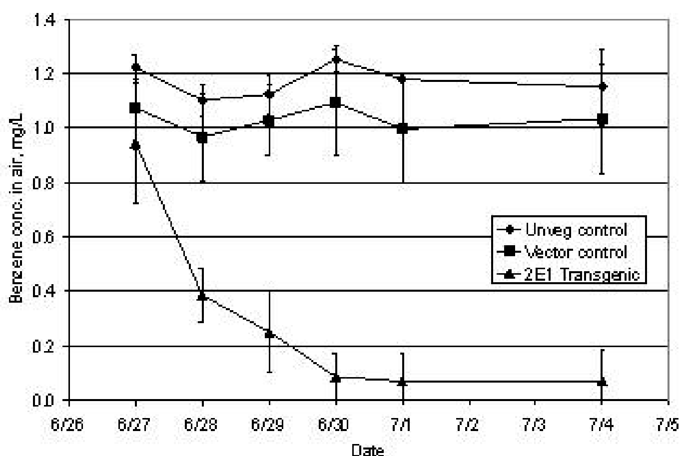
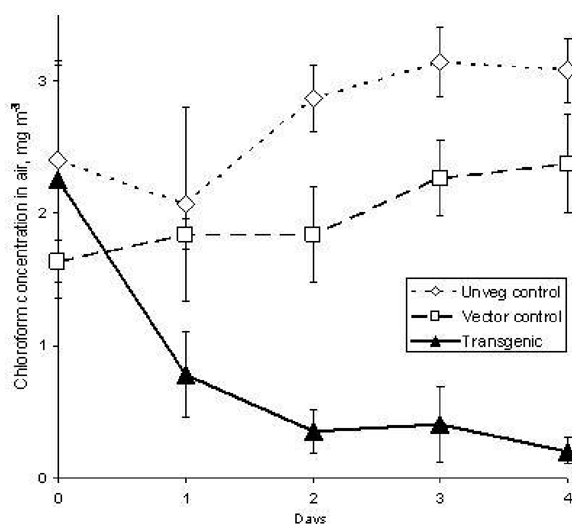


Figure 2
Removal of benzene from the headspace of 40 mL vials containing small tobacco plants growing in hydroponic nutrient solution (10 mL). Transgenic plants expressed the human cytochrome P450 2E1 gene. Vector controls consisted of plants with the selectable marker (kanamycin resistance), but no 2E1 gene. Unvegetated controls contained only the nutrient solution. Headspace samples (300 uL taken through septum valves) were analyzed by GC-FID. Averages and standard deviations of triplicates and quadruplicates are shown.



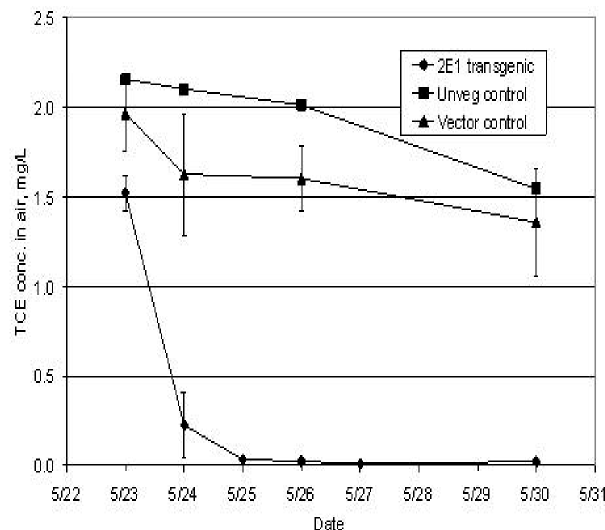


Figure 3. Removal of (a) chloroform and (b) trichloroethylene by tobacco plants transformed with the 2E1 gene. Same technique as in Figure 1, except analysis was by GC-ECD.

The removal of chloroform and trichloroethylene followed similar trends (Figure 2). Note that although the chloroform levels ranged from 200 to 2000 µg m^{-3}, 1000x lower than the previous experiment (though still 10-100x higher than typical indoor air levels in bathrooms and homes [34]), apparent first order kinetics were maintained. Toluene and carbon tetrachloride removal was also increased in the 2E1 transformed plants, but perchloroethylene was not significantly transformed by either the transgenic 2E1 plants or by the vector controls (data not shown). This result is consistent with observations by others that perchloroethylene is not a strong substrate for 2E1 [35].

The time course of the removal of benzene, chloroform and trichloroethylene appear to follow first order kinetics. Accordingly, first order rate constants were calculated for these compounds and are shown in Figure 3. It should be noted that the removal of benzene, chloroform, and trichloroethylene by the untransformed plants was not significantly different from the unvegetated controls, so the calculated rates may be overestimated. These results are consistent with strong activity of cytochrome P450 2E1 in the transgenic plants, as chloroform, benzene and trichloroethylene are strong substrates of 2E1. Bromodichloromethane, another VOC associated with water chlorination, was also rapidly degraded in vials containing the CYP2E1 plants

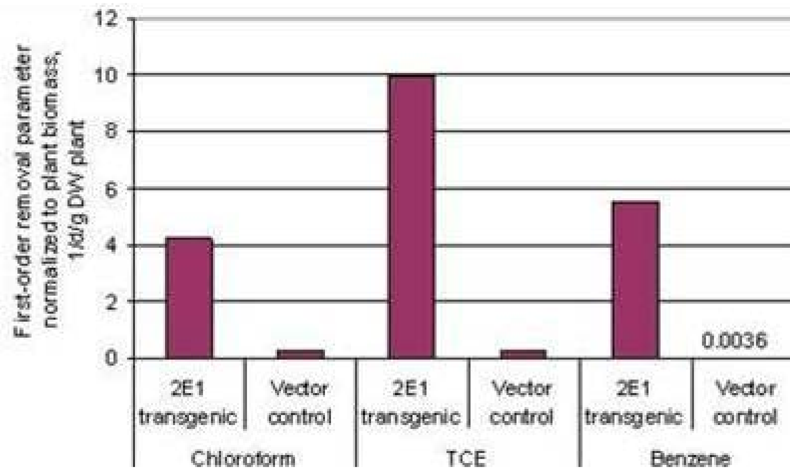


Figure 4. Estimated first-order rate constants for removal of volatile organic compounds by tobacco plants transformed with 2E1 gene, compared to removal rates by vector control (wild-type) plants. Rates are normalized to the average dry weight of the plants. TCE = trichloroethylene.

Transformation of pothos ivy

Pothos ivy will be transformed with *Agrobacterium tumefaciens* following published protocols modified in our lab. *A. tumefaciens* inserts its DNA into the genome of the plant it infects (in this case, cells in pothos plant segments). The 2E1 gene from the rabbit cytochrome P450 will be added into the Agrobacterium as a plasmid which will then be inserted into the pothos genome.

We have constructed binary vectors for *A. tumefaciens* transformation containing the 2E1 gene, the GUS/GFP visual markers, and the hygromycin resistance selection marker. Starting with the pSLD50 vector for transformation of kanamycin sensitive plants (Figure 4), the rabbit 2E1 gene (R2E1) was transformed into the pCAMBIA 1301 vector (Figure 5), including the GUS visual marker gene, producing the pCambia1302+2e1 vector (Figure 6). Separate vectors were constructed with a GFP gene instead of the GUS gene, pCambia1301+2e1. Since pothos is naturally resistance to kanamycin toxicity, but sensitive to hygromycin, hygromycin resistance will be used as the selection marker. The hptII gene confers hygromycin resistance and is incorporated into the pCambia1301+2e1 and pCambia1302+2e1 plasmids.

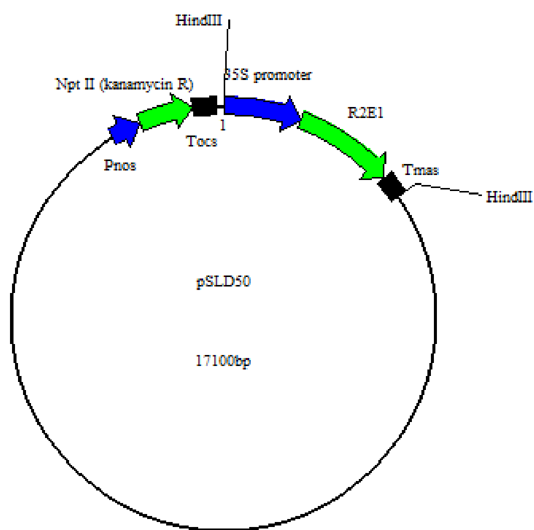


Figure 5. pSLD50 vector containing 2e1 gene.

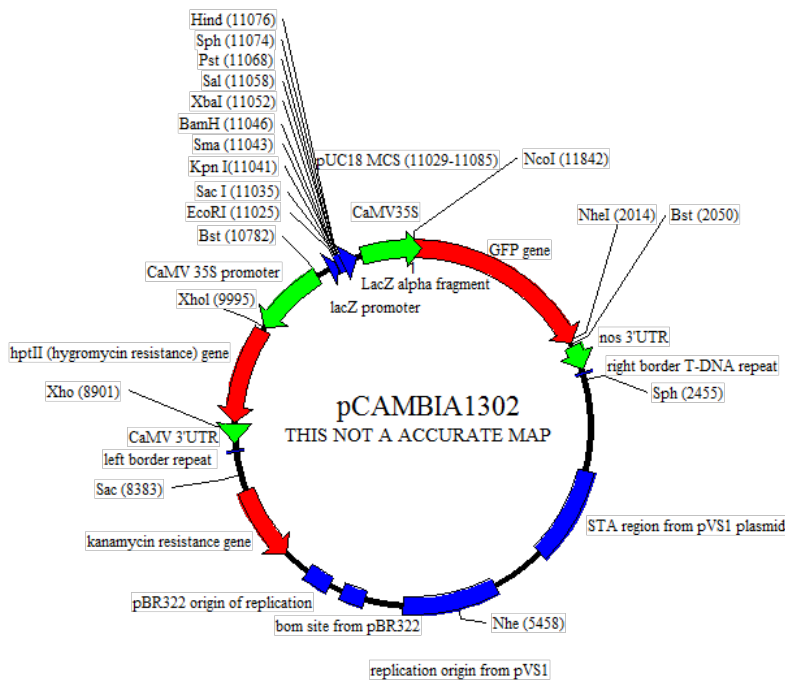


Figure 6. pCambia1302 vector containing kanamycin and hygromcin resistance genes. Note: the project uses the 1301 with GUS, rather than the 1302 vector shown, with the GFP gene.

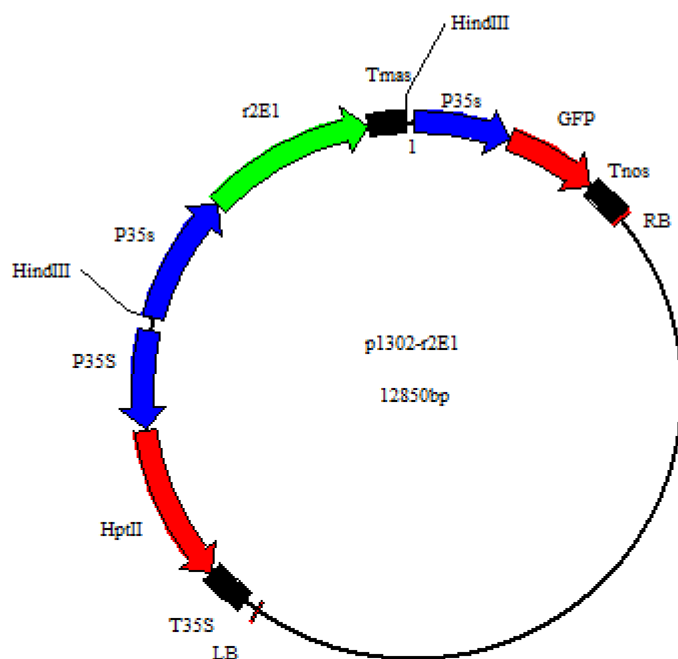


Figure 7. pCambia1302+2e1 plasmid. Note: We are presently using the pCambia1301+2e1 plasmid with the GUS visual marker in place of the GFP gene shown.

Vector construction proceeded as follows. The pSLD50 vector containing the 2E1 gene was cut with HINDIII restriction enzyme to release the 2E1 segment and the pCambia1301 plasmid was cut with HINDIII. These plasmids code for GUS or for GFP as visual markers and, hptII, the hygromycin resistance gene. Then we ligated the 2E1 gene segment and the restricted pCambia1301 or pCambia1302 producing the p1302+2E1 and p1301+2E1 vectors. PCR, sequencing and selection for hygromycin resistance was used to verify the successful construction of this vector. Finally the plasmids were infected into A. tumefaciens and used to transform pothos.

We have made some progress transforming and regenerating pothos. We have modified the procedures in Kotsuka and Tada [36], titrating incubation times, hygromycin concentrations and hormonal conditions during regeneration. At this time we have two candidate pothos plantlets growing on hygromycin selection media that appear to be hygromycin resistant. These plantlets are growing to produce sufficient biomass for DNA and RNA extraction and PCR/sequencing to confirm the presence and expression of 2E1. However, it is important we have many more pothos lines transformed with 2E1 to select from in order to make it likely that we can select the best lines with optimal 2E1 detoxifying activity.

5. Experimental/Research Design and Methods

Transfer 2E1 gene to an appropriate houseplant and test its ability to oxidize chloroform and benzene.

Hypotheses:

1. Cytochrome P450 2E1 from rabbit can be functionally expressed in *Epipremnum aureum*, Jade or Green Pothos Ivy.
2. Pothos ivy, genetically modified with 2E1 can remove chloroform and benzene from air significantly faster than wild type pothos ivy.

Our target houseplant is *Epipremnum aureum* (pothos) cv. Jade or Green Pothos (Linden & Andre) Bunt. Pothos is among the most commonly used houseplant and is tolerant to an indoor low light environment. There is no intellectual property issue associated with Jade Pothos, it does not flower indoors and seldom flowers outside, and Jade Pothos is not invasive except in Hawaii. Methods for transformation and regeneration of pothos ivy have been reported in the literature [36-39]

To efficiently trace which plants are genetically modified, we will also label the 2E1 transgenic plants GUS reporter system, Thus, the transgenic nature of our plant and their progeny will be easily discernible from its formation of a blue color when challenged with the GUS substrate. We will construct our vectors so that the 2E1 gene and the GUS genes are closely linked assuring that there will be little chance of a cross-over event between them during meiosis.

We will screen the transformed tissue cultures for cells with a successful insertion of the gene cassette into the plant chromosome by the resistance of the cells to a selectable marker, such as an antibiotic or herbicide, conferred by the standard transformation vectors. We will also develop a process for screening tissue cultures for their ability to transform 2E1 substrates. Our objective will be to increase the throughput of the small batch whole plant assays we have previously shown to reflect differences in VOC transformation activities of transgenic and wild type plants. We have previously shown that isolated leaves from test plants have the ability to degrade halogenated hydrocarbons. Therefore, we can begin to screen the new transgenic lines as soon as two or more leaves have developed.

Leaves will be incubated in small sealed vials with medium, chloroform and benzene will be injected into the vials at a concentration of 500 ug/m^3, and the disappearance of chloroform and benzene will be followed by gas chromatography of the headspace. The removal rates will be normalized to the leaf biomass and the best plant lines selected. The correlation between activity and transgene expression will be verified by semiquantitative RT-PCR. The best transgenic lines will then be propagated for further analysis.

Methods

Plant transformation

Sterile pothos stems, leaves, and petioles are cut from young pothos plants (Figure 7). Note that Agrobacterium is most effective at infecting wounded sites in plants. The pothos segments are incubated for five days in an infection medium containing the transformed Agrobacterium (Figure 7). Then the pothos is washed in an antibiotic solution to kill the Agrobacterium (Figure 8), and the pothos segments were plated on callus induction media. The pothos segments were incubated for several weeks until callus developed on the plant segment (Figure 9).

The cells of callus are the equivalent of pluripotent mammalian stem cells in plants. These embryogenic calli have the ability to develop into new plants that are genetic clones. The callus

development medium also contains hygromycin to inhibit Agrobacterium growth the pothos cells that were not transformed with the pCambia1301+2E1 vector containing the resistance gene, hptII.

Finally we excise and transfer calli to new medium containing nutrients and plant hormones needed to promote regeneration of the callus to into a fully developed plantlet with roots and shoots.

Figure 8. Pothos leaves (below) and stems (middle) pre-cultured on callus induction medium.

Figure 9. Pothos explants (one above) being washed after co-culture with Agrobacterium.

Plant regeneration

Due to the low efficiency of transformation of pothos by Agrobacterium transformation, thousands of explants need to be transformed to obtain a handful of calli that could survive and grow on the hygromycin selection media. Our initial preliminary efforts screened a few hundred treated pothos tissues and found two plantlets that survived and regenerated on selection medium.

Figure 10. Development of callus on leaf and stem cuttings of pothos ivy and the regeneration of plantlets on hygromycin selection medium

Confirm Transformation and expression

The next step will be to confirm that the plantlets obtained from the calli are transformed, are able to express the 2e1 gene, and have VOC degrading activity.

PCR of 2E1 gene

DNA will be extracted from pothos and tested for housekeeping genes and 18S RNA genes to confirm clean DNA. An amplicon of the 2E1 gene using PCR primers specific to the 2E1 gene will be amplified and sequenced.

GUS assays will be used to test for expression of GUS, the beta-glucouronidase gene, which is in the pCambia1301 vector. Plants lack GUS, which transforms 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to a product with a strong blue color, which can be used to visually select transformed plants. Hygromycin resistance and GUS staining would be strong evidence for the activity of the full cassette of inserted genes.

2E1 gene presence and activity will be confirmed by PCR using 2E1 specific primers and sequencing of the amplified PCR fragment (amplicon) to confirm.

RT-PCR of 2E1 gene

After the presence of the 2E1 gene in the transformed pothos lines is confirmed, we will confirm that the 2E1 gene is express by measuring the message RNA for the transcribed 2E1 gene (2E1 mRNA) using reverse transcription-PCR. mRNA will be extracted from the pothos using established methods, and used to generate the complimentary DNA (cDNA) of the mRNA sequences in the extract. Then 2E1 cDNA will be amplified by PCR using 2E1 primers specific to the 2E1 cDNA sequence (complimentary to the 2E1 gene) and sequenced to confirm. Controls will consist of RT-PCR of housekeeping genes (native plant genes that are always expressed in living plant) and of hptII and GUS genes.

Western blot of 2E1 protein

Translation of the 2E1 mRNA to protein in the putative transformed pothos will be confirmed by extracting proteins and electrophoresis of the extract with staining by antibodies to the rabbit 2E1 (Western blot).

Activity assays

Volatile Chloroform Uptake.

The wild-type and transgenic lines will be screened in triplicate for enhanced metabolism of chloroform following the methods described previously [40, 41]. Small rooted pothos plants in 4-inch pots containing 1:1 ProMix and perlite will be incubated in
10-inch-diameter Pyrex glass desiccators. The desiccators will be sealed with Fluorolube. The sample ports will be valved and additionally sealed with rubber septa. The sample ports will be closed such that the septa will be exposed only during sample collection. Eighty-two microliters of chloroform-saturated water in 10-ml glass beakers was placed in the desiccators before

sealing. Sealed desiccators will be incubated at room temperature with a 16-h photoperiod. The chloroform concentration will be measured daily by withdrawing 500 µL of air from the sample port by using a gas-tight syringe and manually injecting into a PerkinElmer GC-ECD (Supelco PTE-5 Capillary Column); performed in triplicate. Chloroform will be quantified by using external standards. The desiccators will be sealed for the entire incubation period. At least three 2E1 lines will be tested in triplicate and three wild type plants will be evaluated, each in its own sealed dessicator.

At least nine plants and three media samples will analyzed in this experiment, which will be replicated. The average fresh weights of the plants at the end of the experiment will be determined and used to normalize the chloroform removal rates to plant weight.

Volatile Benzene Experiments.

The wild-type and transgenic lines will be screened in triplicate for enhanced metabolism of benzene following the methods described previously [40, 41]. The plant (with 8-12 leaves) will be placed in 225 mL of sterile hydroponic solution (1/2 strength Hoagland's solution) in 250-ml flasks. The mouth of the flask will be plugged with cotton around the plant stems, and the entire flask and the cotton will be covered with aluminum foil. The plants cuttings will be transferred into desiccators. Approximately 200 µL of benzene-saturated water will be placed in a 10-ml beaker inside the desiccators. Sealed desiccators will be incubated at room temperature with a 16-h photoperiod for up to 10 days. Periodically, dessicator air will be analyzed by manually injecting a 500- µL sample into an SRI 8610C GC-flame ionization detector (Alumina Sulfate Plot column; Supelco). The plant weights will be determined and used to normalize benzene degradation rate.

Ecosafety tests

We will select two or three confirmed optimal 2E1 transformed pothos lines and subject them to tests of their ecosafety. These tests can occur only after sufficient plant biomass has been accumulated. We expect that this will be achieved in the second year, after a commercialization agreement has been reached with a horticulture firm. Commercialization will be contingent on whether these plants pass the ecosafety tests. These tests will confirm that addition of the transgenes does not increase the weediness of the transformed pothos, by showing that they do not have increased resistance to herbicides, do not have an increased growth rate, and are not more cold tolerant that wild type pothos.

Herbicide Resistance

The selected 2E1 transformed pothos plants and wild type plants will be exposed to three herbicides, e.g., glyphosate, 2,4-dichlorophenoxyacetic acid (24D), and dicambra at three concentrations, along with plants not exposed to herbicides. All lines will be represented at each exposure by at least four plants. The response of the plants to the herbicides will be documented by weighing and photography. We will document any enhancement of pothos survival of herbicide application.

Growth Rate

Identically sized transformed pothos and wild type pothos plants will be grown under identical lighting, watering, and nutrient conditions for 2-3 weeks, after which plant weights will be determined and the increases compared.

Cold Hardiness

Identically sized transformed pothos and wild type pothos plants will be grown for seven days at room temperature, 10C, 5C, and 0C, after which plant survivals will be determined by regrowth at room temperature and compared.

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