CHARACTERIZATION OF TRANSGENIC PEANUTS EXPRESSING OXALATE OXIDASE FOR GOVERNMENTAL APPROVAL OF THEIR RELEASE FOR CONTROL OF SCLEROTINIA BLIGHT

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ABSTRACT

Sclerotinia minor Jagger is a fungal pathogen of cultivated peanut (*Arachis hypogaea* L.) that can cause crop losses in excess of 50%. Fungicides are not completely effective at controlling the disease and can cost up to $311 per hectare for three applications. The ability to produce oxalic acid is necessary for the pathogenicity of some *Sclerotinia* spp. With little to no naturally occurring resistance to Sclerotinia blight in *Arachis* spp., a biotechnological approach was used to confer resistance to the disease. Peanut plants were transformed with a gene from barley encoding oxalate oxidase, an enzyme that degrades oxalic acid. Transformed peanuts showed resistance to *S. minor* and increased yields under disease pressure compared to the parental lines. Before the resistant varieties can be marketed, they must be reviewed and approved by the governmental regulatory system. Responsibility for regulation of transgenic plants in the U.S. is shared among the U.S. Department of Agriculture (USDA) through the Animal and Plant Health Inspection Service (APHIS), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA). These agencies require several different data sets including molecular characterization and field studies before each transformation event can be commercialized. This project was designed to characterize three different transformation events, N70, P39 and W171. Molecular characterization included determination of insertion number, copy number, intactness of the expression cassette and stable inheritance of the transgene. N70 was found to have two insertions and two copies while W171 had one insertion with one copy. The P39 event has two

insertions and two or more copies. Each of the three events was stable over multiple generations. Phenotypic comparisons of each transgenic line to the parent cultivar were carried out in field studies. Characteristics such as oxalate oxidase expression, yield and quality, hay quality, disease occurrence, aflatoxin content and plant height were assessed. Transgenic peanuts showed few differences from the parent cultivar other than resistance to Sclerotinia blight and yield under disease pressure. Outcrossing studies were completed to determine the rate and distance of cross pollination. Outcrossing rates in N70, P39 and W171 were less than 2.5% and occurred up to 19 rows or 17.4 m from the nearest transgenic row. The molecular characterization and field performance of N70, P39 and W171 have been assembled into a document to petition APHIS for determination of non-regulated status.

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INTRODUCTION

ARACHIS HYPOGAEA

BACKGROUND

Cultivated peanuts, also called groundnuts, originated in South America. Evidence for the earliest known use of peanuts comes from an archaeological site in Peru and dates to about 3500 years ago (Hammons, 1994). Eighty different species belonging to the genus *Arachis* have been described, but with the exception of some primitive land races in South America, only *A. hypogaea* L. is grown for human consumption.

With the exploration of the New World beginning in the 16th century, peanut quickly spread from South America to Africa and Asia along with other new world crops such as bean, squash, potato, tobacco and corn (Hammons, 1994). Peanuts came to North America from west Africa with the slave trade.

Peanut is an allopolyploid (4X=40) meaning that the plant was derived from distinct A and B genomes. Several *Arachis* spp. have been proposed as the donor of each genome. The most recent evidence suggests that a cross between *A. ipanesis* and *A. duranensis* is the most likely origin. There would have been a genome duplication after hybridization occurred to produce fertile offspring (Favero et al., 2006). This conclusion was reached by making crosses between probable A genome parents and probable B genome parents and applying colchicine. The only other species capable of naturally outcrossing with *A. hypogaea* is *A. monticola*, which is the only other polyploid species.

Peanuts are not true nuts but are a member of the family *Fabaceae*, which is comprised of the leguminous plants. These plants are capable of forming associations with nitrogen fixing bacteria in the soil. Peanut flowers are self fertile with outcrossing rates of 2 to 8% (Knauft et

al., 1992). Geotrophic pegs form about a week after fertilization and grow toward the ground. Seeds develop in pods on the ends of the pegs underground.

A. hypogaea is divided into two subspecies, *hypogaea* and *fastigiata* (Figure 1) (Singh and Simpson, 1994; Anonymous, 2003). The *hypogaea* subspecies contains the *hypogaea* (VA-type) and *hirsuta* (runner-type) botanical varieties. The runner types are grown most often in the U.S. and used mainly for the production of peanut butter. The Virginia types are grown in southeastern Virginia, in the Carolinas and in parts of Texas and Oklahoma. Virginia-type peanuts produce large kernels and are sold as gourmet peanuts. They are also roasted in the shell. The *fastigiata* subspecies consist of four botanical varieties, but only *fastigiata* (Valencia) and *vulgaris* (Spanish) are grown in the U.S. The Valencia type is not widely grown and is often eaten boiled in the southwestern states. Spanish peanuts, grown in Texas and Oklahoma, are used primarily to produce peanut oil. All four varieties can be found in cans of mixed nuts.

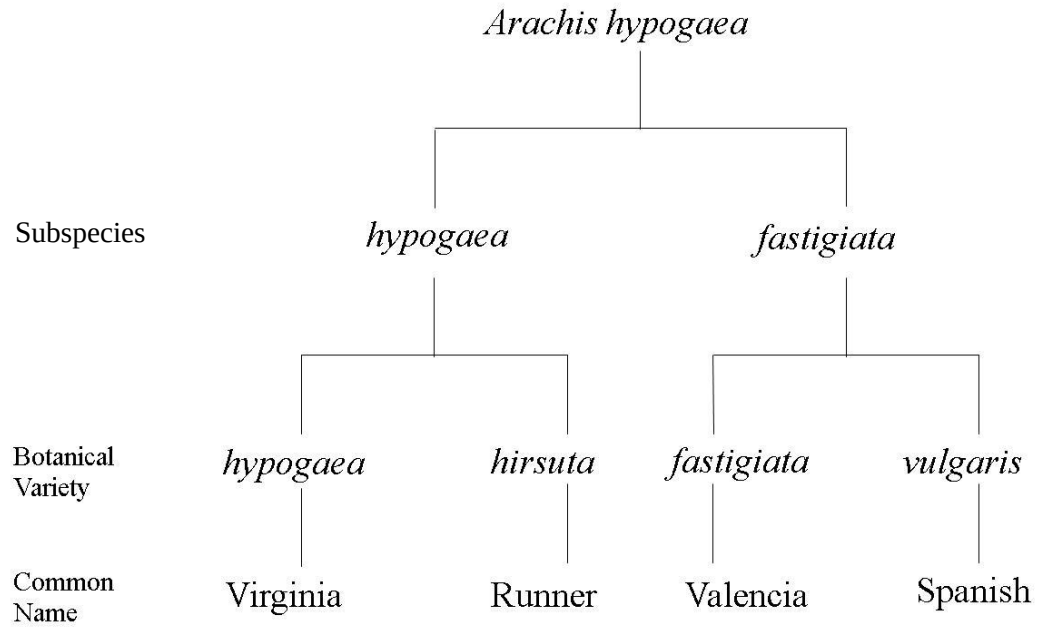

Figure 1. Peanut varieties grown in the U.S.

PEANUT PRODUCTION AND DISEASE

Peanuts are produced around the world between 40°N latitude and 40°S latitude (Anonymous, 2003). Many of those are produced for subsistence living. In 2003, the countries producing the most peanuts were China (42%), India (20%), Nigeria (7%), the U.S. (5%), Sudan (3%), and Senegal (2%).

Beginning with the 2002 Farm Bill, the peanut quota system was discontinued and U.S. prices dropped from an average of $623 per metric ton (1982-2001) to $379 per metric ton in 2006 (Anonymous 2002; USDA, 2007). From the late 1950's until the mid 1990's, Virginia produced an average of about 40,500 hectares of peanuts (Figure 2). Production dropped slightly with the 1996 Farm Bill to 31,160 hectares. Production has steadily dropped since the 2002 Farm Bill to an all time low of 6,475 hectares in 2006. Peanut yield has remained steady at 3.4 metric tons per hectare for the past three decades while the price to produce peanuts has risen due to increases in costs for equipment, fuel, labor and agrichemicals.

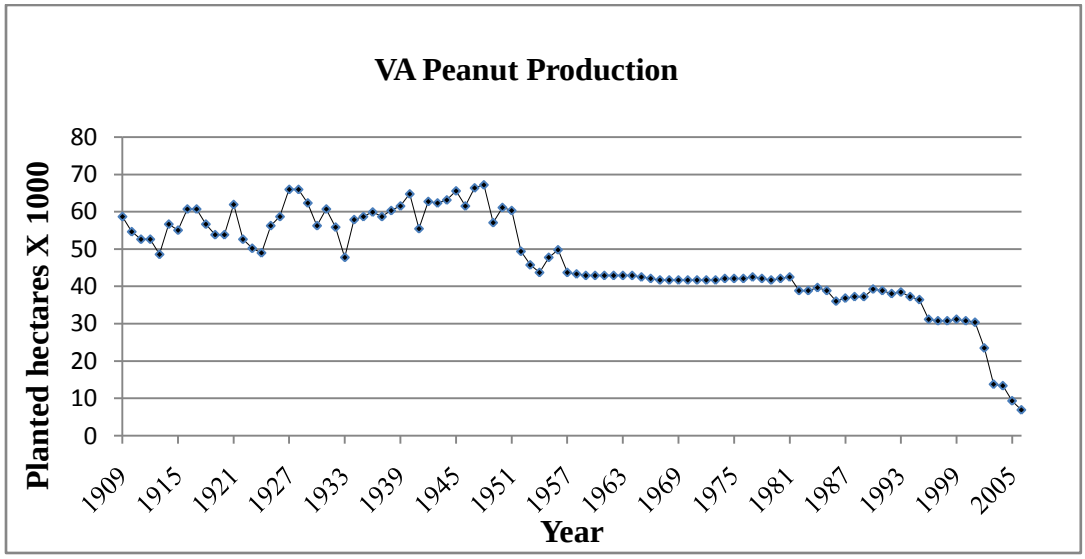

Figure 2. Virginia peanut production from 1909 until 2006 (USDA, 2007).

Peanuts in the Virginia-North Carolina Area are affected by many diseases including early and late leaf spot, southern stem rot, Cylindrocladium black rot, web blotch, tomato spotted wilt virus (TSWV), nematodes, and Sclerotinia blight. Sclerotinia blight, caused by *Sclerotinia minor* Jagger, is a major disease of peanut and other crops, such as common bean, and lettuce. *Arachis* has little natural resistance to this pathogen (Porter and Melouk 1997). The necrotrophic pathogen produces cottony white mycelia and oxalic acid which predispose the plant to invasion by the fungus. Mutants of a closely related pathogen, *S. sclerotiorum,* that are unable to produce oxalic acid, cannot infect plants (Godoy et al., 1990). There are several proposed mechanisms for how oxalic acid functions in infection, including the following:

1. Oxalic acid can accumulate in plant tissues and lower the pH to optimal levels for the functioning of cell wall-degrading enzymes (Favaron, 2004). The lowered pH has also been implicated in the regulation of certain pathogenesis-related genes in the fungus (Rollins and Dickman, 2001; Chen et al., 2004).
2. Oxalic acid is capable of disrupting the integrity of plant cell walls by chelating calcium ions. This allows enzymes to hydrolyse cell wall components, specifically pectates (Bateman and Beer, 1965).
3. Cessna et al. (2000) were able to show that oxalic acid is able to suppress the oxidative burst, an early defence response.
4. Guimaraes and Stotz (2004) showed that oxalic acid induces guard cell opening and inhibits abscisic acid-induced closure. This causes foliar wilting.
5. Recently, Kim et al. (2008) suggested that oxalic acid is used as a signalling molecule to kill the plant tissue by initiating programmed cell death through the plant's endogenous systems.

When the fungus encounters a nutrient-limited environment, desiccation-resistant over-wintering structures, called sclerotia, are formed when hyphae aggregate and produce melanin (Bolton et al., 2006). Sclerotia are able to remain viable in the soil for up to 8 years. When conditions are favorable, the sclerotia germinate to either directly infect plants (*S. minor*) or produce ascospores (*S. sclerotiorum*), which become airborne to spread the fungus and cause disease throughout a field. *Sclerotinia* sp. produce ascospores in fruiting structures called apothecia. *S. minor* produces apothecia only occasionally and only during the winter or early spring before peanuts are planted in the field (Phipps, personal communication).

Sclerotinia blight causes major crop losses and is very difficult and expensive to control. Two fungicides are used for control of Sclerotinia blight on peanut: fluazinam (Omega 500 from Syngenta) and boscalid (Endura 70WG from BASF). These fungicides offer partial control of the fungus, and are marketed at a similar cost of $104 per hectare for a single application.

OXALATE OXIDASE

PROTEIN CHARACTERIZATION

Oxalate oxidase (Oxox) is an enzyme that catalyzes the degradation of oxalic acid into carbon dioxide and hydrogen peroxide. Barley oxalate oxidase has been purified and used in the medical field for many years to test blood oxalate levels. In order to determine the DNA sequence encoding the enzyme, purified antibody was used to screen a barley expression library and the cDNA was identified (Dumas et al., 1993). The DNA sequence was found to have 98% homology to a protein from wheat known as germin. Germin was then tested and found to have Oxox activity (Lane et al., 1993).

Germin was first observed in 1980 during experiments designed to identify proteins associated with the early stages of germination of wheat embryos (Thompson and Lane, 1980). In this research, wheat embryos were germinated in water and RNA samples were extracted at different time points. The proteins were synthesized from the mRNA in a cell-free rabbit reticulocyte system in media containing radiolabeled methionine. Proteins extracted at the different time points were separated on isoelectric focusing gels and visualized. It was shown that the synthesis of germin mRNA and protein begins between 5 and 10 hours after imbibition and continues to increase through the germination process. The protein is not present in mature plants. The name germin was not given to the protein until 1991 when the cDNA was sequenced (Lane, 1991). At the time, there were no known proteins with homology to germin and no function was determined until the discovery of Oxox from barley. Proteins that have a high degree of homology to germin and posess Oxox activity exist in cereals such as: wheat, barley, rye, oat, corn, and rice (Lane et al., 1992). Proteins that have similar sequences to germin, are known as germin-like proteins (GLPs). This family of proteins will be discussed later in the thesis. The germin and barley Oxox proteins each have 224 amino acid residues, 23 of which make up an apoplastic targeting sequence that is cleaved. The mature monomers are about 25 kDa in size. Barley Oxox and wheat germin are 88.8% identical and 98% homologous.

The Oxox from barley has been crystallized and the structure determined (Woo, 2000). The enzyme is a homohexamer, meaning that it is composed of six identical monomers. The homohexamer is arranged as a trimer of dimers. Two Oxox monomers are attached to each other to form a dimer. Three dimers are then locked together by their C-terminal alpha-helices to form the trimer of dimers. Oxox is very stable because each monomer folds into a beta-barrel or beta-jellyroll structure and is glycosylated.

Barley Oxox has two putative glycosylation sites—Asn 47 and Asn 52. There is no evidence for the glycosylation of Asn 52 (Woo, 2000; Opaleye et al., 2006). In wheat, it is known that Asn 47 has an N-linked glycan that exists in at least two different forms (Jaikaran et al., 1990).

Oxox belongs to the cupin superfamily of proteins. The word cupin, from the Latin "cupa" meaning small barrel, refers to the beta-barrel structure that is the hallmark of the superfamily. Members of the cupin superfamily include: phosphomannose isomerases, polyketide synthases, dioxygenases, spherulins, germin-like proteins, AraC-type transcription factors, oxalate decarboxylases, sucrose-binding proteins and seed storage proteins, as well as many others (Dunwell et al., 2000).

Oxox is a member of the germin-like protein (GLP) family. This family is divided into five different subgroups based on phylogenetic analysis (Bernier and Berna, 2001). The germin group, which includes Oxox, includes any proteins that have 90% or higher homology to wheat germin. The other four groups have between 30 to 70% identity to wheat germin. GLPs have been found in all angiosperm and gymnosperm families, as well as in mosses. The functions of many GLPs are unknown. GLPs with known functions include enzymes, structural proteins and receptor proteins.

It has been demonstrated that the germin isolated from barley is a bifunctional enzyme. The protein has both oxalate oxidase activity and superoxide dismutase activity (Woo, 2000). The active site of Oxox requires a metal ion, manganese, which is held by four amino acid residues: histidine 88, histidine 90, glutamate 95, and histidine 137. Manganese is not a common cofactor in other oxidizing enzymes but it is the cofactor in many microbial superoxide dismutases. Superoxide dismutase enzymes are found in microorganisms, as well as in the

mitochondria of eukaryotes where its function is to destroy superoxide (O^{2-}), a toxic byproduct of respiration, to produce dioxygen and hydrogen peroxide. Barley Oxox dismutase activity is only 25% of that found in other manganese-containing enzymes and is much lower than the activity of enzymes that contain copper and zinc cofactors (Woo, 2000). Oxox shows no significant sequence similarity to other superoxide dismutases.

Several catalytic mechanisms have been proposed for oxalate oxidase activity. The mechanism has only recently been elucidated. It has been shown that manganese is the only metal ion present in active enzyme and that the ion exists in the 2+ oxidation state (Requena and Bornemann, 1999). A second study was able to determine the amino acid residues involved in the mechanism by crystallizing native and mutated enzymes with substrate bound and by performing biochemical assays on the mutated enzymes. Asparagine 75, asparagine 85 and glutamate 139 are involved in the catalytic mechanism (Opaleye et al., 2006).

EXPRESSION OF OXALATE OXIDASE

Germin-like proteins have been implicated in many different developmental processes in plants (Table 1). Three main functions have been assigned to the GLPs that have been discovered so far. GLPs are known to function as enzymes, in the case of Oxox, as structural proteins, and as receptors (Bernier and Berna, 2001)

Table 1. Developmental processes that involve GLPs .*

Developmental process	Species
Germination	*Triticum aestivum*
Floral induction	*Arabidopsis thaliana*
	Sinapis alba
	Pharbitis nil
	Raphanus stivus
	Nicotiana plumbaginifolia
Fruit ripening	*Fragaria ananassa*
	Malus domestica
	Citrus reticulata
Seed development	*Piper nigrum*
Wood development	*Pinus taeda*
	Populus balsamifera
Embryogenesis	*Pinus caribaea*
	Pinus radiata
	Solanum tuberosum
	Lupinus
Nodulation	*Pisum sativum*

*Information taken from a review by Dunwell et al., 2000.

The enzymatic activity of Oxox produces hydrogen peroxide that is thought to cause cell wall cross-linking in germinating seeds (Caliskan and Cuming, 1999). It has been suggested that the roles of GLPs in wood development are as structural proteins. Nodulation involves GLPs functioning as receptor proteins for the cell surface proteins called rhicadhesins which are produced by *Rhizobium* spp.. This was observed in pea (Swart et al., 1994). Auxin-binding GLP receptors have been described in peach, but no physiological function has been assigned (Ohmiya et al., 1998)

GLPs have been found to be up-regulated in plants exposed to abiotic stresses (Table 2). The best study of GLP response to abiotic stress involved transgenic tobacco plants containing a GUS reporter gene under the control of the germin promoter from wheat (Berna, 1997). This

research showed that GUS could be detected under several abiotic stresses, including high salt and aluminum.

Table 2. GLPs upregulated during abiotic stress of plants.*

Abiotic Stress	Species
Salt stress	*Hordeum vulgaris*
	Mesembryanthemum crystallinum
	Germin promoter induced in transgenic Tobacco
Water stress	*Populus tomentosa*
	Populus popularis
Manganese deficiency	Tomato
Heavy metals	Germin promoter induced in transgenic Tobacco
Aluminum	Germin promoter induced in transgenic Tobacco
	Triticum aestivum
Plant growth regulators	Germin promoter induced in transgenic Tobacco

*Information from reviews by Dunwell et al., 2000; and Bernier and Berna, 2001

OXALATE OXIDASE AS A RESISTANCE GENE

Oxox is known to be up-regulated in wheat (Hurkman and Tanaka, 1996) and barley (Zhang et al., 1995) upon infection with powdery mildew. There are several theories as to why Oxox is needed in defense:

1. It is thought that the hydrogen peroxide produced by the enzymatic oxidation of oxalate acts as a messenger to activate other defense genes and the hypersensitive response in the infected cell or in surrounding cells (Lane, 1994; Dunwell et al., 2000).
2. The hydrogen peroxide may be involved in cross-linking of cell walls for defense (Zhang et al., 1995) or it may act as an antimicrobial agent.
3. Degradation of oxalate contained in calcium salts will result in the release of calcium ions, which are known to be important in signaling pathways.

Barley has a GLP that is expressed during powdery mildew infection and is involved in papilla formation (Wei et al., 1998). There is also evidence that a GLP in *Brassica napus* may

be involved in plant defense. The protein was detected in plants infected with *Leptosphaeria maculans* (Dunwell et al., 2000).

Oxalic acid production has been found in all classes of fungi (Bolton et al., 2006). Oxalate producers include *S. minor* (Hollowell et al., 2001), *S. sclerotiorum* (Bolton et al., 2006), *Sclerotium rolfsii* (Bateman and Beer, 1965), *Septoria musiva* (Liang et al., 2001), and *Penicillium oxalicum* (Ikotun, 1984), among others. These pathogens are able to infect a wide range of crops, with *S. sclerotiorum* infecting over 400 species of plants worldwide (Bolton et al., 2006). These pathogens do not normally infect cereals which may be due to the presence of Oxox, which degrades oxalic acid and prevents infection (Bernier and Berna, 2001).

Oxox is a powerful defense compound against oxalic acid-producing pathogens and has been used to genetically engineer several crops (Table 3). In all cases where it was tested, the transgenic plants showed greatly enhanced resistance to oxalate-producing pathogens when compared to non-transgenic parent lines.

Table 3. Plant species genetically modified to contain the gene for Oxox.

Transgenic Species	Reference
Oilseed rape	(Thompson et al., 1995)
Tobacco	(Berna and Bernier, 1999)
Poplar	(Liang et al., 2001)
Soybean	(Donaldson et al., 2001)
Sunflower	(Hu et al., 2003)
Peanut	(Livingstone et al., 2005)

Transgenic corn expressing wheat germin has also been shown to have reduced corn borer herbivory compared to non-transgenic plants (Ramputh et al., 2002). This may be due to changes in plant cell walls or the up-regulation of defense genes caused by hydrogen peroxide or to the effects of hydrogen peroxide on the insects themselves. GLPs have also been implicated in tobacco's defense against insects (Lou and Baldwin, 2006).

DEVELOPMENT OF TRANSGENIC PEANUT LINES

TISSUE CULTURE AND TRANSFORMATION

Because Oxox can degrade oxalic acid, a necessary pathogenicity factor for *S. minor*, peanut plants were genetically modified to express the gene for Oxox at Virginia Tech (Livingstone et al., 2005). Transformation and regeneration were performed as previously described (Livingstone and Birch, 1999). Embryogenic callus was generated from three popular peanut cultivars: NC7, Wilson and Perry. The pOxox transformation construct (Figure 3) contained the gene for Oxox from barley and a hygromycin resistance gene as the selectable marker. The construct was introduced by particle bombardment and the plants were regenerated.

The transgenic peanut lines were tested for Oxox expression and for resistance to oxalic acid and colonization by *S. minor*. Oxox expression was assayed using a coupled colorometric reaction (Livingstone et al., 2005). Plants expressing Oxox showed significantly smaller lesion sizes compared to non-transgenic plants of the same line when exogenous oxalic acid was applied to wounded leaflets. The same observation was made with leaflets challenged with *S. minor*.

Figure 3. Representation of the pOxox transformation construct.
AmpR, ampicillin resistance; Ori, bacterial origin of replication; 35S, DE, dual enhanced 35S Cauliflower Mosaic Virus Promoter; E, Tobacco Etch Virus translational enhancer sequence; Hyg R, hygromycin resistance gene; T, CaMV 35S terminator; Oxox, oxalate oxidase; a-T, terminator from the alpha' subunit of the beta-conglycinin gene from soybean.

SELECTING LINES FOR MARKETING

Transgenic lines were propagated and tested in the greenhouse for gene expression in each generation at the Tidewater Agricultural Research and Extension Center (TAREC) in Suffolk, VA. Based on results in the 2004 and 2005 field years, several lines were selected for preliminary genetic characterization. These lines were: P39, P53, P97, P98, P99, N99, W14, W59, N70, W171 and W73. W83 and W51 were lines that did not express Oxox in field trials. Based on the Southern blot in Figure 4, it appears that the transgene was lost during propagation rather than another cause such as gene silencing.

The information gained in the preliminary Southern blot indicated that the P39, P97, P98 and P99 lines may have originated from the same transformation event. N70 and N99 appear to be from the same transformation event, and W171 and W73 also appear to be from the same event. The best performing transgenic lines were selected from each parent cultivar to continue testing for marketing and governmental deregulation purposes. The lines selected were N70, P39 and W171. For regulatory purposes, all lines from the same original transformation event were considered to be the same line.

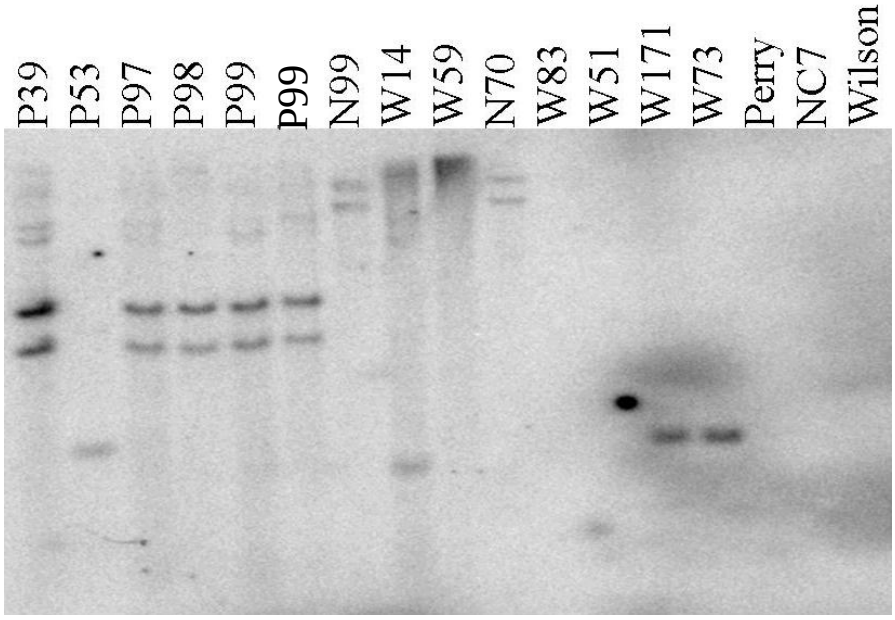

Figure 4. Preliminary genetic characterization of superior lines in field trials using Perry, NC7 and Wilson as negative controls.
DNA was digested with BstXI and hybridized to probe 5, see 'Genetic Characterization Experimental Design' in Chapter 2.

GOVERNMENTAL REGULATION OF TRANSGENIC PLANTS

The policy that addresses deregulation of transgenic plants was formally established in 1986 and is known as the Coordinated Framework for Regulation of Biotechnology. This framework was based on the following health and safety laws: The Plant Protection Act, The Federal Food, Drug and Cosmetic Act, The Federal Insecticide, Fungicide and Rodenticide Act

(FIFRA) and The Toxic Substances Control Act. These laws have been formulated to regulate food and traditional chemical pesticides. No new laws have been passed to regulate products derived from modern biotechnology. The Coordinated Framework involves three different governmental agencies: the U.S. Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA).

U.S. Department of Agriculture

Under the Plant Protection Act and the Plant Quarantine Act, the USDA is responsible for preventing the introduction and dissemination of plant pests. This function is carried out by the Biotechnology Regulatory Service (BRS) arm of the Animal and Plant Health Inspection Service (APHIS), a division of the USDA. APHIS considers all transgenic plants to be regulated articles until it can be shown that they pose no risk to agriculture. The Code of Federal Regulations Title 7 (7 CFR) section 340.6 provides a means for petitioning the agency to declare that a regulated article poses no risk to agriculture and should no longer be regulated.

APHIS uses the concept of 'substantial equivalence' when evaluating transgenic plants. All of the required data are intended to show that other than the intended function of the transgene, there is no difference between transgenic and non-transgenic plants.

Food and Drug Administration

The FDA is responsible for the safety of human and animal foods under the Federal Food, Drug and Cosmetic Act. The agency does not require that transgenic plants undergo review before marketing, but offers a voluntary consultation program. This program is designed to ensure that foods are unlikely to harm human or animal health before they are marketed and that the foods meet all labeling requirements. All transgenic plants on the market for human

consumption have voluntarily undergone FDA review. The safety assessment is carried out according to the *Codex Alimentarius,* the guidelines for food safety set forth by the United Nation's (UN) Food and Agriculture Organization (FAO) and the World Health Organization (WHO). These guidelines are also based on the concept of 'substantial equivalence'.

Before transgenic plants can be exported as food, they must be evaluated by the FDA's analogue in the country where they will be imported. Fortunately, many countries have adopted FAO and WHO guidelines.

ENVIRONMENTAL PROTECTION AGENCY

The EPA is required by FIFRA to evaluate all pesticides for detrimental environmental effects and to regulate their usage. By agency guidelines, Oxox is considered to be a Plant Incorporated Protectant (PIP). A PIP is any substance introduced into a plant through breeding or biotechnology that is intended to "prevent, destroy, repel or mitigate any pest." All PIPs introduced into plants via modern biotechnology must be registered through the EPA as pesticides. The EPA defines modern biotechnology as manipulating the genetic makeup of an organism using new techniques. "Old techniques" are defined as conventional breeding while an example of "new techniques" is genetic engineering. This definition has been left ambiguous to account for any new technologies that become available. The registration requirements for PIPs are the same as those for microbial pesticides and can be found in the Code of Federal Regulations Title 40 (40 CFR) section 158.740.

Pesticide registration usually costs between $100,000 and 400,000 for large companies and universities. Because Virginia Tech is considered a 'governmental entity', the EPA has agreed to waive the fees.

Submitting Data for Governmental Review

Preparing the documents necessary for deregulation and registration is not a straightforward process. Each of the three agencies is interested in different data and requires a different format for submission. Each agency provides a list of data that could be required for a petition to be successful, but adds a disclaimer that the data required depends on the specific plant species and introduced traits. In consultation with the agencies, including two meetings where all three agencies were represented, I have compiled a list of the data requirements of each agency (Table 4).

Table 4. Data required by each governmental agency.

	Requirement	USDA	FDA	EPA
Literature Review	Background on peanut	✓	✓	
	Background on barley		✓	
	Description of transformation system	✓	✓	✓
	Description of donor genes	✓	✓	✓
	Description of regulatory sequences	✓	✓	✓
	Background on oxalate oxidase		✓	✓
Data	Genetic characterization of insertion	✓	✓	✓
	Oxalate oxidase expression	✓	✓	✓
	Agronomic performance	✓		
	Gene flow (Outcrossing)	✓		✓
	Effects on other diseases	✓		
	Allergenicity		✓	✓
	Aflatoxin content		✓	
	Nutrient content analysis		✓	
	Persistence in soils			✓
	Acute oral toxicity			✓
	Acute dermal toxicity			✓
	Acute inhalation toxicity			✓
	Avian oral toxicity			✓
	Avian injection toxicity			✓
	Freshwater fish toxicity			✓
	Freshwater invertebrate toxicity			✓
	Nontarget plant toxicity			✓
	Nontarget insect toxicity			✓
	Honey bee toxicity			✓
Discussion	Rationale for development	✓		
	Formation of other substances (H_2O_2)	✓	✓	✓
	Positive environmental impact	✓		✓
	Negative environmental impact	✓		✓
	Formulation and production			✓

Each agency requires certain legal statements to be made about the release of confidential business information to the public and about the agency's legal right to review the data presented and grant deregulated status or complete registration. The FDA and USDA request that a single

document be sent containing all of the required information but they do not have any particular formatting requirements. The EPA requests that each data requirement be presented separately and sent in triplicate, with at least two copies printed and bound in distinct volumes. The EPA also requests that all supporting scientific papers be sent as print copies. The documents sent to the EPA must follow the formatting guidelines presented in 40 CFR section 158.32.

OBJECTIVES

Field trials of transgenic peanuts have been underway for 4 years and non-segregating lines have been developed by making single plant selections of plants positive for Oxox expression. Based on field data from 2004-2006 and genetic characterization of the insert, we selected two lines with good market characteristics as candidates for the initial review process. These lines are N70 and W171. This project consists of two main objectives as follows:

Objective 1: *To characterize the transgenic lines at the molecular level.*

Characterization of the insertion pattern, gene copy number and protein expression patterns were determined for transgenic peanuts expressing Oxox.

Objective 2: *To assemble data for governmental review.*

Data gathered in the first objective along with field data were compiled into a document to petition APHIS for deregulation of transgenic peanuts with resistance to *S. minor*.

In order to demonstrate completion of Objective 2, Chapter 2 is written in the general format of a petition to be submitted to APHIS for de-regulation of events N70, P39 and W171.

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CHAPTER 2

PETITION FOR THE DETERMINATION OF NON-REGULATED STATUS

Content is presented in the general format of a petition to be submitted to the USDA

Petition for the Determination of Nonregulated Status For Sclerotinia Blight Resistant Transgenic Peanut Events N70, P39 and W171

OECD Unique Identifiers:
VPI-NØØ7Ø-5
VPI-WØ171-7

No CBI

Submitted Date 2008

The undersigned submits this petition under 7 CFR 340.6 to Request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the articles should not be regulated under 7 CFR part 340.

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SUMMARY

Peanuts (*Arachis hypogaea* L.) are a major source of vegetable protein and oil around the world. There are six market types of peanut, four of which are commonly grown in the U.S.: Runner, Valencia, Spanish and Virginia type. This petition will specifically consider the Virginia-type (VA-type) peanut which are large seeded and marketed primarily roasted in the shell and for gourmet products. VA-type peanuts are important to the economy of Virginia, North Carolina and South Carolina where they are primarily grown. Additional limited acreage is grown in Oklahoma and Texas.

Under the last two Farm Bills, peanut prices have fallen from approximately $662 per metric ton to as low as $368 per metric ton. This, combined with increasing fuel, labor and agrichemical costs has caused peanut production in Virginia to decline from a fifty year average of 40 thousand hectares to only 6.5 thousand hectares in 2006.

The most important disease of the VA-type peanut is Sclerotinia blight, caused by the fungal pathogen *Sclerotinia minor* Jagger. Chemical control of Sclerotinia blight costs growers from $207 to 311 per hectare for two or three fungicide applications that have proven to be only partially effective. A major pathogenicity factor of the fungus is the secretion of oxalic acid (OA). It has been shown in related species such as *S. sclerotiorum* that mutants lacking the ability to produce OA are unable to infect a plant (Godoy et al., 1990). It has also been shown that the presence of an enzyme that can degrade OA will prevent infection.

Two enzymes have been used for this purpose: germin from wheat and oxalate oxidase (Oxox) from barley. Homologous enzymes are found naturally in other cereals such as rice, rye, oats and corn. These enzymes are members of the cupin superfamily of proteins and are normally expressed in cereals during germination and during infection by fungal pathogens. Related cupin

proteins are ubiquitous in plants and serve a multitude of functions including enzymes, structural proteins and receptors.

Three different cultivars of VA-type peanut (NC 7, Perry and Wilson) were transformed to express an oxalate oxidase enzyme from barley. Tissue cultures of each cultivar were subjected to particle bombardment with the Oxox gene and the selectable marker hygromycin B phosphotransferase (*aph4*). After regeneration of plants, breeding for seed production occurred by allowing plants to naturally self pollinate. Only those plants expressing Oxox, as determined by a colorimetric activity assay, were selected. Based on early field trial results and genetic characterization, three elite lines, N70, P39, and W171, were selected for further analysis.

These lines have been tested in the field for 4 years with certain tests being completed at two locations in the final year. Results have shown a significant reduction in disease incidence caused by *S. minor* and significantly higher yields under disease conditions. Our observations have indicated that Oxox expression does not affect the incidence of unrelated diseases such as early and late leaf spot, web blotch, tomato spotted wilt virus or Cylindrocladium black rot in peanut. No significant yield reduction under non-disease conditions has been found. Transgenic plants are phenotypically indistinguishable from the non-transgenic parents for the parameters tested. Outcrossing studies have demonstrated that gene flow between peanut plants in the field is low in the transgenic lines, occurring at less than 2.5% in the N70, P39 and W171 lines. *A. hypogaea* is genetically distinct from wild relatives and does not naturally cross with them. Wild relatives of peanut are only found in South America, making the possibility of gene flow from cultivated fields into wild species unlikely. We believe that new transgenic peanut varieties with resistance to Sclerotinia blight will benefit growers by eliminating the need for expensive fungicides for disease control and increasing yields.

Based on the information and data contained in this petition, Virginia Tech requests that USDA-APHIS make a determination of non-regulated status for *S. minor* resistant transformation events N70, P39 and W171 and any progeny derived from crosses between these and other peanut varieties.

Difference from a Typical Petition

In order to make this petition fit into the format of a thesis, the following items were omitted:

- Omitted-Certification of completeness of data: consists of a statement that the person submitting the petition has included all data, even that which is negative, and their signature
- Omitted-List of Tables: to avoid duplication of thesis table
- Omitted-List of Figures: to avoid duplication of thesis table
- Omitted-List of Abbreviations: a specific requirement of APHIS
- Omitted-Table of Contents: to avoid duplication of thesis table

RATIONALE FOR THE DEVELOPMENT OF TRANSGENIC PEANUTS WITH SCLEROTINIA BLIGHT RESISTANCE

THE BASIS FOR THE REQUESTING NONREGULATED STATUS

Under the Plant Protection Act and the Plant Quarantine Act, the U.S. Department of Agriculture (USDA) has the responsibility to prevent the introduction and dissemination of plant pests into the U.S. This responsibility is carried out by the Animal and Plant Health Inspection Service (APHIS) division of the USDA. The right of an applicant to petition APHIS to evaluate submitted data to determine that a regulated article does not present a plant pest risk and should no longer be regulated is detailed in 7 CFR Part 340.6. If APHIS determines that a particular regulated article does not present a risk to U.S. agriculture, then the petition is granted and the unrestricted introduction of the article is allowed.

RATIONALE FOR THE DEVELOPMENT OF N70, P39 AND W171

This section provides background and rationale for the development of transgenic peanut lines expressing Oxalate Oxidase (Oxox).

EFFECTS OF THE 2002 FARM BILL ON VA-TYPE PEANUT PRODUCERS AND THE BENEFITS OF USING TRANSGENIC PLANTS

Since the adoption of the 2002 Farm Bill, the market value of VA-type peanuts has fallen from ~$600 per metric ton to less than $390 per metric ton in 2006. This 35% drop in price along with increased fuel, labor and agrichemical costs has reduced the combined peanut acreage in North Carolina and Virginia from an average of 100 thousand hectares prior to 2002 to only 42 thousand hectares in 2007 (Figure 5). This drastic loss of production has resulted in closings of several buying stations, warehouses and shelling plants in the area and has caused heavy losses of revenue by farmers and rural communities.

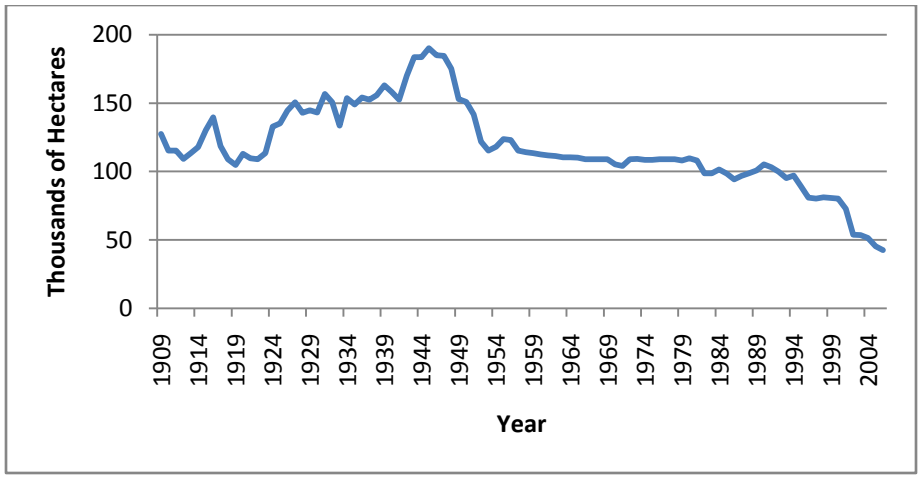


Figure 5. VA-type peanuts harvested in North Carolina and Virginia from 1909 to 2007 (USDA, 2007).

Peanut growers in the Virginia-Carolina region have the highest production costs among U.S. growers because VA-type peanuts require supplemental calcium for pod development and intensive use of chemicals for the control of diseases that are not prevalent in other peanut growing regions of the country. The 2007 Virginia Peanut Production Guide (Roberts, 2007) estimates the grower's cost for peanut production at between $1235 and $1482 per hectare. The average farm produces 3.4 metric tons of peanuts per hectare (USDA, 2007). At the 2006 price of $390 per metric ton, the average gross is $1326 per hectare. After deduction of production costs, many growers were left with little profit while others were showing a loss.

One of the major diseases of peanut in this region is Sclerotinia blight caused by the fungal pathogen *Sclerotinia minor* Jagger, which can cause yield losses in excess of 50% in severely affected fields (Porter and Melouk, 1997). There is little natural resistance to Sclerotinia blight in peanuts and breeding for resistance has not produced lines that would negate the need for fungicide sprays. Currently, growers attempt to mitigate the disease with crop rotation and by the use of fungicides. Crop rotation is not highly effective because the sclerotia (overwintering structures) produced by *S. minor* can persist in the soil for more than 8 years.

Chemical control for *S. minor* is currently the best option for growers but the cost is very high—between $207 and $311 per hectare. This is a substantial cost considering the current peanut prices.

The commercialization of N70, P39 and W171 peanuts will provide substantial benefits to growers. The increased resistance to Sclerotinia blight will help to reduce input costs and increase yields making peanuts a more profitable crop.

Rationale for Using Oxalate Oxidase to Control Sclerotinia minor

Research has demonstrated that *S. minor* and other related fungi produce oxalic acid, a compound that predisposes plants to infection. Research by Godoy et al. (1990) showed that *S. sclerotiorum* mutants that are unable to produce oxalic acid cannot infect bean plants. This indicates that oxalic acid is a necessary pathogenicity factor for *S. sclerotiorum* and may also be a necessary pathogenicity factor for other pathogens that secrete the compound.

Cereal crops are not susceptible to oxalic acid producing *Sclerotinia* spp. This may be due to the presence of an enzyme known as oxalate oxidase. This enzyme catalyzes the break-down of oxalic acid into carbon dioxide and hydrogen peroxide. Oxalate oxidase, also known as germin, has been identified in wheat, barley, rye, rice, oats and corn and is known to be expressed, along with other related proteins, during germination, plant stress and pathogen infection (Bernier and Berna, 2001). The Oxox proteins from wheat and barley are the most extensively characterized and the genes have been transformed into several different plant species (Table 5). All of the transgenic plants expressing Oxox that have been tested exhibit significantly higher resistance to oxalic acid producing pathogens.

Table 5. Some plant species transformed to express oxalate oxidase.

Transgenic Species	Reference
Tobacco	(Berna and Bernier, 1999)
Poplar	(Liang et al., 2001)
Soybean	(Donaldson et al., 2001)
Sunflower	(Hu et al., 2003)
Potato	(Schneider et al., 2002)
Peanut	(Livingstone et al., 2005)

ADOPTION OF N70, P39 AND W171

Currently, peanut growers depend on conventional chemical methods for control of Sclerotinia blight. Many growers are eager to have a biotechnological alternative to spraying chemicals. Adoption of peanuts with resistance to Sclerotinia blight will be in areas of the country where VA-type peanuts are grown and Sclerotinia blight is a major disease. This limits the expected growing area to southeastern Virginia, eastern North Carolina and parts of Texas and Oklahoma. Sclerotinia has not been reported to occur in South Carolina.

SUBMISSIONS TO OTHER REGULATORY AGENCIES

SUBMISSION TO THE FDA

In 1992, the U.S. Food and Drug Administration outlined its policy concerning food and feed products derived from new plant varieties, including those produced through genetic engineering (FDA, 1992). Virginia Tech has initiated contact with the FDA and will voluntarily complete the consultation process before commercialization and distribution of transgenic peanuts. A safety and nutritional assessment of transformation events N70, P39 and W171 will be submitted to the FDA in the near future.

Submission to the EPA

The Environmental Protection Agency (EPA) requires all pesticides, including Plant Incorporated Protectants (PIPs), be registered through their agency. A PIP is defined in 40 CFR Part 152 as any substance produced by a plant that can "prevent, destroy, repel or mitigate" any pest, whether it evolved naturally in a plant or was introduced through conventional breeding or through biotechnology (EPA, 2001). Under this definition, oxalate oxidase is a PIP and will be registered by the EPA. Virginia Tech will submit an application for registration to the EPA in the near future.

Submissions to Foreign Governments

Canada is the leading importer of peanuts and peanut products from the U.S. and represents 35% of the export market (Boriss and Kreith, 2006). Submission for import approval will be made to the Canadian Food Inspection Agency (CFIA) after submissions to the FDA have been completed. As appropriate, submissions will be made to other countries that import VA-type peanuts.

THE PEANUT FAMILY

This section covers the taxonomy and biology of peanuts. The history of peanut and its use as a crop will also be summarized.

TAXONOMY

Peanut (*Arachis hypogaea* L.) belongs to the Stylosanthinae sub tribe and Aeschynomeneae tribe which are included in the sub family, Faboideae, a member of the legume family Fabaceae (Table 6). There are 80 described species belonging to the genus *Arachis* (Favero et al., 2006). With the exception of a few primitive landraces, only two of the 80 species are cultivated—*A. hypogaea* L. which is produced primarily for human consumption and *A. glabrata* Benth, which is grown solely for the production of hay for animal feed.

Cultivated peanuts are further categorized into subspecies and botanical varieties. In the U.S., two subspecies are grown: *hypogaea* and *fastigiata*. The *hypogaea* subspecies is comprised of two distinct botanical varieties, *hypogaea* (Virginia-) and *hirsuta* (Runner-). The *fastigiata* subspecies contains the *fastigiata* (Valencia-) and *vulgaris* (Spanish-) varieties.

Table 6. Taxonomic classification of cultivated peanut grown in the U.S.

Family: Fabaceae (Leguminosae; Papilionaceae)
Sub Family: Faboideae (Papilionoideae)
Tribe: Aeschynomeneae
Sub Tribe: Stylosanthinae
Genus: *Arachis*
Species: *hypogaea*
Subspecies: *hypogaea*
Variety: *hypogaea* (Virginia)
Variety: *hirsuta* (Runner)
Subspecies: *fastigiata*
Variety: *fastigiata* (Valencia)
Variety: *vulgaris* (Spanish)

PEANUT AS A CROP

Peanut (*Arachis hypogaea* L.), also commonly known as groundnut, is grown throughout the world as a major source of edible oil and digestible protein. Statistics gathered by the Food and Agriculture Organization (FAO, 2002) of the United Nations show that peanut is the world's 13th most important food crop, the 4th most important source of edible oil and the 3rd most important source of vegetable protein. The crop can be grown in much of the world between 40°N latitude and 40°S latitude where there is sufficient rain or irrigation and well drained soils. Eighty percent of the world's peanuts are produced by six countries: China (40%), India (20%), Nigeria (8%), the U.S. (5%), Indonesia (4%) and Sudan (3%) (FAO, 2004).

A. glabrata Benth. is grown in Florida solely for the purpose of producing hay for animal feed. This species is a perennial plant and cannot cross pollinate with *A. hypogaea*.

Virginia-type peanuts produce the largest kernels and are marketed as gourmet nuts as well as peanuts that are roasted in the shell. Significant numbers of this variety are grown in Virginia and the Carolinas with smaller numbers grown in other states, notably Texas and Oklahoma. The runner type makes up the majority of the peanuts that are grown in the U.S. This type of peanut is cultivated throughout the Southeast and in Texas and Oklahoma. Runner-type peanuts are most commonly eaten in the form of peanut butter. Spanish peanuts, grown in Texas and Oklahoma, are used primarily in the production of peanut oil. The Valencia type is not widely grown and is often eaten boiled in the southwestern U.S. All four types can be found in mixed nuts, candy and peanut butter.

ORIGIN AND HISTORY OF PEANUT

Peanut is native to South America and most likely evolved in the higher elevations of Brazil and was then distributed by the downward movement of water and soil into the areas where wild peanuts now grow (Singh and Simpson, 1994). The crop was probably domesticated in the Paraná and Paraguay river valleys (Hammons, 1994). Peanut hulls have been recovered from archaeological sites in Peru dating to 3750-3900 years ago but there are no good estimates of when the plant was first domesticated. Archaeological findings such as golden peanut pods and paintings depicting peanuts have led anthropologists to believe that peanuts were an important crop to many pre-Columbian South American cultures.

Beginning in the 1500's with European exploration of the Americas, groundnut was dispersed throughout the world along with other New World crops such as bean, squash, corn, potato and tobacco (Hammons, 1994). Peanut was adopted in Asia and in Africa where it became a major source of protein while production in Europe was limited. The first peanuts to gain a large market were introduced into the southeastern U.S., most probably from Africa or the

Caribbean with the slave trade and were likely of the runner variety. Spanish and Valencia varieties were most likely introduced from Europe by early European settlers. It is documented that TB Rowland introduced a Spanish cultivar into Norfolk, VA in 1871 from Spain. The Virginia type may have been in cultivation in Virginia as early as 1844, but its origins are unclear. The variety may have originated from a chance hybrid or have been introduced from Africa.

GENETICS OF PEANUT

Arachis hypogaea is an allotetrapolid (2n=4x=40) derived from distinct A and B genomes. These genomes differ by one pair of small chromosomes. Several species have been proposed as the progenitors of modern groundnut, including *Arachis monticola*, the only wild species capable of interbreeding with *A. hypogaea*. Recently, a cross between *Arachis duranensis* (A genome) and *Arachis ipaensis* (B genome) has been shown to produce progeny with the same chromosomal and botanical characteristics as *A. hypogaea*. This cross only produced fertile offspring when treated with colchicine to create a tetrapolid plant. The hybrid plants were then crossed with *A. hypogaea* and fertile offspring were produced. These results indicate that *A. duranensis* and *A. ipaensis* are the most likely progenitors of the modern cultivated peanut (Favero et al., 2006).

There is little genetic polymorphism in *A. hypogaea* compared to that of other plants. This is due to the polyploidization event in the evolution of the species. Polyploidization causes plants to be sexually isolated from the parent population restricting gene flow. A long history of domestication and selection by humans has further limited the gene pool (Favero et al., 2006).

POLLINATION OF PEANUT

Peanut flowers have one unilocular ovary which contains 1 to 3 ovules. Each flower can contain up to 10 anthers, some of which are usually sterile. The pollen matures 6 to 8 hours prior to anthesis. The stigma and anthers are enclosed by the keel, which induces self fertilization. Pollination occurs simultaneously with anthesis, which also promotes self pollination (Holbrook and Stalker, 2003).

Bees are known to visit groundnut flowers and outcrossing has been attributed to bee pollination. In the U.S., bees from four different families, Halictidae, Megachilidae, Apidae and Bombidae have been identified as carriers of peanut pollen. Natural outcrossing rates in the field have been reported to be up to 8% (Knauft et al., 1992). In Virginia, outcrossing rates from 0 to 2.8% have been reported (Coffelt, 1989). Outcrossing is dependent on the peanut cultivar in question as well as environmental conditions.

WEEDINESS OF PEANUT

Groundnut seeds that remain in the field after harvest can remain dormant during the winter and germinate the following year. These volunteer plants are commonly killed by herbicides or land cultivation either before or after the next crop is planted. Peanut seeds are not easily dispersed and are only known to travel long distances when carried from the field by birds. Only one species, *A. monticola*, can outcross with domesticated peanut and it is not found in the U.S. No reference to *A. hypogaea* or any of its relatives as a weedy species has been found.

CHARACTERISTICS OF RECIPIENT PEANUT MATERIAL

Three VA-type peanut cultivars were used to create the transgenic peanut plants with resistance to Sclerotinia blight.

NC 7

The NC 7 cultivar was developed by the North Carolina Agriculture Research Service and released in 1978 (Wynne et al., 1979). The cultivar was selected from the fourth generation of a cross between the Fla393 and NC 5 peanut cultivars.

The plants have a decumbent growth habit with a main stem height of 33.8 cm. NC 7 produces an average of 89% fancy pods and 50% extra large kernels. NC 7 has a tan seed coat.

WILSON

The Wilson cultivar was jointly released by The Virginia Agricultural Experiment Station of Virginia Tech and the USDA-ARS in 2002. Wilson was selected from a thirteenth generation cross between the breeding line VA 781621 and PI 781621, a plant introduction from China with resistance to early leaf spot. VA 781621 was developed from a cross between NC-Fla 14 and PI 476823 from Israel (Mozingo et al., 2004).

Wilson has a main stem height of 32.5 cm and an intermediate-runner growth habit. The seed coat is described as pink to light pink. The cultivar produces an average of 75% fancy pods and 39% extra large kernels. Wilson is moderately resistant to early leaf spot. This cultivar matures early but is able to maintain yield at later harvesting dates (Mozingo et al., 2004).

PERRY

The Perry cultivar was released in 2000 by North Carolina Agricultural Research Service (Isleib et al., 2003). It is a fourth generation selection from the breeding line X8140. X8140 was produced from a cross between the breeding line N90021 and an unnumbered progeny from a cross between NC 7 and Florigiant. N90021 was produced from a cross between NC 2 and NC3033.

Perry has a running growth habit with an alternate branching pattern and produces seed with pink testa. The average seed size is 93g per 100 seeds and the cultivar produces 49% fancy pods and 46% extra large kernels. Perry has resistance to *Cylindrocladium parasiticum*, the causative agent of Cylindrocladium Black Rot (CBR).

DEVELOPMENT OF TRANSGENIC PEANUT PLANTS

This section describes the plasmid and transformation system that were used to develop transgenic peanut plants with enhanced resistance to Sclerotinia blight. The origin and function of specific genes and regulatory sequences will be discussed.

DESCRIPTION OF THE TRANSFORMATION SYSTEM

Transgenic peanut plants were produced by microprojectile bombardment in the Fralin Biotechnology Center at Virginia Tech (Livingstone et al., 2005). Embryos from mature seed of the NC7, P39 and Wilson peanut cultivars were excised and the radical end was removed. Embryogenic callus was induced and maintained on MP3 media in the dark at 28°C.

Transformation and regeneration were performed as previously described (Livingstone and Birch, 1999). Embryogenic callus was incubated on osmoticum for several hours before and after bombardment. Tungsten particles were coated with the pOxox transformation vector and bombarded into embryogenic callus using Bio-Rad's Particle Delivery System-1000 pressurized with helium. The callus was then transferred to MP3 maintenance medium for 2 weeks. Transformed cells were selected by transferring the callus onto selection medium containing hygromycin B. Plants were regenerated on MS medium for one month followed by one month of desiccation and transfer back to MS medium. Embryos that did not differentiate or turn green were discarded.

Regenerated peanut plants (T_0) were grown in a greenhouse and leaflets were tested for Oxox expression as well as for resistance to oxalic acid and infection by *S. minor* (Livingstone et al., 2005). Seeds (T_1) were harvested from plants that were shown to express Oxox and have enhanced resistance Sclerotinia blight. The T_1 plants were grown in the greenhouse at the Tidewater AREC in Suffolk, VA and seeds (T_2) were collected from plants expressing the

transgene. The T_2 plants and subsequent generations were grown in the field and greenhouse at the Tidewater AREC in Suffolk, VA. A schematic representation of the development of the transgenic peanut lines follows (Figure 6). Several lines with the high yields of seed and low disease were tested in advanced generations and three were chosen for deregulation and release.

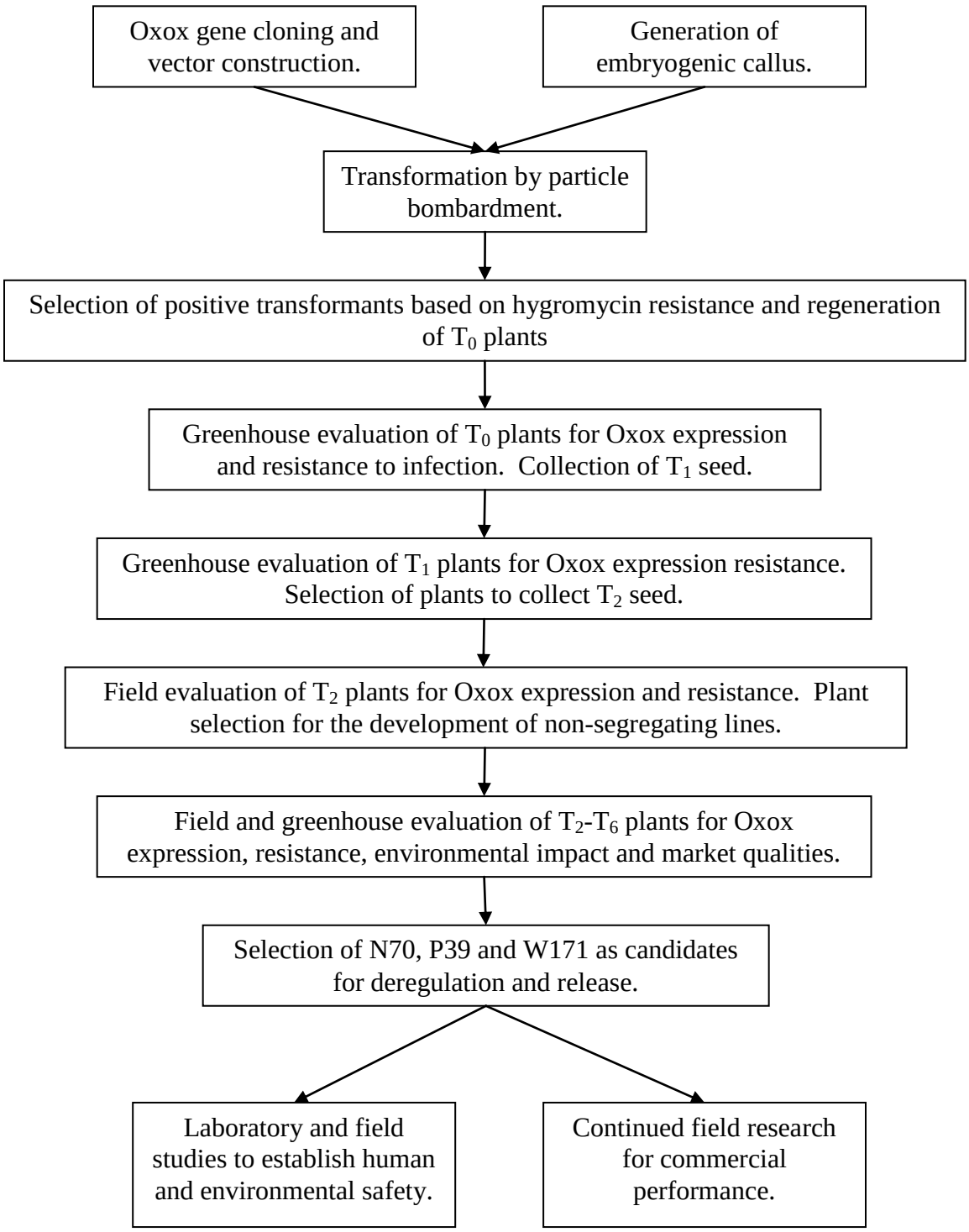


Figure 6. Schematic of the development of transgenic peanut lines N70, P39 and W171.

Description of the Transformation Vector

The plasmid vector pOxox (Figure 7) was used for the transformation of embryogenic peanut callus to produce peanut cultivars with enhanced resistance to Sclerotinia blight. The plasmid vector utilized was originally constructed by Li et al. (1997) to express a phytase gene in soybean. The phytase ORF was removed and replaced with the Oxox ORF for use in peanut and the vector was sequenced. The vector was constructed in a pUC19 backbone, which contains all of the sequences necessary for replication in bacteria. A list of all of the functional genetic elements and their origins is included (Table 7).

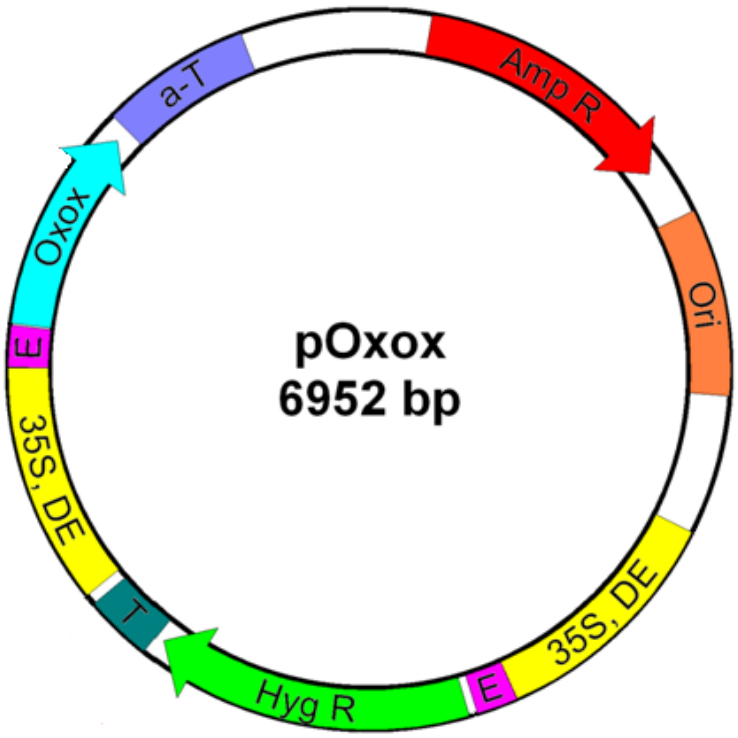


Figure 7. Plasmid map of vector pOxox.
See description of genetic components in Table 7.

Table 7. Functional genetic elements in the pOxox transformation vector.

Genetic Element	Location (bp)	Size (kb)	Description	Reference
AmpR	191-1061	0.871	ß-lactamase ORF. Bacterial gene conferring resistance to Ampicillin which is used as a selectable marker for maintaining the plasmid in *E. coli*.	(Yanisch-Perron et al., 1985)
Ori	1232-1819	0.588	Origin of Replication. Permits DNA replication of the plasmid in bacterial cultures such as *E. coli*.	(Yanisch-Perron et al., 1985)
35S, D	2256-3012 4466-5222	0.757	Cauliflower Mosaic Virus 35S dual enhanced promoter. Constitutive plant promoter driving transcription of HygR.	(Kay et al., 1987; Fang et al., 1989)
E	3013-3143 5223-5353	0.133	Tobacco etch virus (TEV) translational enhancer sequence.	(Carrington and Freed, 1990)
HygR	3169-4194	0.980	Hygromycin B phosphotransferase APH4. Provides resistance to hygromycin in plants and is used as a selectable marker.	(Waldron, 1997)
T	4221-4432	0.212	Cauliflower Mosaic Virus 35S terminator. Terminates transcription of HygR and directs poly-adenylation.	(Pietrzak et al., 1986)
Oxox	5360-6034	0.675	Oxalate oxidase ORF. Gene obtained from barley cDNA.	(Livingstone et al., 2005)
a-T	6080-6551	0.472	Terminator from the ß-conglycinin α'-subunit in *Glycine max*. Terminates transcription of Oxox and directs poly-adenylation.	(Chen et al., 1986)

BACTERIA SPECIFIC SEQUENCES

All of the bacterial sequences in pOxox originated from pUC19, a common cloning vector (Yanisch-Perron et al., 1985). The plasmid includes a bacterial origin of replication for proliferation in bacteria such as *E. coli*. pOxox also contains a selectable marker, the ß-lactamase gene, which provides resistance to the antibiotic ampicillin. There is also a screenable

marker, the lacZ gene, located in the plasmid. The multiple cloning site of pUC19 is located in the lacZ gene allowing for blue/white screening. With the introduction of the plant specific sequences, the lacZ gene was rendered non-functional. Due to the nature of the transformation system, the entire plasmid can be incorporated into the plant's genome.

PLANT SPECIFIC REGULATORY SEQUENCES

The constitutive 35S promoter from the Cauliflower mosaic virus (CaMV) was used to drive transcription of both the oxalate oxidase and hygromycin phosphotransferase genes *in planta*. The promoter is dual enhanced, meaning that two promoter sequences were inserted in tandem. At the 3' end of the dual enhanced promoter, a translational enhancer sequence from the tobacco etch virus (TEV) was attached. The entire promoter cassette was then inserted at the 5' end of each gene. This cassette was obtained from the pRTL2 plant transformation vector (Rastrepo et al., 1990).

The Hyg^R ORF is followed by the CaMV 35S terminator (T). This sequence is responsible for terminating transcription and specifying poly-adenylation. The Hyg^R sequence was also taken from the pRTL2 plasmid.

The terminator used with the Oxox ORF was obtained from the α' subunit of the soybean ß-conglycinin gene (a-T).

HYG^R CODING SEQUENCE

Hyg^R encodes the enzyme hygromycin B phosphotransferase (*aph4*). The enzyme is able to phosphorylate the antibiotic hygromycin and confer antibiotic resistance to the plant. The coding region was derived from *E. coli* (Waldron, 1997). The EPA has declared this protein exempt from the requirement of tolerance levels.

OXOX CODING SEQUENCE

mRNA from germinating barley seed was isolated and the Oxox coding region was amplified by reverse-transcription PCR (Livingstone et al., 2005) using primers designed from the published sequence (accession no. Y14203; (Zhou et al., 1998)). The enzyme that is produced degrades oxalic acid and produces carbon dioxide and hydrogen peroxide enhancing the plant's resistance to oxalic acid producing pathogens.

GENETIC CHARACTERIZATION OF N70, P39 AND W171

Southern blot analyses were performed to characterize the plasmid insertions in N70, P39 and W171. Several characteristics of the individual transformation events were analyzed, including the following:

- The number of insertions in each transformation event
- The stability of the trait during propagation
- The intactness of the expression cassettes in each transformation event
- The intactness of the plasmid backbone in each transformation event
- The approximate point of recombination at each insertion
- The number of copies of the plasmid inserted into the genome in each transformation event

GENETIC CHARACTERIZATION EXPERIMENTAL DESIGN

The restriction enzymes used for genetic characterization of the insert are depicted in Figure 8 and the probes are defined in Table 8.

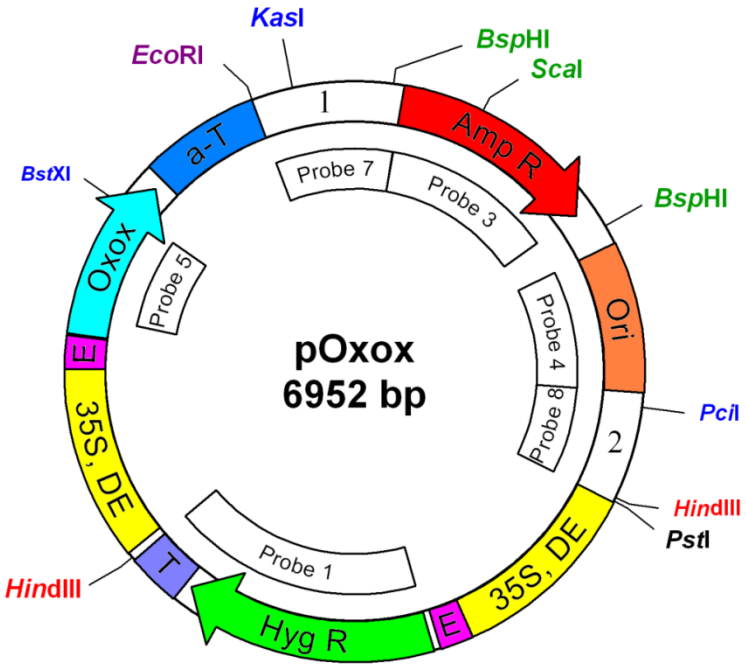


Figure 8. Diagram showing the restriction enzymes important to Southern analysis as well as the probes that were used.

Table 8. Uses of probes and restriction enzymes in Southern analysis.

Figure #	Probe #	Restriction Enzymes	Band Size (bp)	Purpose
Figure 9	Probe 5	BstXI	Variable	To determine the number of insertions and stability during breeding
Figure 10	Probe 1	HindIII	2212	To determine the intactness of the Hyg R gene
Figure 11	Probe 5	HindIII + KasI	2268	To determine the intactness of the Oxox gene
Figure 12	Probe 3	BspHI	1008	To determine the intactness of the Amp R coding region
Figure 13	Probe 4	BspHI + PciI	720	To determine the intactness of the Ori region
Figure 14	Probe 7	EcoRI + ScaI	907	To determine the intactness of section 1 of the plasmid backbone
Figure 15	Probe 8	PstI + BspHI	1095	To determine the intactness of section 2 of the plasmid backbone

The parent lines, NC7, Perry and Wilson, were used as negative controls. Digested pOxox plasmid was used as a positive control when beginning each experiment (not shown in all blots).

RESULTS OF GENETIC CHARACTERIZATION

The number of insertions in each transformation event as well as the stability of the insertions during propagation (Figure 9) were determined using DNA from two different generations of each transgenic line. DNA was digested with BstXI and evaluated with probe 5 (Figure 8, Table 8). The presence of two bands in N70 and P39 and one band in W171 indicated the presence of two insertions in N70 and P39 and one in W171. The same banding pattern in two generations in each of the three lines indicates stability of the insertions during propagation.

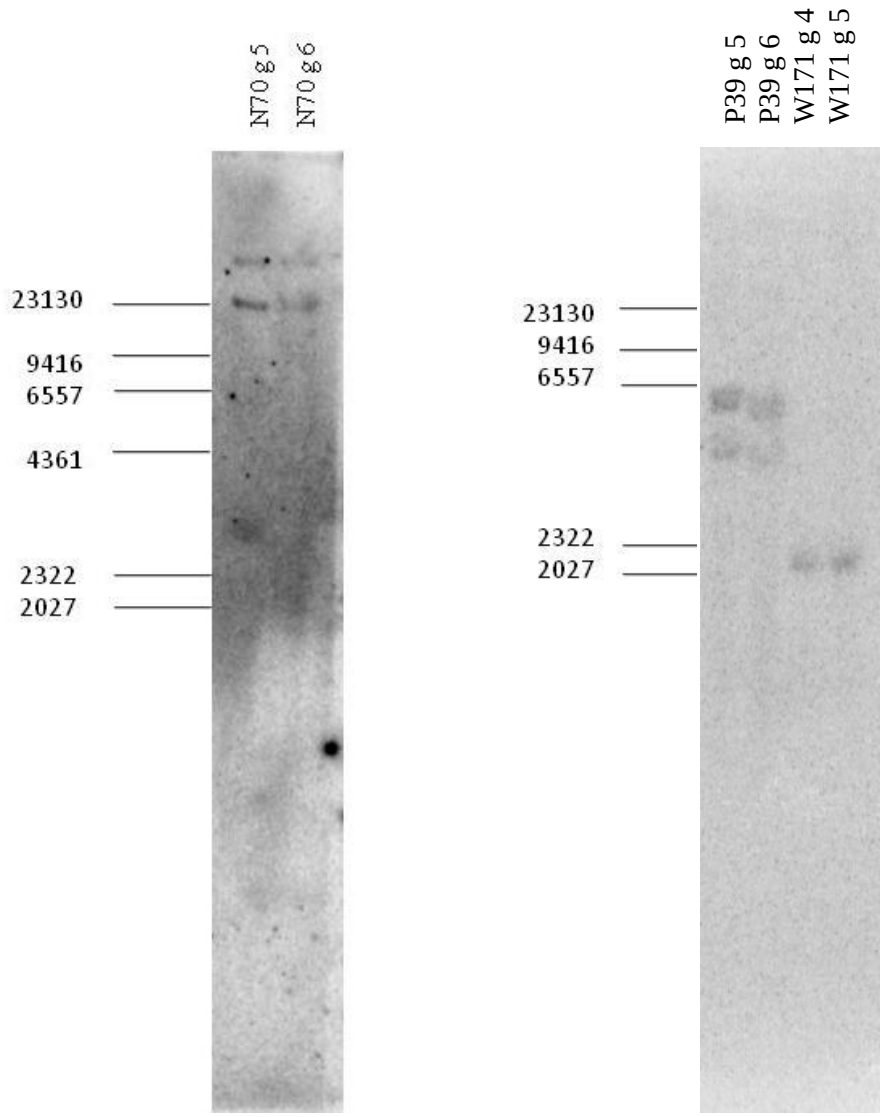


Figure 9. Southern blots showing the number of insertions in transgenic peanut lines N70, P39 and W171 and stability of the insertions in different generations.
Genomic DNA was digested with restriction endonuclease BstXI. Blots were hybridized with radiolabeled probe 5 from within the OxOx coding sequence. 'g' refers to the generation of plant used for analysis. Size markers were HindIII digested lambda DNA.

Evidence for the intactness of the Hyg R cassette in N70 and W171 is presented in (Figure 10). DNA was digested with HindIII and probe 1 (Figure 5, Table 8). The presence of only one band in the N70 lane shows that every copy of the Hyg^R gene is intact in the N70 transformation event. The presence of one band of the correct size in the P39 event indicates that at least one copy of the Hyg^R gene is intact. A faint band lower than the expected size in the P39 even may indicate the presence of recombination in this region of the plasmid.

The presence of two bands in the W171 transformation event, neither of which are the correct size for an intact Hyg R gene, shows that recombination occurred within the Hyg R region for this transformation event. The recombination did not occur in the coding region of the Hyg R gene because the gene produces a functional phosphotransferase. The probe that was used does not bind to the 35S CaMV promoter (Figure 8), so the presence of two bands shows that recombination did not occur in the promoter region. Based on this evidence, it appears that recombination occurred in the terminator region of the Hyg^R gene in the W171 transformation event. This blot also suggests that there is only one copy of the plasmid incorporated into the genome in transformation event W171. The presence of two plasmids would show more than two bands because it is unlikely that recombination occurred in the same region in every plasmid.

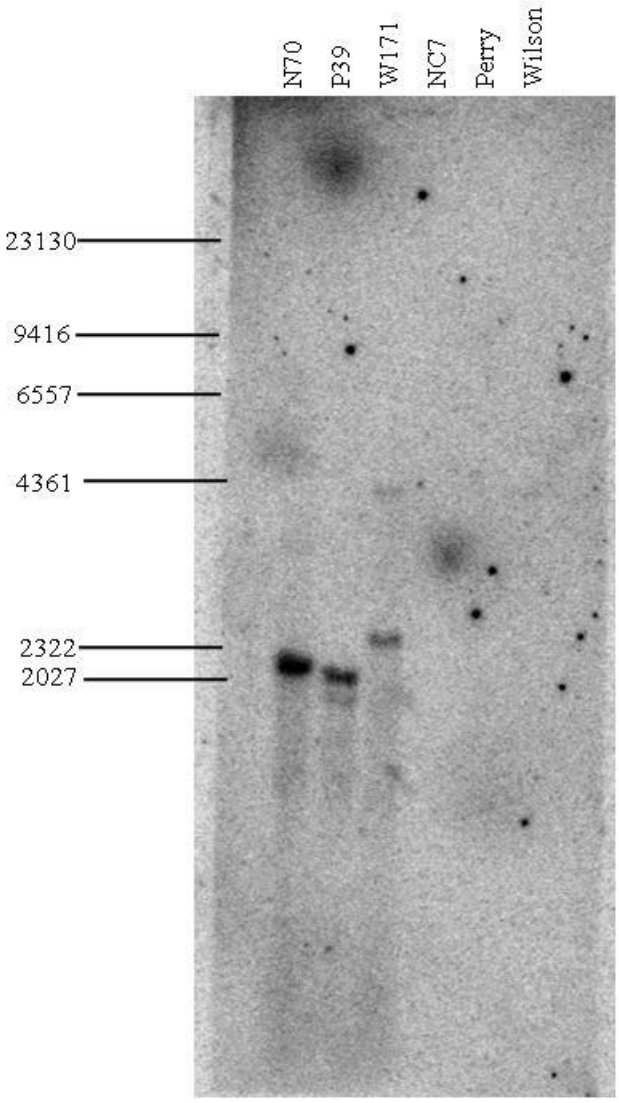


Figure 10. Southern blot showing intactness of the Hyg ^{R} gene in transgenic peanut lines N70, P39 and W171.
Genomic DNA was digested with restriction endonuclease HindIII. The blot was hybridized with radiolabeled probe 1 from the hygromycin resistance cassette. Size markers were HindIII-digested lambda DNA.

DNA was digested with HindIII and KasI and probe 5 was used to detect the Oxox cassette. The presence of one band of the correct size to be the Oxox expression cassette (2268 bp) indicates that every copy of the Oxox gene is intact in the N70 and W171 transformation events (Figure 11). Additional bands higher than the expected size in the P39 transformation event indicates the presence of at least two copies of the plasmid insert, one of which recombined in the Oxox region.

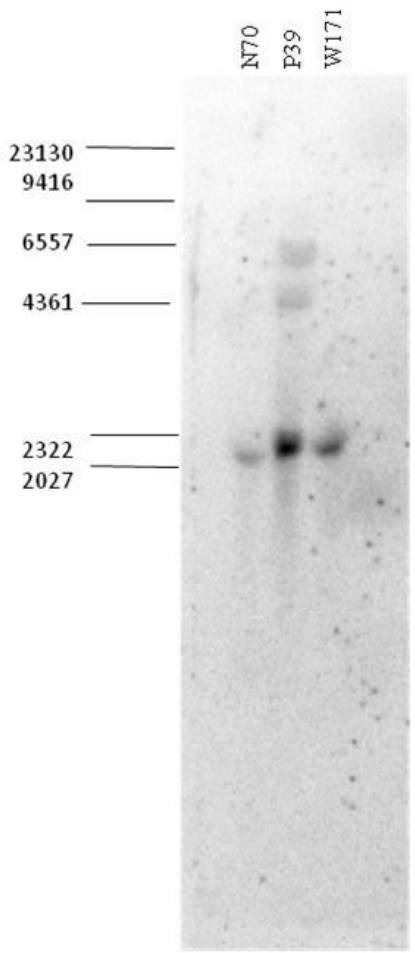


Figure 11. Southern blot showing intactness of the Oxox gene in transgenic peanut lines N70, P39 and W171.
Genomic DNA was digested with restriction endonucleases HindIII and KasI. The blot was hybridized with radiolabeled probe 5 from the Oxox expression cassette. Size markers were HindIII-digested lambda DNA.

Figure 12 shows that every copy of the Amp^R gene is intact in the P39 and W171 transformation events. The presence of three bands in the N70 transformation event, one of which is the correct size for an intact Amp R gene (1008bp), shows that there were at least two copies of pOxox inserted into the genome. Based on the enzyme and probe that were used (Figure 8, Table 8), recombination for one copy of the plasmid occurred in the middle of the Amp^R gene. Faint bands in N70 are likely due to incomplete digestion.

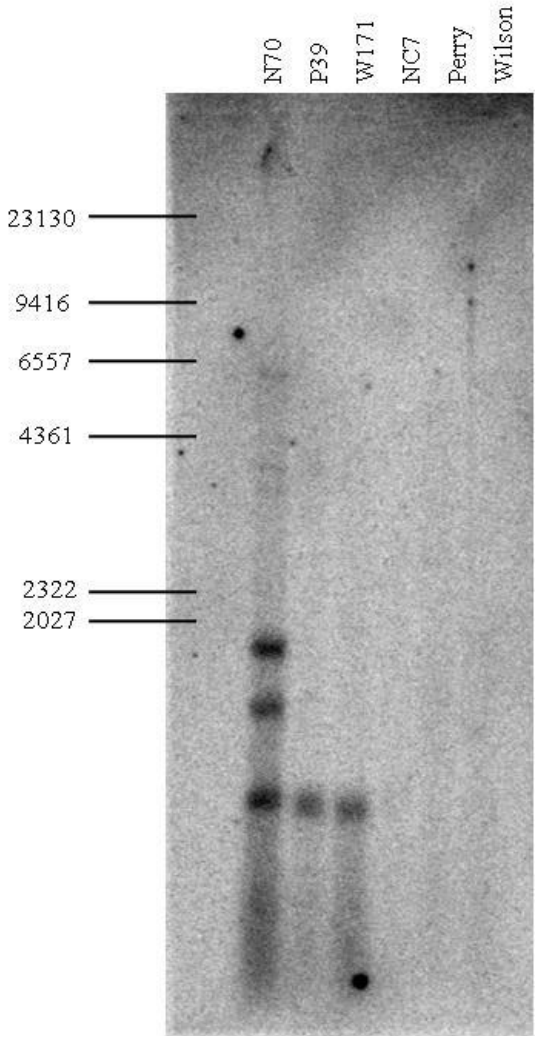


Figure 12. Southern blot showing intactness of the Amp^R gene in transgenic peanut lines N70, P39 and W171
Genomic DNA was digested with restriction endonuclease BspHI. The blot was hybridized with radiolabeled probe 3 from the Amp^R region. Size markers were HindIII-digested lambda DNA.

The Ori region of the plasmid backbone is intact in every copy in the P39 and W171 transformation events (Figure 13). The presence of multiple bands in the N70 transformation event, one of which is the correct size for an intact Ori region (720bp), showed that there were at least two copies of pOxox inserted into the genome. Based on the enzymes and probe that were used (Figure 8, Table 8), recombination for one copy of the plasmid occurred in the Ori region.

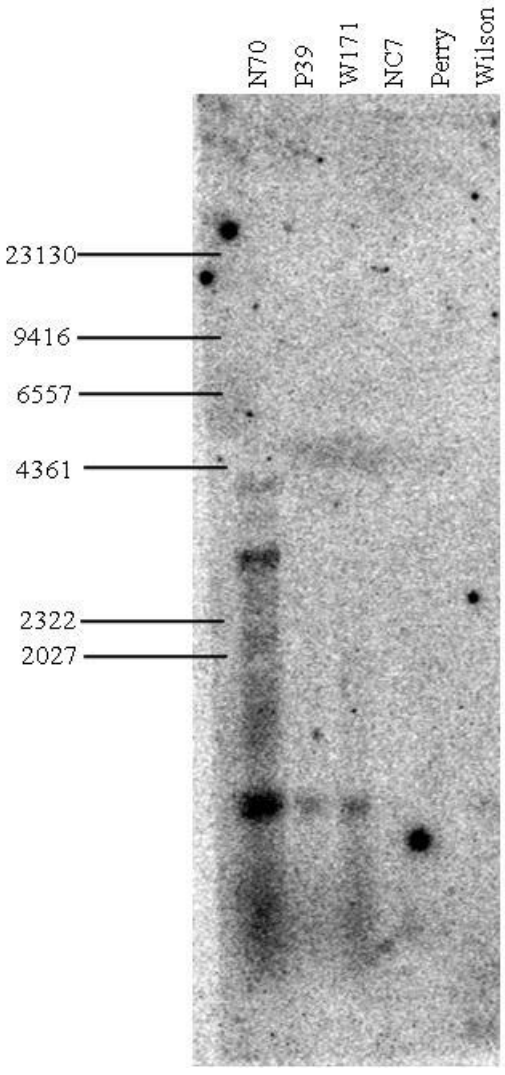


Figure 13. Southern blot showing intactness of the Ori region in transgenic peanut lines N70, P39 and W171.
Genomic DNA was digested with restriction endonucleases BspHI and PciI. The blot was hybridized with radiolabeled probe 4 from the Ori region. Size markers were HindIII-digested lambda DNA.

To address the intactness of region 1 of the plasmid backbone, DNA was digested with EcoRI and ScaI and evaluated with probe 7. The presence of one band (Figure 14) at the expected size of 907bp indicated that section 1 of the plasmid backbone was intact in the N70, P39 and W171 transformation events. Additional bands lower than the expected size is present in the P39 transformation event. This indicates that recombination occurred in region 1 in the P39 event.

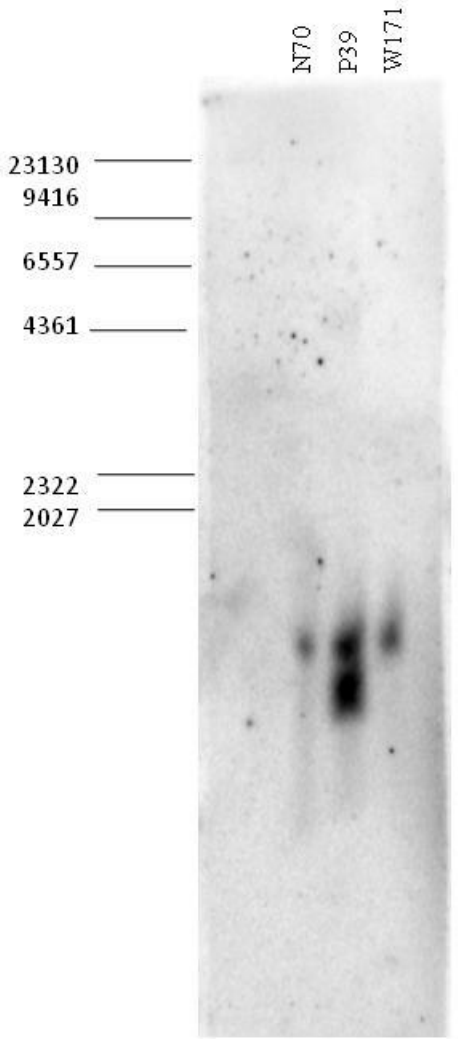


Figure 14. Southern blot showing intactness of region 1 of the plasmid vector backbone in transgenic peanut lines N70, P39 and W171.
Genomic DNA was digested with restriction endonucleases EcoRI and ScaI. The blot was hybridized with radiolabeled probe 7 from region 1 of the plasmid backbone. Size markers were HindIII-digested lambda DNA.

Section 2 of the plasmid backbone was intact in both the N70 and W171 events (Figure 15). A single band of the expected size of 1095bp was present in the W171 line. The higher band in the N70 lane corresponds to the recombination that occurred in the Ori region, which was due to the combination of restriction enzymes used (Figure 8, Table 8). Region 2 of the plasmid backbone was not intact in the P39 transformation event, indicating that recombination occurred in this region. Further analysis would be needed to determine the recombination pattern in this region in the P39 event.

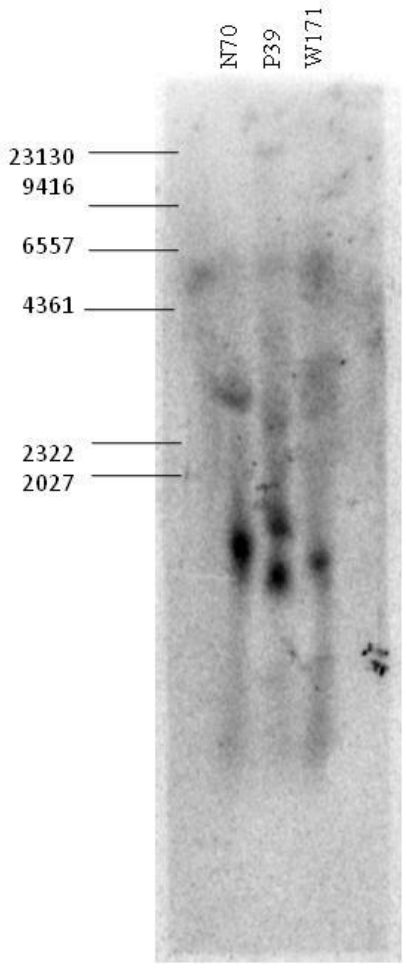


Figure 15. Southern blot showing intactness of region 2 of the plasmid vector backbone in transgenic peanut lines N70, P39 and W171.
Genomic DNA was digested with restriction endonucleases BspHI and PciI. The blot was hybridized with radiolabeled probe 8 from region 2 of the plasmid backbone. Size markers were HindIII-digested lambda DNA.

SUMMARY OF GENETIC CHARACTERIZATION

The N70 transformation event has two insertions with one copy of the pOxox plasmid at each insertion. One copy recombined in the Ori region and the second copy recombined in the Amp^R region. The W171 transformation event has one insertion and one copy of the pOxox plasmid at that insertion. The single copy recombined in the terminator region of the Hyg^R cassette, but did not interfere with the function of that gene. The results of the genetic characterization are summarized in Table 9 and illustrated in Figure 16.

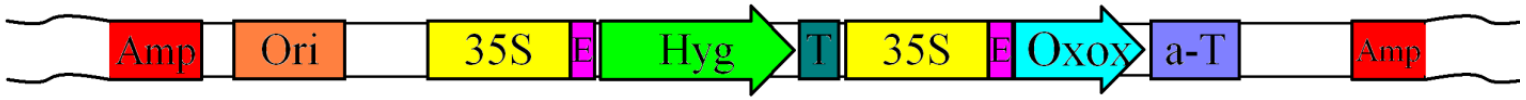
The P39 transformation event has two insertions with more than one copy of the pOxox plasmid at one or both of the insertions. Recombination appears to have occurred in the Oxox open reading frame, region 1 and region 2 of the plasmid backbone and possibly in the Hyg^R cassette (Table 9). These recombination events may represent full copies of the pOxox plasmid or partial copies. Further testing would need to be done to fully characterize this transformation event.

Table 9. Summary of results for the genetic characterization of transformation events N70, P39 and W171*.

Transformation Event	Number of Insertions	Total Number of Copies	Intactness						Point of Recombination	Stable Over Multiple Generations
			Hyg^R	Oxox	Amp^R	Ori	Plasmid Backbone 1	Plasmid Backbone 2		
N70	2	2	✓	✓	✓	✗	✓	✓	Amp^R region	Yes
			✓	✓	✗	✓	✓	✓	Ori region	
P39	2	3 +	?	✓	✓	✓	✓	✓	Possible Hyg^R region	Yes
			✓	✗	✓	✓	✓	✓	Oxox ORF	
			✓	✓	✓	✓	✗	✓	Plasmid Backbone 1	
			✓	✓	✓	✓	✓	✗	Plasmid Backbone 2	
W171	1	1	✗	✓	✓	✓	✓	✓	Hyg^R terminator region	Yes

*A ✓ symbol denotes intactness of the region; An ✗ symbol denotes interruption of the region; A ? denotes insufficient information to make a clear judgment about the intactness of the region.

N70 Insertion 1



N70 Insertion 2

W171 Insertion 1

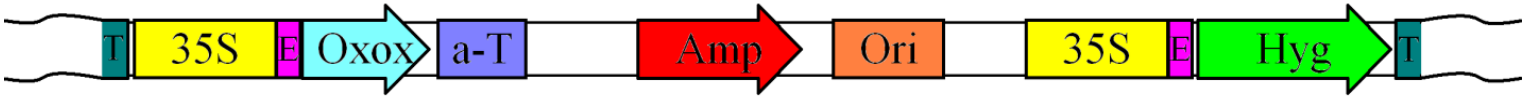


Figure 16. Representations of the pOxox plasmids incorporated into the peanut genome in N70 and W171 transformation events. Amp, ampicillin resistance gene; Ori, origin of replication; 35S, CaMV 35S promoter; E, enhancer; Hyg, Hygromicin resistance open reading frame; Oxox, Oxalate oxidase open reading frame; a-T, terminator.

CHARACTERIZATION OF THE INTRODUCED OXALATE OXIDASE AND HYGROMYCIN B PHOSPHOTRANSFERASE PROTEINS

This section describes the mechanism of action of both the Oxox enzyme and the hygromycin phosphotransferase enzymes. A western blot was used to demonstrate the presence and size of the Oxox protein produced in peanut seeds.

OXALATE OXIDASE

LITERATURE REVIEW

Oxalate oxidase (EC 1.2.3.4) is an enzyme that catalyzes the degradation of oxalic acid into carbon dioxide and hydrogen peroxide (Figure 17). Germin, another name for the Oxox from wheat, was first observed in 1980 during experiments designed to identify proteins associated with the early stages of germination of wheat embryos (Thompson and Lane, 1980). It was shown that the synthesis of germin mRNA and protein begins between 5 and 10 hours after imbibition and continues to increase through germination.

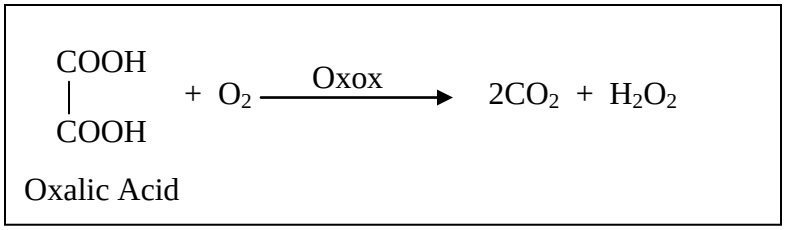


Figure 17. Oxalate oxidase catalyzes the reaction to degrade oxalic acid into carbon dioxide and hydrogen peroxide.

Proteins with a high degree of homology to germin with Oxox activity exist in all of the cereals that have been tested: wheat, barley, rye, oat, corn, and rice (Lane et al., 1992). Proteins that have similar sequences to germin but are not oxalate oxidases are known as germin-like proteins (GLPs). GLPs have been found in all angiosperm and gymnosperm families as well as

in mosses. The functions of many GLPs are unknown. GLPs with known functions include enzymes, structural proteins and receptor proteins. Examples include: phosphomannose isomerases, polyketide synthases, dioxygenases, spherulins, germin-like proteins, AraC-type transcription factors, oxalate decarboxylases, sucrose-binding proteins and seed storage proteins as well as many others (Dunwell et al., 2000).

Barley Oxox has 224 amino acid residues, the first 23 of which make up an apoplastic targeting sequence. The monomers that make up the mature protein are about 25kDa in size. The Oxox from barley has been crystallized and the structure determined (Woo, 2000). The enzyme is a homohexamer, meaning that it is composed of six identical monomers. Oxox is known to have an N-linked glycan at Asn 47 (Woo, 2000; Opaleye et al., 2006).

It has been demonstrated that the germin isolated from barley is a bifunctional enzyme. The protein has both oxalate oxidase activity and superoxide dismutase activity (Woo, 2000). The active site of Oxox requires a manganese ion, which is held by four amino acid residues: histidine 88, histidine 90, glutamate 95, and histidine 137. It has been shown that manganese is the only metal ion present in active enzyme and the ion exists in the 2+ oxidation state (Requena and Bornemann, 1999). In a second study researchers were able to determine the amino acid residues involved in the mechanism by crystallizing native and mutated enzymes with substrate bound and by performing biochemical assays on the mutated enzymes. Asparagine 75, asparagine 85 and glutamate 139 are involved in the catalytic mechanism (Opaleye et al., 2006).

Characterization of Oxox in Transgenic Peanuts

The Oxox gene for use with transgenic peanuts was amplified from the barley cultivar 'Steptoe' (Muir and Nilan, 1973; Livingstone et al., 2005). The deduced protein sequence is defined in Figure 18. Because Oxox has been extensively characterized by others, a rabbit-anti-

germin antibody was available and was generously donated by Drs. Byron Lane and Andrew Cuming. Western blots were performed to show the relative amounts of Oxox present in seeds of each transformed line as well as to compare the size of the exogenous protein from transgenics to the naturally occurring barley protein. A western blot was performed using protein extracts from individual seeds of each transgenic line. Figure 19 shows that the protein produced in the transgenic peanuts is the same size as the protein produced in barley seeds.

```
M G Y S K N L G A G L F T M L L L A P A I M A T D P D P L Q D F
C V A D L D G K A V S V N G H T C K P M S E A G D D F L F S S K
L T K A G N T S T P N G S A V T E L D V A E W P G T N T L G V S
M N R V D F A P G G T N P P H I H P R A T E I G M V M K G E L L
V G I L G S L D S G N K L Y S R V V R A G E T F V I P R G L M H
F Q F N V G K T E A Y M V V S F N S Q N P G I V F V P L T L F G
S D P P I P T P V L T K A L R V E A G V V E L L K S K F A G G S
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Figure 18. Protein sequence for oxalate oxidase from the barley cultivar 'Steptoe' (224 amino acids).

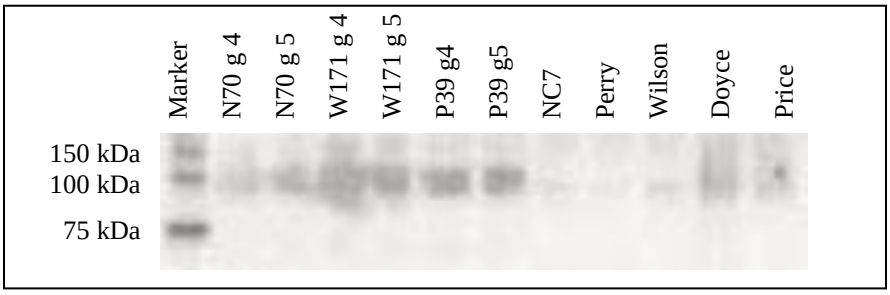

Figure 19. Western blot showing Oxox protein from two generations of each transformation event, negative controls and Oxox that is expressed in germinating barley seeds.
Barley cultivars 'Doyce' and 'Price' were positive controls; 'g' defines generation of plant in analysis.

HYGROMYCIN PHOSPHOTRANSFERASE

CHARACTERIZATION OF HYGROMYCIN B PHOSPHOTRANSFERASE IN TRANSGENIC PEANUTS

The protein sequence in Figure 20 is the deduced amino acid sequence of the hygromycin B phosphotransferase (*aph4*) protein predicted from the known gene sequence. No further characterization was undertaken because full characterization has been completed by others. One previous example would be the petition for the determination of non-regulated status of Syngenta's Cot 102 line. The EPA has also ruled this protein exempt from the requirement of tolerance (40 CFR part 180).

```
M K K P E L T A T S V E K F L I E K F D S V S D L M Q L S E G E
E S R A F S F D V G G R G Y V L R V N S C A D G F Y K D R Y V Y
R H F A S A A L P I P E V L D I G E F S E S L T Y C I S R R A Q
G V T L Q D L P E T E L P A V L Q P V A E A M D A I A A A D L S
Q T S G F G P F G P Q G I G Q Y T T W R D F I C A I A D P H V Y
H W Q T V M D D T V S A S V A Q A L D E L M L W A E D C P E V R
H L V H A D F G S N N V L T D N G R I T A V I D W S E A M F G D
S Q Y E V A N I F F W R P W L A C M E Q Q T R Y F E R R H P E L
A G S P R L R A Y M L R I G L D Q L Y Q S L V D G N F D D A A W
A Q G R C D A I V R S G A G T V G R T Q I A R R S A A V W T D G
C V E V L A D S G N R R P S T R P R A K E
```

Figure 20. Deduced sequence for hygromycin B phosphotransferase.

PHENOTYPIC ASSESSMENT OF TRANSGENIC LINES

This section will address the phenotypic and compositional analysis of N70, P39, W171 and other transgenic lines as compared to the non-transgenic parent varieties. Phenotypic analysis includes traits such as yield, disease occurrence, peanut size (grading) and blanchability. Compositional analysis has been performed and includes characteristics such as nutrient content, fatty acid profiles and aflatoxin content (Table 10). Stand counts, flowering time and plant height measurements have been taken and no significant differences were found between transgenic and non-transgenic lines (data not shown).

Table 10. Summary of phenotypic and compositional analysis data included in this document along with the years in which the experiments were completed.*

	Year				
Analysis Completed	2004	2005	2006	2007	Tables
Oxalate Oxidase Expression	✓	✓	✓	✓	Table 11 to Table 14
Sclerotinia Blight Resistance	✓	✓	✓	✓	Table 11 to Table 14
Yields in Non-Treated Fields	✓	✓	✓	✓	Table 11 to Table 14
Yields in Fungicide Treated Fields				✓	Table 13 to Table 14
Occurrence of Other Diseases			✓	✓	Table 15 and Table 16
Peanut Grade and Value		✓	✓	✓	Table 17 to Table 20
Pod Brightness		✓	✓	✓	Table 21 to Table 24
Blanchability		✓	✓	✓	Table 25 to Table 30
Nutrient Analysis		✓	✓	✓	Table 31 to Table 33
Hay Protein and Fiber			✓		Table 34
Fatty Acid Analysis		✓	✓	✓	Table 35 to 37
Aflatoxin Analysis		✓	✓	✓	Table 38

*Data provided by D. Partridge-Telenko, J. Hu, and P. M. Phipps

OXALATE OXIDASE EXPRESSION, SCLEROTINIA BLIGHT RESISTANCE AND YIELD

Oxalate oxidase expression is determined using a colorimetric assay that detects hydrogen peroxide produced during the degradation of oxalic acid. Using this assay and the western blot analysis, we have shown that N70, P39 and W171 produce the enzyme while the non-transformed parent lines do not (Table 11 to Table 14) This is expected because peanuts are not known to have an endogenous enzyme to degrade oxalic acid. Under normal growing conditions and disease pressure from Sclerotinia blight in large plots from 2004 through 2006, transgenic varieties yielded significantly more than their non-transgenic parents (Table 11 and Table 12).

In 2007, transgenic and non-transgenic plants were grown in fields that were sprayed with and without fungicide for control of Sclerotinia blight (Table 13 to Table 14). In spite of irrigation in 2007, high temperatures and drought stress limited disease development and yield losses. Under these conditions, transgenic lines and their corresponding non-transgenic parents produced similar yields. These results indicate that the presence of Oxox gene provides a yield advantage only in situations of disease pressure caused by *S. minor*. Some data in this section has been published previously by Partridge et al. (2005; 2006) and Hu et al. (2007).

Table 11. Oxalate oxidase expression, disease resistance and yield of non-transformed parent and T_3 transgenic lines with the barley oxalate oxidase gene in Virginia, 2005.

	Oxalate oxidase expression[x]		Sclerotinia blight[y]				Yield
Parent, and lines	17 Aug	29 Sep	9 Sep	4 Oct	27 Oct	AUDPC	(lb/A)[z]
NC7 (non-transformed) .	-0.005 b	-0.008 b	9.3 a	33.0 a	35.8 a	1318.8 a	2619
N70-8-B.....	0.106 a	0.237 a	0.3 b	4.5 b	9.8 b	223.3 b	3661
N70-6-B.....	0.031 ab	0.081 b	0.5 b	6.3 b	7.3 b	239.6 b	3725
LSD (*P*≤0.05).....	*0.044*	*0.097*	*7.0*	*16.9*	*15.2*	*624.1*	*n.s.*
Perry (non-transformed)	-0.005 d	-0.011 d	2.8 a	20.0 a	25.8 a	810.5 a	3711 d
P53-26-B	0.092 a-c	0.195 bc	0.0 b	1.3 b	2.3 b	55.9 b	3732 d
P53-4-B	0.075 bc	0.300 ab	0.5 b	4.5 b	7.0 b	194.8 b	4368 ab
P53-30-B	0.064 c	0.212 a-c	0.3 b	5.0 b	4.8 b	177.8 b	3825 cd
P53-28-B	0.043 cd	0.151 c	0.0 b	0.5 b	2.5 b	40.8 b	4068 b-d
P53-27-B	0.069 bc	0.213 a-c	0.0 b	3.3 b	6.0 b	147.0 b	4146 a-c
P39-7-B	0.126 a	0.320 a	0.8 b	2.3 b	6.8 b	141.0 b	4503 a
P39-8-B	0.115 ab	0.230 a-c	0.3 b	3.8 b	6.8 b	170.8 b	4332 ab
LSD (*P*≤0.05).....	*0.049*	*0.120*	*1.3*	*6.4*	*6.6*	*232.6*	*408*
Wilson (non-transformed)	-0.020 c	-0.008 d	7.0 a	25.3 ab	31.3 a	1052.9 ab	3166 b
W73-27-B.....	0.037 b	0.087 bc	0.3 b	2.5 c	3.8 c	106.3 d	4290 a
W171-17-B.....	0.096 a	0.235 a	0.3 b	5.0 c	6.8 bc	200.8 dc	4141 a
W83-7-B.....	0.000 c	0.014 cd	2.0 b	19.5 b	26.3 a	794.9 b	2889 b
W59-10-B.....	0.000 c	0.018 cd	3.0 b	21.0 b	29.8 a	883.6 ab	2903 b
W59-11-B.....	0.054 b	0.153 b	2.3 b	9.8 c	14.0 b	423.1 c	3187 b
W51-9-B.....	-0.012 c	-0.009 d	3.0 b	30.3 a	33.3 a	1145.9 a	2654 b
LSD (*P*≤0.05).....	*0.028*	*0.078*	*2.9*	*7.6*	*7.8*	*276.1*	*636*

[x]Oxalate oxidase expression was performed on leaf samples from 10 plants per plot using a colormetric detection method (Livingston, et al. 2005 Plant Phys. 137:1354) to measure hydrogen peroxide released from oxalic acid substrate using a microtiter plate reader at 540 nm. [y]Counts of infection centers in the two rows of each plot or a total of 50 ft of row. An infection center was a point of active growth by *Sclerotinia minor* and included 6 in. on either side of that point. AUDPC is area under disease progress curve. [z]Yields are weights of peanuts with 7% moisture. Peanuts were dug on 1 November and harvested on 9 November 2005. Means in each group followed by the same letter(s) are not significantly different (LSD, *P*=0.05); n.s. denotes differences within a group are not significant. Data previously published (Partridge et al. 2006. B&C Tests Report 21:FC051. DOI: 10.1094/BC21).

Table 12. Oxalate oxidase expression, yield and disease resistance for non-transformed and T_4 transgenic lines with the barley oxalate oxidase gene in Virginia, 2006.

	Oxalate oxidase expression[z]		Sclerotinia blight[y]				Yield[x]
Parent, and lines	8 Aug	11 Sep	23 Aug	20 Sep	9 Oct	AUDPC	(lb/A)
NC7 (non-transformed)	0.018 b[w]	0.026 d	5.50 a	23.8 a	35.5 a	1047.1 a	3296 c
N70-8-24-B	0.246 a	0.764 a	0.00 b	1.5 b	2.0 c	54.3 b	5518 a
N99(P60)-29-10-B.....	0.260 a	0.834 a	0.25 b	1.3 b	3.5 bc	69.4 b	5009 b
N70-8-B-B.....	0.188 a	0.512 b	0.00 b	2.8 b	7.5 bc	135.9 b	4966 b
N70-6-B-B.....	0.097 b	0.291 c	0.25 b	6.0 b	8.8 b	230.9 b	4675 b
LSD ($P \leq 0.05$)	*0.084*	*0.141*	*2.57*	*5.3*	*6.7*	*241.5*	*455*
Perry (non-transformed)	0.017 d	0.020 d	1.50	9.8 a	18.8 a	451.0 a	5645 bc
P98(N6)-1-10-B.....	0.304 b	0.561 a	0.25	0.3 b	1.5 b	26.9 b	5885 ab
P97(N6)-2-8-B.....	0.307 b	0.579 a	0.00	0.0 b	1.5 b	14.3 b	6124 a
P39-7-9-B	0.283 b	0.590 a	0.00	0.0 b	0.5 b	4.8 b	5960 ab
P53-30-21-B	0.058 d	0.195 c	0.00	0.0 b	0.8 b	7.1 b	5406 c
P99(N6)-4-14-B.....	0.377 a	0.528 a	0.00	0.0 b	0.3 b	2.4 b	6304 a
P53-28-B-B	0.050 d	0.194 c	0.00	1.0 b	2.8 b	49.6 b	5286 c
P39-8-B-B	0.199 c	0.334 b	0.00	0.8 b	4.5 b	60.4 b	5915 ab
LSD ($P \leq 0.05$)	*0.059*	*0.100*	*n.s.*	*3.9*	*4.7*	*162.2*	*423*
Wilson (non-transformed)	0.015 c	0.019 d	2.75 a	31.5 a	48.0 a	1273.8 a	3036 e
W14-10-2-B.....	0.269 a	0.441 a	0.25 b	2.0 c	9.0 d	139.3 d	4714 ab
W59-8-2-B.....	0.087 b	0.299 b	0.75 b	9.5 b	11.8 d	355.1 c	3313 de
W171-17-15-B.....	0.075 b	0.237 bc	0.50 b	13.5 b	23.5 b	554.0 b	4247 bc
W73-27-B-B.....	0.064 b	0.183 c	0.00 b	3.0 c	9.5 d	160.8 d	5138 a
W171-17-B-B.....	0.061 b	0.222 bc	0.50 b	10.5 b	18.0 c	431.3 bc	3999 dc
LSD ($P \leq 0.05$)	*0.038*	*0.101*	*1.01*	*5.9*	*5.2*	*180.1*	*699*

[z] Oxalate oxidase expression was performed with leaf samples from 10 plants per plot using a colormetric detection method to measure hydrogen peroxide released from oxalic acid substrate using a microtiter plate reader at 550 nm (Livingston, et al. 2005 Plant Phys. 137:1354). [y] Counts of infection centers in the two rows of each plot or a total of 60 ft of row. An infection center was a point of active growth by *Sclerotinia minor* and included 6 in. on either side of that point. AUDPC is Area Under Disease Progress Curve from 28 July to 9 October. [x] Yields are weights of peanuts with 7% moisture. Peanuts were dug on 19 October and harvested on 31 October. [w] Means in each parent group and column followed by the same letter(s) are not significantly different (LSD, P=0.05). Data previously published (Partridge et al. 2007. PDMR Report 1:FC004. DOI: 10.1094/PDMR01).

Table 13. Oxalate oxidase expression, disease resistance, and yield of non-transformed parent lines and T_5 transgenic lines with the barley oxalate oxidase gene in Virginia, 2007.

Variables	Oxalate oxidase expression [z]		Sclerotinia blight [y]			AUDPC[x]	Yield [w] (lb/A)
	26 Jul	18 Sep	3 Sep	23 Sep	10 Oct		
Parent and T_5 line							
NC7 (non-transformed)	0.009 d	0.011 d	1.4 a	6.6 a	8.9 a	238 a	3959 c
N70-8-24-B-B	0.681 a	0.733 a	0.0 b	0.0 d	0.0 b	3 b	4209 bc
N70-6-B-B-B	0.230 c	0.283 c	0.4 b	0.5 cd	0.5 b	21 b	4209 bc
Wilson (non-transformed)	0.011 d	0.017 d	1.4 a	4.9 ab	6.4 a	185 a	4467 bc
W14-10-2-B-B	0.559 b	0.585 b	0.1 b	0.0 d	0.0 b	2 b	4842 ab
W73-27-B-B-B	0.189 c	0.284 c	0.0 b	0.0 d	0.0 b	0 b	5136 a
Perry (non-transformed)	0.010 d	0.007 d	0.5 b	3.5 bc	6.4 a	135 a	4584 a-c
P39-7-9-B-B	0.579 b	0.613 ab	0.0 b	0.0 d	0.0 b	0 b	4525 a-c
P53-30-21-B-B	0.186 c	0.227 c	0.0 b	0.1 d	0.4 b	8 b	4548 a-c
LSD	*0.087*	*0.125*	*0.8*	*3.0*	*4.6*	*111*	*635*
Fungicide							
Non-treated control	na	na	0.6	2.4	3.4	89	4418
Omega 500 l pt/A	na	na	0.3	1.1	1.6	43	4577
LSD	na	na	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Split-plot analysis (*P* value)							
Cultivar	na	na	0.0003	0.0001	0.0002	0.0001	0.0236
Fungicide	na	na	0.2908	0.1976	0.1889	0.2380	0.8074
Fungicide x cultivar	na	na	0.0724	0.3343	0.5755	0.3740	0.5492

[z] Oxalate oxidase expression was determined by assay of leaflets from 10 plants/plot in non-treated main plots using a colormetric detection method to measure hydrogen peroxide released from oxalic acid substrate using a microtiter plate reader at 550 nm (Livingston, *et al*. 2005 Plant Phys. 137:1354).
[y] Counts of diseased plants in two 30-ft rows/plot on each date. [x]AUDPC is area under disease progress curve from July to harvest. [w]Yields are weight of peanuts with 7% moisture. Peanuts were dug in Virginia on 17 October and harvested on 23-24 October. Means in each parent group and column followed by the same letter(s) are not significantly different according to Fisher's Protected LSD (LSD, *P*=0.05); n.s. denotes differences within a group are not significant; na denotes not applicable because oxalate oxidase expression was measured only in non-treated plots. Data previously published (Hu et al. 2008. PDMR Report 1:FC093. DOI: 10.1094/PDMR02).

Table 14. Oxalate oxidase expression, disease resistance and yield of non-transformed parents and T_5 transgenic lines with the barley oxalate oxidase gene in North Carolina, 2007.

Variables	Oxalate oxidase expression [z] 21 Jul	Sclerotinia blight [y] 31 Aug	14 Sep	15 Oct	AUDPC[x]	Yield [w] (lb/A)
Parent and T_5 line						
NC7 (non-transformed)	0.010 f	0.5	0.8	12.8 a	220 a	3447 a
N70-8-24-B-B	0.560 a	0.0	0.0	0.3 c	4 c	2647 bc
N70-6-B-B-B	0.163 de	0.3	0.4	5.0 bc	89 bc	2991 ab
Wilson (non-transformed)	0.006 f	0.0	0.0	12.4 ab	186 ab	3403 a
W14-10-2-B-B	0.334 b	0.1	0.1	0.9 c	19 c	2498 cd
W73-27-B-B-B	0.239 cd	0.1	0.3	0.9 c	22 c	3403 a
Perry (non-transformed)	0.009 f	0.0	0.0	8.4 ab	126 a-c	3148 a
P39-7-9-B-B	0.272 bc	0.1	0.1	0.5 c	12 c	2154 d
P53-30-21-B-B	0.095 e	0.0	0.0	0.6 c	9 c	3507 a
LSD	*0.083*	*n.s.*	*n.s.*	*7.4*	*123*	*485*
Fungicide						
Non-treated control	na	0.1	0.1	3.9	63	3070
Omega 500 l pt/A	na	0.2	0.2	5.3	90	3076
LSD	na	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Split-plot analysis						
Cultivar	na	0.6920	0.2974	0.0006	0.0010	0.0001
Fungicide	na	0.1942	0.0577	0.3772	0.3131	0.9049
Fungicide x cultivar	na	*0.4431*	*0.6963*	*0.5231*	*0.5893*	*0.8432*

[z] Oxalate oxidase expression was determined by assay of leaflets from 10 plants/plot in non-treated main plots using a colorimetric detection method to measure hydrogen peroxide released from oxalic acid substrate using a micro titer plate reader at 550 nm (Livingston, *et al*. 2005 Plant Phys. 137:1354). [y] Counts of diseased plants in two 30-ft rows/plot on each date. [x]AUDPC is area under disease progress curve from Jul to harvest. [w]Yields are weight of peanuts with 7% moisture. Peanuts were dug in Virginia and North Carolina on 17 Oct and harvested on 23-24 Oct. Means in each parent group and column followed by the same letter(s) are not significantly different according to Fisher's Protected LSD (LSD, *P*=0.05); n.s. denotes differences within a group are not significant; na denotes not applicable because oxalate oxidase expression was measured only in non-treated plots. Data previously published (Hu et al. 2008. PDMR Report 1:FC093. DOI: 10.1094/PDMR02).

Effects on Other Diseases

The incidence of other diseases in plots was recorded each year in field trials of transgenic and non-transgenic plants. Diseases present included tomato spotted wilt virus (TSWV), Cylindrocladium black rot (CRB), web blotch, early leaf spot and Sclerotinia blight. The transgenic lines did not exhibit increased levels of susceptibility or resistance of concern or value in control of diseases other than Sclerotinia blight compared to their corresponding non-transgenic parent line (Table15 and Table 16). Data in this section has been previously published by Partridge et al. (2007) and Hu et al. (2008).

Table 15. Susceptibility of non-transformed and T_5 transgenic lines with the barley oxalate oxidase gene to common peanut diseases in Virginia, 2006.

Parent and T_5 lines	Oxalate oxidase expression[z] (19 Sep)	TSWV[y] (23 Aug)	% early leaf spot[x] (4 Oct)	% web blotch[x] (4 Oct)	% defoliation[w] (4 Oct)	CBR[v] (4 Oct)	Southern stem rot[u] (4 Oct)	Sclerotinia blight[t] AUDPC	Yield[s] (lb/A)
Wilson (non-transformed)	0.026 b	5.25	42.5	75.0	40.0 b	2.8 b	0.5	571 a	4192
W73-27-B-B.....	0.173 a	3.50	47.5	72.5	72.5 a	2.8 b	0.0	88 b	4521
W171-17-B-B.....	0.147 a	4.00	33.8	81.3	63.8 a	7.8 a	0.0	155 b	4179
LSD.....	*0.069*	*n.s*	*n.s.*	*n.s.*	*9.2*	*3.4*	*n.s.*	*176*	*n.s.*
Perry (non-transformed)	0.019 c	5.75	52.5	56.3	20.0 b	2.0 b	0.0	384 a	4642 a
P53-28-B-B	0.162 b	4.25	52.5	63.8	68.8 a	5.5 a	1.0	58 b	3814 b
P39-8-B-B	0.389 a	5.25	53.8	43.8	25.0 b	2.0 b	0.0	57 b	4703 a
LSD.....	*0.109*	*n.s.*	*n.s.*	*n.s.*	*15.7*	*2.2*	*n.s.*	*188*	*212*
NC 7 (non-transformed)	0.021 b	7.25	38.8	63.8	36.3	5.8	0.3	809 a	4021 b
N70-8-B-B.....	0.247 a	6.25	42.5	71.3	38.8	5.0	0.0	115 b	4703 a
N70-6-B-B.....	0.175 a	4.75	47.5	68.8	32.5	5.8	0.0	88 b	4630 a
LSD.....	*0.112*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*418*	*457*

[z] Oxalate oxidase expression was measured in leaf samples from 8 plants/plot using a colorimetric detection method to measure hydrogen peroxide released from oxalic acid substrate using a microtiter plate reader at 550 nm (Livingston, et al. 2005 Plant Phys. 137:1354). [y]Counts of plants per plot with symptoms. [x] Leaf spot/web blotch rating scale 0= none, 100= spots or blotches on all leaflets. [w] Defoliation rating scale: 0=none, 100= no leaves on plants. [v]Number of plants with symptoms and signs of Cylindrocladium black rot (CBR). [u]Counts of infection centers/plot with symptoms and signs of southern stem rot. An infection center was a point of active fungal growth and included 6 in. on either side of that point. [u] Number of infection centers/plot with symptoms and signs of Sclerotinia blight from 14 July to 4 October were used to calculated Area Under Disease Progress Curve (AUDPC). [s] Yields are weight of peanuts with 7% moisture. Peanuts were dug on 4 October and harvested on 12 October. Means in each parent group and column followed by the same letter(s) are not significantly different (LSD, *P*=0.05); n.s. denotes differences within a group are not significant. Data previously published (Partridge et al. 2007. PDMR Report 1:FC035. DOI: 10.1094/PDMR01).

Table 16. Susceptibility of non-transformed and T_5 transgenic lines with the barley oxalate oxidase gene to common peanut diseases in Virginia, 2007.

Variables	Oxalate oxidase expression[z] (18 Sep)	TSWV[y] (14 Aug)	% early leaf spot[x] (10 Oct)	% web blotch[x] (10 Oct)	% defoliation[w] (10 Oct)	CBR[v] (3 Sep)	Sclerotinia blight AUDPC[u]	Yield[t] (lb/A)
Parent and T_5 line								
NC 7 (non-transformed)	0.010 e [s]	4.25	43.38	0.38	18.00	1.75	81	3936
N70-8-24-B-B	0.778 a	2.00	44.63	0.50	25.00	0.38	4	4547
N70-6-B-B-B	0.394 c	3.13	40.88	0.25	16.63	0.50	11	4113
Wilson (non-transformed)	0.009 e	2.38	44.13	0.75	18.50	1.13	41	4343
W14-10-2-B-B	0.588 b	2.25	40.25	2.00	16.13	0.75	1	4255
W73-27-B-B-B	0.245 d	2.50	45.00	2.50	19.75	1.13	12	4520
Perry (non-transformed)	0.011 e	1.50	45.25	0.38	18.75	0.63	95	4741
P39-7-9-B-B	0.804 a	1.88	47.38	0.25	18.63	1.25	2	4503
P53-30-21-B-B	0.148 d	2.38	46.75	2.00	18.63	0.75	4	4379
LSD	*0.112*	*n.s.*	*n.s.*	*n.s.*	--	*n.s.*	--	--
Fungicide								
Non-treated control	na	2.89	82.36 a	1.556	36.11	0.72	14	4083
Treated with Bravo Weather Stik	na	2.06	6.00 b	0.444	1.67	1.11	42	4659
LSD	na	*n.s.*	*2.30*	*n.s.*	--	*n.s.*	--	--
Split-plot analysis								
Cultivar	na	0.0886	0.0737	0.6732	0.0019	0.2153	0.0001	0.0006
Fungicide	na	0.5379	0.0001	0.1086	0.0009	0.1098	0.2553	0.0257
Fungicide x Cultivar	na	0.1424	0.1049	0.8738	0.0100	0.3846	0.0255	0.0030

[z] Oxalate oxidase expression was measured in leaf samples from 10 plants/plot using a colormetric detection method to measure hydrogen peroxide released from oxalic acid substrate using a microtiter plate reader at 550 nm (Livingston, et al. 2005 Plant Phys. 137:1354). [y] Counts of plants/plot with symptoms. [x] Leaf spot/web blotch rating scale 0= none, 100= spots or blotches on all leaflets. [w] Defoliation rating scale: 0=none, 100= no leaves on plants. [v] Number of symptomatic and/or dead plants/plot. [u] AUDPC is area under disease progress curve from 14 Jul to 10 Oct. [t] Yields are weight of peanuts with 7% moisture. Peanuts were dug on 17 Oct and harvested on 2 Nov. [s] Means in each parent group and column followed by the same letter(s) are not significantly different (LSD, P=0.05); n.s. denotes not significant; -- denotes combined analysis not valid due to significant interaction between fungicide and cultivar; na denotes not applicable because oxalate oxidase expression was measured only in non-treated plots. Data previously published (Hu et al. 2008. PDMR Report 1:FC094. DOI: 10.1094/PDMR02).

Quality Assessment

The quality of transgenic peanuts was compared with that of non-transgenic peanuts. The differences can be attributed to a significant reduction of Sclerotinia blight in the transgenic plants. Quality was assessed by grading (Table 17 to Table 20), assessing pod brightness (Table 21 to Table 24), and blanching (Table 25 to Table 30). The difference in value is due to the increased yield under disease pressure from Sclerotinia blight (Table 17 to Table 19). In the absence of Sclerotinia blight, there were no differences in the value between transgenic and non-transgenic lines (Table 20).

Table 17. Grade characteristics, yield and value of non-transformed parent lines and T_3 transgenic peanut lines containing barley oxalate oxidase gene in Virginia, 2005.

Parent and T_3 lines		%*								Total kernels	¢/lb	Yield (lb/A)	Value ($/A)
		Jumbo	Fancy	Moisture	ELK	SS	OK	DK	SMK				
NC 7	non-transformed	69.0 a	88.8	6.10	52.8	2.6	1.9	3.9	64.5	72.8	16.35	2619	428
	N70-8-B	69.3 a	91.0	5.90	51.8	1.9	1.7	4.9	63.5	72.0	14.97	3661	559
	N70-6-B	59.3 b	91.0	5.85	53.8	2.6	1.3	3.4	66.8	74.1	17.34	3725	650
	N99(P60)-4-B	66.8 a	89.5	6.18	55.3	1.6	1.1	3.6	66.3	72.6	17.00	3683	625
	LSD	*5.7*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	non-transformed	35.5 a	78.5 a-c	6.05 cd	53.8 a	2.6 ab	1.2 c	2.1 a-c	69.7 ab	75.7 a	18.34 a-c	3711 de	683 d
	P53-26-B	7.0 e	50.3 e	6.15 bc	38.0 d	2.7 a	1.1 c	1.4 b-d	68.6 bc	73.7 b	18.23 a-d	3732 de	680 d
	P53-4-B	26.0 c	78.8 ab	6.18 a-c	46.5 bc	1.7 b-d	1.2 c	0.7 d	69.6 ab	73.2 bc	18.46 a-c	4368 ab	806 a-c
	P53-30-B	19.8 d	76.3 bc	6.23 a-c	43.5 c	1.5 cd	1.3 bc	1.0 cd	68.5 bc	72.3 cd	18.10 a-d	3825 cd	692 d
	P53-28-B	27.3 bc	77.8 a-c	6.40 a	43.8 c	1.3 d	1.2 c	1.2 b-d	68.8 b	72.4 cd	18.06 a-d	4069 b-d	735 cd
	P53-27-B	26.3 bc	79.8 ab	6.33 ab	45.8 bc	1.7 a-d	1.2 c	1.6 b-d	67.7 bc	72.2 d	17.93 b-d	4147 a-c	743 b-d
	P39-7-B	23.0 cd	76.5 bc	6.25 a-c	47.0 bc	1.6 cd	1.3 c	1.1 b-d	71.7 a	75.6 a	18.91 ab	4503 a	851 a
	P39-8-B	10.3 e	59.5 d	6.15 bc	43.5 c	2.7 a	1.3 c	0.8 d	71.5 a	76.2 a	19.12 a	4332 ab	828 ab
	P97(N6)-2-B	21.5 cd	73.3 c	5.88 de	47.0 bc	1.9 a-d	1.3 bc	3.1 a	67.7 bc	74.0 b	17.43 cd	4047 b-d	706 d
	P98(N6)-1-B	36.5 a	82.8 a	5.75 e	48.8 b	1.8 a-d	1.7 ab	2.3 ab	68.1 bc	73.9 b	17.98 b-d	3997 b-d	720 cd
	P99(N6)-4-B	32.3 ab	80.5 ab	5.88 de	46.8 bc	2.4 a-c	1.9 a	2.9 a	66.4 c	73.5 b	17.18 d	3418 e	585 e
	LSD	*6.1*	*5.3*	*0.24*	*3.7*	*0.9*	*0.43*	*1.2*	*2.3*	*0.9*	*1.07*	*402*	89
Wilson	non-transformed	43.8 a	85.5 ab	6.05 c	43.5 ab	2.2 a	1.5 ab	3.9	62.1	69.6 bc	15.23	3166 b	478 b
	W73-27-B	45.0 a	89.3 a	6.05 c	46.3 a	1.6 a-c	0.9 c	2.1	66.4	71.0 ab	15.23	4291 a	730 a
	W171-17-B	33.3 bc	85.0 ab	6.08 bc	44.0 ab	1.4 a-c	1.0 bc	2.6	66.5	71.4 a	17.03	4140 a	710 a
	W83-7-B	12.8 d	69.5 c	6.38 ab	33.0 d	1.2 bc	1.6 ab	2.0	66.6	71.4 a	17.26	2889 b	498 b
	W59-10-B	39.5 ab	86.8 ab	6.63 a	38.5 c	0.9 c	1.6 ab	1.8	65.4	69.7 bc	17.03	2903 b	496 b
	W59-11-B	35.0 b	83.3 b	6.08 bc	40.5 bc	1.1 c	1.7 a	4.2	62.3	69.2 c	15.08	3187 b	480 b
	W51-9-B	25.3 c	74.3 c	6.55 a	38.3 c	1.8 ab	1.8 a	2.6	65.2	71.5 a	17.06	2654 b	454 b
	LSD	*8.5*	*5.9*	*0.31*	*3.7*	*0.8*	*0.6*	*n.s.*	*n.s.*	*1.7*	*n.s.*	*636*	*169*

*FAN=fancy sized in-shell, ELK=extra large kernels, SS=sound splits, OK=other kernels, DK=damaged kernels, SMK=sound mature kernels. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 18. Grade characteristics, yield and value of non-transformed parent lines and T_4 transgenic lines containing barley oxalate oxidase gene in Virginia, 2006.*

Parent and T_4 lines		% LSK	FM	Jumbo	Fancy	Moisture	ELK	SS	OK	DK	SMK	Total kernels	¢/lb	Yield (lb/A)	Value ($/A)
NC 7	non-transformed	0.03	0.23	66.8 b	88.5	7.91	56.3	1.3	1.2	0.3	69.4	72.2	18.44	3296 c	610 c
	N70-8-24-B	0.05	0.33	69.8 b	89.3	7.95	55.0	0.9	1.1	0.1	70.8	72.8	18.63	5518 a	1032 a
	N99(P60)-29-10-B	0.10	0.30	58.3 c	87.8	7.98	55.3	0.7	1.5	0.1	70.6	72.8	18.56	5009 b	930 b
	N70-8-B-B	0.03	0.40	74.5 a	90.3	8.22	57.3	0.5	0.9	0.2	70.4	71.8	18.45	4966 b	918 b
	N70-6-B-B	0.08	0.38	61.3 c	87.5	8.17	51.8	1.1	1.3	0.2	69.4	71.9	18.30	4675 b	856 b
	LSD	*n.s.*	*n.s.*	*4.3*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*455*	*94*
Perry	non-transformed	0.03	0.20	33.5 a	79.3 ab	7.30	53.0 a	1.9	1.1	0.0	71.1 a	74.1 a	18.93 a	5645 bc	1068 bc
	P98(N6)-1-10-B	0.00	0.35	32.3 a	77.8 a-c	7.46	51.5 ab	1.9	1.7	0.2	70.2 ab	74.0 a	18.73 ab	5885 ab	1102 ab
	P97(N6)-2-8-B	0.05	0.25	17.3 b	74.0 bc	7.58	52.3 a	1.8	1.5	0.0	71.0 a	74.3 a	18.88 a	6124 a	1158 a
	P39-7-9-B	0.08	0.33	14.5 bc	72.5 c	7.56	47.5 cd	1.2	1.6	0.0	71.9 a	74.6 a	18.88 a	5960 ab	1125 ab
	P53-30-21-B	0.03	0.23	9.5 c	72.0 cd	8.05	50.8 a-c	1.7	1.2	0.0	68.9 b	71.7 b	18.29 bc	5406 c	990 cd
	P99(N6)-4-14-B	0.03	0.33	31.3 a	81.3 a	8.06	52.8 a	1.3	1.6	0.2	71.0 a	74.1 a	18.79 a	6304 a	1186 a
	P53-28-B-B	0.03	0.20	17.8 b	76.0 a-c	7.72	46.0 d	1.3	1.3	0.0	69.1 b	71.8 b	18.19 c	5286 c	962 d
	P39-8-B-B	0.00	0.18	10.5 c	66.0 d	7.27	48.8 b-d	1.5	1.5	0.1	72.0 a	75.1 a	18.99 a	5915 ab	1124 ab
	LSD	*n.s.*	*n.s.*	*5.3*	*6.1*	*n.s.*	*3.4*	*n.s.*	*n.s.*	*n.s.*	*1.8*	*1.5*	*0.45*	*423*	*84*
Wilson	non-transformed	0.05	0.68	31.5 a	83.8 a	7.64	40.3	1.0	2.0	1.9	64.2	69.1	16.77	3036 e	510 d
	W14-10-2-B	0.05	0.85	11.5 b	76.8 b	7.92	38.3	0.7	2.4	1.1	67.8	72.0	17.66	4715 ab	829 ab
	W59-8-2-B	0.13	0.95	35.8 a	86.5 a	7.40	41.0	1.2	1.5	0.9	64.5	68.2	16.97	3314 de	562 d
	W171-17-15-B	0.08	1.33	13.5 b	75.8 b	7.71	41.5	1.2	1.7	1.4	66.1	70.5	17.29	4247 bc	734 bc
	W73-27-B-B	0.08	0.63	19.3 b	83.0 a	7.65	44.0	1.0	1.6	1.3	66.6	70.5	17.43	5137 a	896 a
	W171-17-B-B	0.05	0.53	11.8 b	73.8 b	7.84	40.8	1.1	1.9	1.7	65.6	70.3	17.13	4000 d	685 c
	LSD	*n.s.*	*n.s.*	*8.8*	*5.2*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*699*	*108*

* FAN=fancy sized in-shell, ELK=extra large kernels, SS=sound splits, OK=other kernels, DK=damaged kernels, SMK=sound mature kernels. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 19. Grade characteristics, yield and value of non-transformed parent lines and T_5 transgenic peanut lines containing barley oxalate oxidase gene in North Carolina trial, 2007.*

Treatment, parent and T_5 line	%										Total Kernels	¢/lb	Yield (lb/A)	Value ($/A)
	LSK	FM	Jumbo	Fancy	Moisture	ELK	SS	OK	DK	SMK				
Non-treated														
NC7 (non-transformed)..	6.0	2.2	53.5	31.5	7.1	50.0 a	3.8	0.9	0.6	65.8	71.0	17.1	3336 a	571 a
N70-8-24-B-B.....	5.3	2.4	67.0	23.3	6.5	43.0 b	3.1	0.8	0.4	64.8	69.1	16.5	2737 b	451 b
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*5.4*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*596*	*88*
Perry (non-transformed)	3.7	2.2	42.0	41.5	6.6	39.8 a	2.6	1.3	0.4	64.8 a	69.0 a	16.9	3141 a	527 a
P39-7-9-B-B.....	5.5	6.1	26.3	49.5	6.5	22.8 b	2.1	2.3	0.8	58.5 b	63.6 b	15.5	1496 b	235 b
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*6.1*	*n.s.*	*n.s.*	*n.s.*	*4.1*	*5.2*	*n.s.*	*564*	*73*
Wilson (non-transformed).....	3.6	2.0	42.8	40.8	7.5	26.1	3.4 a	1.1	0.9	63.0	68.4 a	16.8	3410	573
W73-27-B-B-B	2.2	1.3	52.0	35.5	6.8	30.5	2.5 b	1.3	0.5	62.3	66.5 b	16.2	3365	547
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.4*	*n.s.*	*n.s.*	*n.s.*	*0.4*	*n.s.*	*n.s.*	*n.s.*
Treated with Omega 500														
NC7 (non-transformed)...	4.9	2.1	56.0	31.8	7.6	47.0	4.0	1.0	0.4	64.6	70.0	16.7	3560	593
N70-8-24-B-B.....	5.3	2.5	61.0	28.0	7.6	45.5	3.3	0.6	0.9	65.8	70.5	16.7	2557	427
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed) .	3.8	2.3	42.3	40.5	7.6	43.3 a	2.0	1.1	0.8	66.4 a	70.3 a	17.5	3156 a	553 a
P39-7-9-B-B.....	3.3	4.5	30.0	43.3	7.7	25.5 b	2.5	1.7	0.4	60.8 b	65.3 b	16.0	1765 b	284 b
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*7.9*	*n.s.*	*n.s.*	*n.s.*	*3.6*	*3.2*	*n.s.*	*927*	*189*
Wilson (non-transformed).....	2.2	1.4	41.3	41.8	7.3	32.0	2.7	1.1	0.6	62.9	67.3	16.8	3395	572
W73-27-B-B-B	2.8	1.3	49.0	38.0	7.7	33.5	2.4	1.0	0.6	62.6	66.6	16.3	3440	562
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*FAN=fancy sized in-shell, ELK=extra large kernels, SS=sound splits, OK=other kernels, DK=damaged kernels, SMK=sound mature kernels. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

Table 20. Grade characteristics, yield and value of non-transformed parent lines and T_5 transgenic peanut lines containing barley oxalate oxidase gene in Virginia trial, 2007.*

Treatment, parent and T_5 line	% LSK	FM	Jumbo	Fancy	Moisture	ELK	SS	OK	DK	SMK	Total kernels	¢/lb	Yield (lb/A)	Value ($/A)
Non-treated														
NC7 (non-transformed)....	0.3	0.8	62.3 b	24.5 a	7.6	47.0	2.3	1.4	0.8	65.8	70.1 b	16.5	3532	583
N70-8-24-B	0.4	1.4	74.5 a	17.5 b	7.6	52.8	2.8	1.1	0.6	67.5	72.0 a	17.0	3944	672
LSD.....	*n.s.*	*n.s.*	*9.9*	*6.8*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*1.4*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)...	0.3	1.7	38.5	41.8 b	7.8	49.3	3.3	1.4	0.8	67.0	72.5	18.0	4460	801
P39-7-9-B.....	0.2	1.5	28.8	51.0 a	7.7	44.5	3.8	1.4	0.8	67.3	73.3	18.3	4533	831
LSD.....	n.s.	n.s.	n.s.	4.6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*n.s.*	*n.s.*	*n.s.*
Wilson (non-transformed)	0.1	0.5	48.5	39.0	7.8	43.3	2.6	1.5	0.9	64.3	69.2	17.1	4238	727
W73-27-B-B.....	0.2	0.7	47.0	38.0	7.8	34.8	2.3	1.5	1.0	65.5	70.3	16.7	5151	862
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*		*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Treated with Omega 500														
NC7 (non-transformed)	0.2	1.0	64.3 b	22.8	7.8	50.5	2.8	1.3	0.5	66.8	71.3	16.9	4385	739
N70-8-24-B	0.5	0.7	71.5 a	18.8	7.8	54.0	2.3	1.0	1.1	67.6	72.0	16.9	4473	760
LSD.....	*n.s.*	*n.s.*	*6.3*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)	0.3	1.6	45.5 a	34.3 b	7.8	49.0	1.5	2.0	1.3	68.0	72.8	*17.1 b*	*4710*	804
P39-7-9-B.....	0.5	0.8	26.8 b	51.5 a	7.6	36.0	2.8	0.9	0.6	69.3	73.5	18.6 a	4518	834
LSD.....	*n.s.*	*n.s.*	*12.6*	*9.4*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.4*	*n.s.*	*n.s.*
Wilson (non-transformed).	0.1	1.1	47.3	36.3	7.8	41.5	2.8	1.8	1.3	62.5	68.3	16.4	4695	773
W73-27-B-B.....	0.1	0.5	45.5	41.3	7.8	45.5	2.3	1.5	0.7	65.0	69.4	16.9	5121	877
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

* FAN=fancy sized in-shell, ELK=extra large kernels, SS=sound splits, OK=other kernels, DK=damaged kernels, SMK=sound mature kernels. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

Table 21. Pod brightness of non-transformed parent lines and T_3 transgenic peanut lines containing barley oxalate oxidase gene in Virginia, 2005.*

		Jumbo pod color			Fancy pod color		
Parent	Parent and T_3 line	L score	a score	b score	L score	a score	b score
NC 7	non-transformed	45.71	3.07	14.00	43.42	2.69	12.88
	N70-8-B	45.56	3.10	13.98	43.38	2.88	12.74
	N70-6-B	46.30	3.10	14.08	43.33	3.28	12.71
	N99(P60)-4-B	45.25	2.98	13.91	43.96	3.11	13.07
	LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	non-transformed	49.33 a	2.70 bc	15.44 a	46.04 c-e	2.60 bc	13.97 cd
	P53-26-B	44.17 d	2.22 c	13.06 e	49.17 a	2.99 ab	15.46 a
	P53-4-B	47.50 a-c	3.31 a	15.10 a-c	47.70 a-c	3.06 ab	15.05 ab
	P53-30-B	48.59 ab	2.69 bc	15.06 a-d	47.35 b-d	2.50 c	14.48 bc
	P53-28-B	48.38 a-c	2.64 bc	15.33 a	47.86 ab	2.44 c	14.81 a-c
	P53-27-B	48.35 a-c	2.60 bc	15.28 ab	47.70 ab	2.75 a-c	14.92 ab
	P39-7-B	47.80 a-c	2.58 bc	14.86 a-d	47.31 b-d	2.40 c	14.66 a-c
	P39-8-B	46.36 c	2.15 c	14.19 d	47.21 b-d	2.85 a-c	14.33 b-d
	P97(N6)-2-B	46.86 bc	3.12 c	14.38 b-c	45.94 de	3.01 ab	14.18 b-d
	P98(N6)-1-B	47.04 bc	3.03 ab	14.23 cd	43.90 f	3.11 a	12.93 e
	P99(N6)-4-B	46.56 bc	3.04 ab	14.42 b-d	45.08 ef	2.99 ab	13.49 de
	LSD	*2.15*	*0.56*	*0.19*	*1.73*	*0.48*	*0.80*
Wilson	non-transformed	49.20	2.46	15.04	47.46	2.60	14.20
	W73-27-B	48.76	2.80	14.87	48.02	2.63	14.50
	W171-17-B	47.94	2.82	14.35	48.18	2.63	14.47
	W83-7-B	45.96	1.88	13.45	47.57	2.09	14.07
	W59-10-B	47.46	2.70	14.47	46.95	2.49	14.39
	W59-11-B	46.30	3.16	14.43	46.19	2.97	13.92
	W51-9-B	48.35	2.15	14.67	47.14	2.27	14.08
	LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*Pod color was measured using a Hunterlab D25 colorimeter equipped with the D25-2RL reduced area viewing for L optial sensor (51-mm diameter sample area).L score measured brightness with a score of 0 indicating complete blackness and 100 indicating perfect whiteness; a score measured color on a red green scale with positives scores indicating redness and negative scores indicating greenness; b score measured color on a blue-yellow scale with positive scores of greater magnitude indicating more intense yellow color (Billmeyer and Saltzmann, 1981). Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 22. Pod brightness of non-transformed parent lines and T_4 transgenic peanut lines containing barley oxalate oxidase gene in Virginia, 2006.*

Parent and T_4 line		Jumbo pod color			Fancy pod color		
		L score	a score	b score	L score	a score	b score
NC 7	non-transformed	44.48	2.91 bc	13.26	40.38	2.71	11.34 a
	N70-8-24-B	43.46	2.64 c	12.67	38.58	2.73	10.53 bc
	N99(P60)-29-10-B ...	43.11	3.18 a	12.76	39.44	2.51	10.53 bc
	N70-8-B-B.....	42.66	2.83 bc	12.42	37.95	2.65	10.14 c
	N70-6-B-B.....	42.62	2.92 ab	12.45	39.64	2.82	11.02 ab
	LSD.....	*n.s.*	*0.27*	*n.s.*	*n.s.*	*n.s.*	*0.76*
Perry	non-transformed	44.96	2.72	13.59	44.96 bc	2.61	13.47
	P98(N6)-1-10-B	44.18	2.91	13.51	43.95 c	3.11	13.34
	P97(N6)-2-8-B	46.06	3.00	14.24	45.08 a-c	2.97	13.71
	P39-7-9-B.....	44.71	3.05	13.88	45.94 ab	2.92	14.21
	P53-30-21-B.....	44.27	2.90	13.80	46.57 a	3.10	14.32
	P99(N6)-4-14-B	44.59	3.26	13.58	43.85 c	2.98	13.33
	P53-28-B-B	45.02	3.05	13.96	45.94 ab	2.85	14.30
	P39-8-B-B	44.49	2.80	13.66	45.81 ab	2.82	14.10
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*1.58*	*n.s.*	*n.s.*
Wilson	non-transformed	46.22 a	2.75	13.97 a	46.48 a	2.69	13.78
	W14-10-2-B	41.58 c	2.84	11.75 b	41.96 b	2.60	12.05
	W59-8-2-B	43.76 b	3.03	13.18 a	43.17 b	2.76	12.81
	W171-17-15-B	44.89 ab	2.69	13.29 a	43.89 b	2.74	12.88
	W73-27-B-B.....	44.45 b	2.70	13.23 a	43.59 b	2.42	12.55
	W171-17-B-B.....	44.83 ab	2.83	13.54 a	44.25 ab	2.75	13.15
	LSD.....	*1.40*	*n.s.*	*0.94*	*2.44*	*n.s.*	*n.s.*

* Pod color was measured using a Hunterlab D25-PC2 colorimeter equipped with the D25-2RL reduced area viewing for L optical sensor (51-mm diameter sample area).L score measured brightness with a score of 0 indicating complete blackness and 100 indicating perfect whiteness; a score measured color on a red green scale with positives scores indicating redness and negative scores indicating greenness; b score measured color on a blue-yellow scale with positive scores of greater magnitude indicating more intense yellow color (Billmeyer and Saltzmann, 1981). Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 23. Pod brightness of non-transformed parent lines and T_5 transgenic peanut lines containing barley oxalate oxidase gene in North Carolina, 2007.*

Treatment	Parent and T_5 line	Jumbo pod color			Fancy pod color		
		L score	a score	b score	L score	a score	b score
Non-treated							
	NC7 (non-transformed).....	43.3	3.3	15.5	41.7	3.2	14.7
	N70-8-24-B.....	43.4	3.6	15.5	41.2	3.6	14.8
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Perry (non-transformed).....	44.3	3.6 a	16.2	44.2	3.4	16.3
	P39-7-9-B	42.1	3.3 b	15.1	42.3	3.4	15.4
	LSD.....	*n.s.*	*0.2*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Wilson (non-transformed)...	44.8	3.1	16.0	43.4	3.1	15.5
	W73-27-B-B	44.7	3.3	16.6	44.3	3.0	16.5
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Treated with Omega 500							
	NC7 (non-transformed)	45.3	3.3	16.6	43.5 a	3.5	16.2 a
	N70-8-24-B.....	43.1	3.4	15.6	41.7 b	3.5	15.1 b
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*1.1*	*n.s.*	*0.6*
	Perry (non-transformed).....	45.5	3.5	17.1	46.0	3.4	17.1
	P39-7-9-B	43.0	3.3	15.6	42.7	3.3	15.4
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Wilson (non-transformed)...	45.9	3.3	16.9	45.5	3.3	16.9
	W73-27-B-B	46.5	3.2	17.6	45.8	2.7	17.2
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

* Pod color was measured using a Hunterlab D25 colorimeter equipped with the D25-2RL reduced area viewing for L optical sensor (51-mm diameter sample area).L score measured brightness with a score of 0 indicating complete blackness and 100 indicating perfect whiteness; a score measured color on a red green scale with positives scores indicating redness and negative scores indicating greenness; b score measured color on a blue-yellow scale with positive scores of greater magnitude indicating more intense yellow color (Billmeyer and Saltzmann, 1981). Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

Table 24. Pod brightness of non-transformed parent lines and T_5 transgenic peanut lines containing barley oxalate oxidase gene in Virginia, 2007.*

Treatment	Parent and T_5 lines	Jumbo pod color			Fancy pod color		
		L score	a score	b score	L score	a score	b score
Non-treated							
	NC7 (non-transformed)	41.2	2.7	12.3	39.7	2.8	11.4
	N70-8-24-B-B.....	39.2	2.4	10.9	37.0	2.3	10.0
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Perry (non-transformed)	42.6	2.5	12.7	41.4	2.4	12.2
	P39-7-9-B-B	41.2	2.1	12.4	40.2	2.2	11.7
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Wilson (non-transformed) ..	43.2	2.2	12.7	41.3	2.3	11.9
	W73-27-B-B-B	40.9	2.1	11.7	39.9	2.2	11.0
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Treated with Omega 500							
	NC7 (non-transformed)	41.9	2.6	12.5	40.4	2.4	11.9
	N70-8-24-B-B.....	41.3	2.6	12.4	39.9	2.4	11.6
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Perry (non-transformed)	42.7	2.6 b	12.6	41.5	2.8	12.2
	P39-7-9-B-B	43.0	2.8 a	12.9	42.0	2.6	12.5
	LSD.....	*n.s.*	*0.1*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
	Wilson (non-transformed) ..	43.7	2.6	13.3	40.6	2.3	11.6
	W73-27-B-B-B	43.1	2.2	12.8	42.9	2.3	13.0
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

* Pod color was measured using a Hunterlab D25 colorimeter equipped with the D25-2RL reduced area viewing for L optical sensor (51-mm diameter sample area).L score measured brightness with a score of 0 indicating complete blackness and 100 indicating perfect whiteness; a score measured color on a red green scale with positives scores indicating redness and negative scores indicating greenness; b score measured color on a blue-yellow scale with positive scores of greater magnitude indicating more intense yellow color (Billmeyer and Saltzmann, 1981). Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

Table 25. Laboratory sample blanching of extra large kernels of non-transformed parent lines and T_3 seed containing the barley oxalate oxidase gene from Virginia, 2005.*

	Roasting			Blanched			
Parent and T_3 lines	% H_2O before	% H_2O after	% blanching loss	% splits	% whole	% not	% partial
NC 7 (non-transformed)	6.54	3.29	3.25	15.3	72.2	0.0	10.0
N70-8-B	6.60	3.17	3.43	11.6	79.4	0.0	6.2
N70-6-B	6.69	3.34	3.35	14.0	77.4	0.0	5.8
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)	6.56	3.51	3.05	16.1	71.0	0.0	10.5
P53-4-B	6.42	3.26	3.16	17.5	72.0	0.1	7.5
P53-30-B	6.42	3.30	3.13	17.9	72.6	0.0	7.1
P53-27-B	6.48	3.26	3.22	11.4	74.0	0.0	12.1
P39-7-B	6.32	3.40	2.93	14.7	71.2	0.0	11.8
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Wilson (non-transformed)	6.54	3.00 b	3.54	16.8	69.4	0.0	11.4
W73-27-B	6.60	3.24 ab	3.36	15.9	71.6	0.0	9.7
W171-17-B	6.77	3.20 ab	3.57	14.5	68.5	0.0	14.6
W59-11-B	6.76	3.49 a	3.27	14.8	74.8	0.0	7.9
LSD	*n.s.*	*0.32*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*After mechanically cutting each side of the peanut skin, 250 gram samples of peanuts were white roasted at 149°C for 15 min, cooled, and then blanched with air pressure at 27 PSI for 5.5 min in a rotating cylinder. The data represent means of four replicate samples. Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 26. Laboratory sample blanching of medium size kernels of non-transformed parent lines and T_3 seed containing the barley oxalate oxidase gene in Virginia, 2005.*

	Roasting			Blanched			
Parent and T_3 lines	% H_2O before	% H_2O after	% blanching loss	% splits	% whole	% not	% partial
NC 7 (non-transformed)	7.14	3.43	3.71	15.7	48.2	5.1	28.1
N70-8.....	6.20	3.36	2.84	8.6	59.2	8.4	20.8
N70-6.....	6.88	3.20	3.68	13.6	62.9	4.2	15.8
LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)	6.42 b	3.30	3.13 b	18.5	47.2 c	0.3	31.4 a
P53-4	6.75 a	3.40	3.35 b	21.8	56.9 ab	0.1	17.7 b
P53-30	6.77 a	3.40	3.38 b	17.3	62.4 a	0.4	16.9 b
P53-27	6.96 a	3.29	3.67 a	22.3	58.2 ab	0.5	14.6 b
P39-7	6.45 b	3.31	3.15 b	21.8	54.2 bc	0.0	21.1 b
LSD.....	*0.26*	*n.s.*	*0.27*	*n.s.*	*7.6*	*n.s.*	*8.1*
Wilson (non-transformed)	5.92 b	3.24	2.68	18.8	48.1 c	0.8 b	23.9 ab
W73-27.....	6.77 a	3.35	3.42	15.3	60.1 ab	1.2 b	20.3 bc
W171-17.....	6.95 a	3.46	3.49	13.5	54.1 bc	1.6 b	27.9 a
W59-11.....	6.83 a	3.25	3.58	13.5	63.7 a	3.0 a	16.3 c
LSD.....	*0.40*	*n.s.*	*n.s.*	*n.s.*	*9.1*	*1.3*	*5.9*

* After mechanically cutting each side of the peanut skin, 250 gram samples of peanuts were white roasted at 149°C for 17 min, cooled, and then blanched with air pressure at 27 PSI for 6 min in a rotating cylinder. The data represent means of four replicate samples. Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 27. Laboratory sample blanching of extra large kernels of non-transformed parent lines and T_4 transgenic lines containing the barley oxalate oxidase gene in Virginia, 2006.*

		Roasting		%	Blanched			
Parent	Parent and T_4 lines	% H_2O before	% H_2O after	blanching loss	% splits	% whole	% not	% partial
NC 7	NC7 (non-transformed).....	8.53	5.71	2.81	9.13	75.68	0.00	11.93
	N70-8-24-B.....	8.70	6.14	2.56	6.10	80.98	0.00	9.98
	N99(P60)-29-10-B.....	8.41	5.91	2.50	7.30	75.95	0.00	12.58
	N70-8-B-B.....	8.80	6.47	2.33	4.78	76.00	0.00	16.10
	N70-6-B-B.....	8.71	5.88	2.83	7.70	79.25	0.00	9.38
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	Perry (non-transformed).....	8.19	4.97	3.22	11.43	69.63	0.00 b	16.50
	P98(N6)-1-10-B.....	8.15	5.17	2.98	12.85	77.63	0.00 b	6.78
	P97(N6)-2-8-B.....	8.17	4.99	3.18	11.73	74.23	0.00 b	11.55
	P39-7-9-B.....	8.19	5.12	3.07	9.38	77.28	0.00 b	11.05
	P53-30-21-B.....	8.16	5.70	2.47	7.48	69.33	0.35 a	20.15
	P99(N6)-4-14-B.....	8.40	5.33	3.07	10.68	73.88	0.00 b	13.18
	P53-28-B-B.....	8.00	5.70	2.30	8.15	70.33	0.28 a	18.55
	P39-8-B-B.....	7.98	5.28	2.70	11.20	71.83	0.00 b	14.75
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.25*	*n.s.*
Wilson	Wilson (non-transformed)....	8.46	5.49	2.96	7.00	70.85	1.85	17.10
	W14-10-2-B.....	9.75	5.17	4.58	7.58	82.88	1.50	4.70
	W59-8-2-B.....	8.49	5.28	3.21	7.23	78.68	0.75	10.15
	W171-17-15-B.....	9.85	5.53	4.32	7.68	75.65	1.40	12.25
	W73-27-B-B.....	9.20	5.76	3.44	6.00	80.05	2.10	8.28
	W171-17-B-B.....	9.20	5.17	4.03	8.83	77.75	1.18	8.83
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*After mechanically cutting each side of the peanut skin, 250 gram samples of peanuts were white roasted at 149°C for 13 min, cooled, and then blanched with air pressure at 27 PSI for 6 min in a rotating cylinder. The data represent means of four replicate samples. Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 28. Laboratory sample blanching of medium size kernels of non-transformed parent lines and T_4 transgenic lines containing the barley oxalate oxidase gene in Virginia, 2006.*

Parent	Parent and T_4 transgenic line	Roasting % H_2O before	Roasting % H_2O after	% blanching loss	Blanched % splits	Blanched % whole	Blanched % not	Blanched % partial
NC 7	NC7 (non-transformed)	6.83 b	3.45	3.40	6.66	54.09	14.52	16.70
	N70-8-24-B	6.76 b	3.44	3.33	5.58	55.41	8.31	19.27
	N99(P60)-29-10-B	7.71 a	4.28	3.41	4.08	48.23	12.38	21.12
	N70-8-B-B	6.94 b	3.43	3.51	5.24	58.57	11.36	18.59
	N70-6-B-B	6.89 b	3.43	3.46	7.56	61.73	12.32	20.48
	LSD.....	*0.62*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	Perry (non-transformed).....	6.44 d	3.45 c	2.99	9.91	51.76 bc	2.81	24.48 b
	P98(N6)-1-10-B	6.16 d	3.11 c	3.05	15.72	54.42 a-c	4.71	18.25 bc
	P97(N6)-2-8-B	7.31 bc	3.96 bc	3.34	8.69	48.17 c	4.09	31.36 a
	P39-7-9-B.....	7.29 bc	3.95 bc	3.34	12.41	68.75 ab	1.48	18.32 bc
	P53-30-21-B.....	6.79 cd	3.41 c	3.37	9.86	63.94 a	2.99	14.24 c
	P99(N6)-4-14-B	6.74 cd	3.43 c	3.32	13.32	53.93 a-c	2.38	21.08 bc
	P53-28-B-B	8.16 a	4.86 a	3.30	5.53	61.72 ab	5.78	20.83 bc
	P39-8-B-B	7.83 ab	4.50 ab	3.33	9.37	64.77 a	1.56	18.78 bc
	LSD	*0.77*	*0.87*	*n.s.*	*n.s.*	-	*n.s.*	-
Wilson	Wilson (non-transformed).....	8.22	4.52	3.69	7.99	58.03	4.48	25.74
	W14-10-2-B	8.81	5.73	3.08	8.23	60.85	4.83	22.75
	W59-8-2-B	7.75	4.80	2.94	8.62	51.51	3.90	25.60
	W171-17-15-B	8.88	4.93	3.96	6.68	57.73	4.20	24.12
	W73-27-B-B	8.66	5.45	3.21	7.68	57.63	6.17	22.34
	W171-17-B-B	8.73	5.39	3.34	5.38	54.43	6.56	28.23
	LSD.....	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*After mechanically cutting each side of the peanut skin, 250 gram samples of peanuts were white roasted at 149°C for 15 min, cooled, and then blanched with air pressure at 27 PSI for 7 min in a rotating cylinder. The data represent means of four replicate samples. Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant; - denotes unbalanced data means separated using Student-Newman-Keuls Test P=0.05. Data provided by DE Partridge, J Hu and PM Phipps.

Table 29. Laboratory sample blanching of extra large size kernels of non-transformed parent lines and T_5 transgenic lines containing the barley oxalate oxidase gene in Virginia, 2007.*

Parent and T_5 transgenic line	Roasting		%	Blanched			
	% H_2O before	% H_2O after	blanching loss	% splits	% whole	% not	% partial
NC 7 (non-transformed)	5.9	4.6 b	1.3	9.3	79.6	5.1	4.7
N70-8-24-B-B	6.0	4.8 a	1.2	9.7	82.9	2.6	3.5
LSD	*n.s.*	*0.2*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)	6.2	4.8	1.4	12.4	64.5	13.2	9.0
P39-7-9-B-B	6.1	4.8	1.3	15.0	70.3	9.7	3.9
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Wilson (non-transformed)	6.1	4.8	1.3	12.5	65.3 b	14.5	5.8 a
W73-27-B-B-B	6.0	4.9	1.1	10.7	78.6 a	6.6	2.5 b
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*12.4*	*n.s.*	*3.0*

* After mechanically cutting each side of the peanut skin, 250 gram samples of peanuts were white roasted at 149°C for 17 min, cooled, and then blanched with air pressure at 27 PSI for 6 min in a rotating cylinder. The data represent the mean of four replicate samples. Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided J Hu and PM Phipps.

Table 30. Laboratory sample blanching of medium size kernels of non-transformed parent lines and T_5 transgenic lines containing the barley oxalate oxidase gene in Virginia, 2007.*

Parent and T_5 transgenic line	Roasting		%	Blanched			
	% H_2O before	% H_2O after	blanching loss	% splits	% whole	% not	% partially
NC 7 (non-transformed)	6.2	5.1	1.1	12.1	54.5	10.4 a	21.8
N70-8-24-B-B	6.2	5.1	1.1	13.0	56.6	5.6 b	22.9
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*4.2*	*n.s.*
Perry (non-transformed)	6.2	5.0	1.2	18.5	45.5	13.6	20.7
P39-7-9-B-B	6.2	5.0	1.2	13.1	62.0	13.2	10.6
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Wilson (non-transformed)	6.2	5.0	1.2	15.0	53.8 b	10.3	19.3
W73-27-B-B-B	6.3	5.0	1.3	11.3	63.2 a	8.6	15.1
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*5.5*	*n.s.*	*n.s.*

* After mechanically cutting each side of the peanut skin, 250 gram samples of peanuts were white roasted at 149°C for 17 min, cooled, and then blanched with air pressure at 27 PSI for 6 min in a rotating cylinder. The data represent the mean of four replicate samples. Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

Compositional Analysis

The composition of the peanuts was assessed for mineral content (Table 31 to Table 33), fiber and protein content of peanut hay (Table 34) and fatty acid profile (Table 35 to Table 37). There were no consistent significant differences in comparisons of a transgenic line and its corresponding non-transgenic parent. However, some increases of mineral and fatty acid content in transgenic lines were associated with improved plant health as a result of control of Sclerotinia blight.

Table 31. Nutrient analysis of kernels from non-transformed parent lines and T_3 transgenic lines containing the barley oxalate oxidase gene in Virginia, 2005.

		Nutrient concentration (mg/kg)*				
Parent	Parent and T_3 transgenic lines	Ca	K	Mg	P	S
NC 7	NC 7 (non-transformed)	808.1	8080	1843	3819	638.7
	N70-8-B	765.3	7901	1908	3900	524.9
	N70-6-B	726.6	7899	1879	3877	531.4
	N99(P60)-4-B	789.5	7960	1888	3955	540.0
	LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	Perry (non-transformed)	868.8 a-c	7797 c	1854 de	3676 d	612.1 bc
	P53-26-B	953.0 a	8684 a	2033 ab	3990 a-d	840.8 a
	P53-4-B	888.5 ab	8233 b	1882 c-e	3709 d	801.0 a
	P53-30-B	936.9 ab	8325 b	1861 c-e	3826 cd	758.7 a
	P53-28-B	917.5 ab	8155 b	1904 c-e	3712 cd	749.8 a
	P53-27-B	905.4 ab	8139 b	1818 e	3801 d	725.2 ab
	P39-7-B	770.8 d	7738 c	1961 a-c	3880 b-d	565.7 cd
	P39-8-B	732.9 d	7763 c	2051 a	3971 a-d	496.1 c-e
	P97(N6)-2-B	747.5 d	7551 c	1944 b-d	4227 ab	386.6 e
	P98(N6)-1-B	802.0 cd	7724 c	1963 a-c	4100 a-c	440.3 de
	P99(N6)-4-B	861.1 bc	7640 c	1965 a-c	4272 a	393.9 e
	LSD	*84.9*	*336*	*106*	*390*	*134.3*
Wilson	Wilson (non-transformed)	872.6	8273	1919	3899 b	682.7
	W73-27-B	844.8	8543	2053	4350 a	603.1
	W171-17-B	949.1	8233	1906	4153 ab	604.3
	W83-7-B	943.8	8206	1990	4188 ab	570.3
	W59-10-B	877.6	8563	2048	4463 a	589.4
	W59-11-B	904.5	8215	1996	4385 a	511.1
	W51-9-B	977.5	8145	2002	3936 b	623.0
	LSD	*n.s.*	*n.s.*	*n.s.*	*346*	*n.s.*

* Calcium, phosphorus, magnesium, potassium and sulfur were measured by dry-ashing peanut meat, extraction in 0.5 hydrochloric acid, and analysis by inductively coupled plasma by spectrophotometry performed by the Virginia Tech Soil Testing Laboratory. A calcium concentration greater than 420 ppm is needed for germination. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 32. Nutrient analysis of kernels from non-transformed parent lines and T_4 transgenic lines containing the barley oxalate oxidase gene in Virginia, 2006.

		Nutrient concentration (mg/kg)*				
Parent	Parent and T_5 transgenic lines	Ca	K	Mg	P	S
NC 7	NC7 (non-transformed)	662.3	7519	1754	3411	428.9
	N70-8-24-B	658.4	7720	1832	3745	402.8
	N99(P60)-29-10-B	583.5	7586	1882	3784	425.1
	N70-8-B-B	634.3	7568	1841	3720	419.7
	N70-6-B-B	604.1	7441	1791	3620	387.9
	LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	Perry (non-transformed)	621.2 ab	7556 b	1793 d	3518 d	356.3 b
	P98(N6)-1-10-B	538.1 c	7600 b	1823 b-d	3915 a	372.4 b
	P97(N6)-2-8-B	621.7 ab	7368 b	1872 bc	3884 ab	385.8 b
	P39-7-9-B	531.9 c	7402 b	1826 b-d	3738 a-c	356.4 b
	P53-30-21-B	662.7 a	8104 a	1891 b	3629 cd	441.0 a
	P99(N6)-4-14-B	549.5 c	7295 b	1819 cd	3743 a-c	372.4 b
	P53-28-B-B	649.3 ab	7988 a	1882 bc	3709 b-d	392.7 b
	P39-8-B-B	584.1 bc	7406 b	1967 a	3793 a-c	357.1 b
	LSD	*67.7*	*370*	*71*	*206*	*44.8*
Wilson	Wilson (non-transformed)	734.0	7942	1906 c	3705 d	359.2
	W14-10-2-B	683.0	7684	1995 ab	4010 a-c	342.0
	W59-8-2-B	717.9	7931	2009 ab	4173 a	374.6
	W171-17-15-B	712.9	8071	2023 a	4063 ab	376.7
	W73-27-B-B	748.5	7880	2013 ab	3946 bc	371.9
	W171-17-B-B	721.6	7820	1941 bc	3853 cd	386.5
	LSD	*n.s.*	*n.s.*	*73*	*183*	*n.s.*

*Calcium, phosphorus, magnesium, potassium and sulfur were measured by dry-ashing peanut meat, extraction in 0.5 hydrochloric acid, and analysis by inductively coupled plasma by spectrophotometry performed by the Virginia Tech Soil Testing Laboratory. A calcium concentration greater than 420 ppm is needed for germination. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 33. Nutrient analysis of kernels from non-transformed parent lines and T_5 transgenic lines treated with and without Omega fungicide for control of Sclerotinia blight in Virginia, 2007.*

Treatment and line	Nutrient concentration (mg/kg)				
	Ca	K	Mg	P	S
Non-treated					
NC7 (non-transformed)	505.9	8672	1829 b	3913	498.4
N70-8-24-B-B	506.2	8490	1954 a	4163	480.4
LSD	*n.s.*	*n.s.*	*100*	*n.s.*	*n.s.*
Wilson (non-transformed)	605.6	8756	1908	3926	484.3
W73-27-B-B-B	651.8	8517	2005	4101	410.1
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)	551.5	8646	1867 b	3924 b	394.9
P39-7-9-B-B	549.7	8263	2012 a	4359 a	373.8
LSD	*n.s.*	*n.s.*	*173*	*374*	*n.s.*
Treated with Omega 500 1 pt/A (13 Aug, 11 Sep)					
NC7 (non-transformed)	541.8	8447	1890	3949	436.5
N70-8-24-B-B	529.4	8552	1972	4101	440.1
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Wilson (non-transformed)	588.6	8938	1922	4024	441.6 a
W73-27-B-B-B	600.3	8781	1994	4119	414.8 b
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*19.1*
Perry (non-transformed)	505.8	8549	1856	3857	388.2
P39-7-9-B-B	503.1	8384	1975	4256	391.9
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*Calcium, phosphorus, magnesium, potassium and sulfur were measured by dry-ashing peanut meat, extraction in 0.5 hydrochloric acid, and analysis by inductively coupled plasma by spectrophotometry performed by the Virginia Tech Soil Testing Laboratory. A calcium concentration greater than 420 ppm is needed for germination. Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (P=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

Table 34. Evaluation of neutral detergent fiber, acid detergent fiber, total digestible nutrient, crude protein, and dry matter in peanut hay of T_4 transgenic lines and their non-transformed parent lines, 2006.*

Parent and T_4 Line	Neutral detergent fiber (% of DM)	Acid detergent fiber (% of DM)	Total digestible nutrient (% of DM)	Crude protein (% of DM)	Dry matter (%)
NC7 (non-transformed)	52.97	48.68	53.41	8.23	92.65
N70-8-24-B	51.45	47.67	54.07	7.87	92.63
N70-6-B-B	50.39	46.76	54.66	8.19	92.67
N70-8-B-B	48.72	44.93	55.86	8.28	92.55
N99-29-10-B	51.25	47.69	54.06	7.65	92.63
LSD [z]	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry (non-transformed)	50.69	47.71	54.04	7.23	92.63
P97-N6-28-B	47.07	43.74	56.64	7.26	92.41
P98-N6-110-B	46.96	44.23	56.32	7.26	92.63
P99-N6-414-B	47.24	44.09	56.41	7.35	92.48
P39-7-9-B	47.69	44.94	55.86	7.30	92.17
P39-8-B-B	47.41	43.97	56.49	7.52	92.49
P53-28-B-B	58.10	52.01	51.24	7.95	92.92
P53-30-21-B	56.24	52.18	51.13	7.90	92.77
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Wilson (non-transformed)	56.82	54.00	49.94	8.59	92.65
W14-10-2-B	53.42	50.67	52.11	8.30	92.72
W171-17-15-B	58.75	54.03	49.92	8.16	92.96
W171-17-B-B	56.04	52.05	51.21	8.66	92.73
W59-8-2-B	55.75	52.78	50.73	8.10	92.80
W73-27-B-B	57.08	53.56	50.23	7.74	92.97
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

*DM is dry matter. Dunnett's test was used to compare the mean of each transgenic line and its non-transformed parent (LSD, P=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 35. Fatty acid composition, iodine value, oleic/linoleic (O/L) ratio, % total saturated, polyunsaturated/saturated (P/S) ratio, and % total long chain saturated of peanut kernels from non-transformed parent lines and T_3 transgenic lines in Virginia, 2005.

	Fatty acid composition (% of total)								O/L ratio					
Line T_0-T_1-T_2	Palmitic 16:0	Stearic 18:0	Oleic C9 18:1	Linoleic 18:2 (n-6)	Arach-idic 20:0	Eico-senoic 20:1	Benhenic 22:0	Ligno-ceric 24:0	Oleic + linoleic[t]	O/L ratio linoleic[u]	Iodine Value (%)[v]	% total saturated[w]	P/S ratio[x]	Total long chain saturated[y]
NC 7	8.77 c	3.51 a	57.23	24.64	1.54 a	0.97 b	2.34 b	1.00	0.715 b	2.32	92.66	17.16 b	1.44	4.89
N70-8-B	9.30 a[z]	3.12 b	56.29	25.21	1.49 b	1.07 a	2.47 a	1.06	0.750 a	2.24	92.92	17.44 a	1.45	5.01
N70-6-B	9.32 a	3.12 b	56.03	25.41	1.50 b	1.05 a	2.55 a	1.02	0.750 a	2.21	93.02	17.51 a	1.45	5.07
N99(P60)-4-B	9.14 b	3.12 b	56.32	25.45	1.49 b	1.05 a	2.46 a	1.00	0.743 a	2.21	93.34	17.19 b	1.48	4.94
LSD	*0.15*	*0.16*	*n.s.*	*n.s.*	*0.03*	*0.06*	*0.10*	*n.s.*	*0.010*	*n.s.*	*n.s.*	*0.23*	*n.s.*	*n.s.*
Perry	9.73 a	2.45 b-d	49.40 b	32.37 a	1.25 cd	1.19 f	2.42 e	1.21 e	0.800 a-c	1.53 c	99.49 a	17.05 bc	1.90 a	4.87 e
P53-26-B	8.65 cd	2.69 a	53.73 a	27.96 c	1.37 a	1.34 bc	2.82 b	1.45 bc	0.763 f	1.92 a	95.69 c	16.97 b-d	1.65 c	5.63 ab
P53-4-B	8.80 c	2.36 d	52.81 a	28.90 bc	1.29 bc	1.42 a	2.94 a	1.48 ab	0.790 cd	1.83 b	96.59 bc	16.89 cd	1.71 c	5.71 a
P53-30-B	8.69 cd	2.55 a-c	53.04 a	28.78 bc	1.34 ab	1.33 bc	2.84 ab	1.44 bc	0.775 ef	1.85 ab	96.51 bc	16.85 cd	1.71 c	5.61 a-c
P53-28-B	8.53 d	2.59 ab	53.57 a	28.35 bc	1.35 a	1.34 bc	2.82 b	1.45 bc	0.767 f	1.89 ab	96.23 c	16.74 d	1.69 c	5.62 ab
P53-27-B	8.52 d	2.38 cd	53.13 a	29.13 b	1.27 cd	1.39 ab	2.77 bc	1.41 b-d	0.783 de	1.83 b	97.25 b	16.35 e	1.78 b	5.45 bc
P39-7-B	9.35 b	2.37 cd	49.01 b	32.42 a	1.29 bc	1.28 c-e	2.74 bc	1.56 a	0.798 a-c	1.51 c	99.30 a	17.30 a	1.87 a	5.58 a-c
P39-8-B	9.25 b	2.37 cd	49.19 b	32.46 a	1.28 c	1.32 cd	2.68 c	1.46 bc	0.795 b-d	1.52 c	99.57 a	17.04 bc	1.91 a	5.41 c
P97(N6)-2-B	9.78 a	2.27 d	48.69 b	32.95 a	1.22 d	1.24 ef	2.52 de	1.34 d	0.810 a	1.48 c	99.92 a	17.13 ab	1.92 a	5.08 d
P98(N6)-1-B	9.66 a	2.32 d	49.57 b	32.04 a	1.24 cd	1.25 d-f	2.54 d	1.38 cd	0.808 ab	1.55 c	99.12 a	17.13 ab	1.87 a	5.17 d
P99(N6)-4-B	9.75 a	2.34 d	49.24 b	32.39 a	1.24 cd	1.21 ef	2.50 de	1.34 d	0.808 ab	1.52 c	99.40 a	17.17 ab	1.89 a	5.08 d
LSD	*0.18*	*0.18*	*1.01*	*0.96*	*0.05*	*0.07*	*0.11*	*0.09*	*0.014*	*0.08*	*0.90*	*0.23*	*0.07*	*0.20*
Wilson	8.53 d	2.71 a	54.32 a	27.64 d	1.39 a	1.27 b	2.74 cd	1.41 a	0.760 d	1.97 a	95.59 c	16.77 bc	1.65 d	5.54 a-c
W73-27-B	8.74 bc	2.71 a	53.27 ab	28.70 c	1.35 a	1.27 b	2.64 d	1.32 c	0.763 cd	1.86 b	96.53 b	16.76 bc	1.71 c	5.31 d
W171-17-B	8.86 bc	2.61 ab	52.25 bc	29.63 bc	1.34 ab	1.29 b	2.69 cd	1.34 bc	0.773 cd	1.77 c	97.27 b	16.84 b	1.76 bc	5.37 cd
W83-7-B	8.73 c	2.34 c	51.83 c	30.24 ab	1.27 c	1.39 a	2.79 bc	1.42 a	0.790 a	1.71 cd	98.03 a	16.55 c	1.83 a	5.48 b-d
W59-10-B	8.88 b	2.58 ab	50.71 d	30.85 a	1.35 ab	1.31 b	2.93 a	1.39 ab	0.775 bc	1.65 d	98.08 a	17.14 a	1.80 ab	5.68 a
W59-11-B	8.85 bc	2.59 ab	52.13 c	29.49 bc	1.37 a	1.31 b	2.85 ab	1.42 a	0.773 cd	1.77 bc	96.94 b	17.08 a	1.73 c	5.64 ab
W51-9-B	9.21 a	2.47 bc	50.65 d	30.85 a	1.30 bc	1.31 b	2.79 bc	1.43 a	0.788 ab	1.64 d	98.02 a	17.20 a	1.80 ab	5.52 a-c
LSD	*0.14*	*0.19*	*1.06*	*0.93*	*0.05*	*0.05*	*0.11*	*0.07*	*.014*	*0.09*	*0.74*	*0.23*	*0.05*	*0.17*

[t]Oleic/Linoleic (O/L) ratio= % oleic / (sum of relative % of oleic + linoleic). [u]Oleic/Linoleic (O/L) ratio = % oleic / % linoleic. Higher O/L ratio indicates longer shelf life. [v]Iodine value= % oleic (0.8601) + % linoleic (1.7321) + % eicosenoic (0.7854). Lower iodine value indicates longer shelf life. [w]Percent total saturated = % palmitic + % stearic + % arachidic + % benhenic + % lignoceric. [x]Polyunsaturated/saturated (P/S) ratio = % polyunsaturated (linoleic)/% total saturated. [y]Percent total long chain saturated = % arachidic + % benhenic + % lignoceric. [z] Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 36. Fatty acid composition, iodine values, oleic/linoleic (O/L) ratio, % total saturated, polyunsaturated/saturated (P/S) ratio, and % total long chain saturated of peanut kernels from non-transformed parent lines and T_4 transgenic lines in Virginia, 2006.

	Fatty acid composition (% of total)								O/L ratio					
Line T_0-T_1-T_2-T_3	Palmitic 16:0	Stearic 18:0	Oleic C9 18:1	Linoleic 18:2 (n-6)	Arach-idic 20:0	Eico-senoic 20:1	Benh-enic 22:0	Ligno-ceric 24:0	Oleic + Linoleic[t]	O/L ratio Linoleic[u]	Iodine Value (%)[v]	% total saturated[w]	P/S ratio[x]	Total long chain saturated[y]
NC7	8.82	3.44	55.55	25.64	1.61	1.09	2.71	1.16	0.684	2.17	93.04	17.73	1.45	5.47
N70-8-24-B	9.33	3.13	55.25	25.79	1.53	1.16	2.71	1.13	0.682	2.15	93.09	17.81	1.45	5.36
N99(P60)-29-10-B	9.10	3.28	55.15	25.75	1.60	1.15	2.77	1.21	0.682	2.14	92.94	17.96	1.44	5.57
N70-8-B-B	9.11	3.20	55.30	25.84	1.56	1.12	2.71	1.78	0.682	2.14	93.20	17.74	1.46	5.45
N70-6-B-B	9.23	3.14	54.77	26.24	1.55	1.16	2.78	1.13	0.676	2.09	93.46	17.84	1.47	5.46
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s*
Perry	10.07 a	2.30 b	48.78 c	32.82 a	1.18	1.23	2.37	1.23	0.598 c	1.49 b	99.78 ab	17.16 a	1.91 ab	4.78
P98(N6)-1-10-B	9.93 a	2.30 b	49.26 bc	32.29 a	1.19	1.34	2.43	1.32	0.604 bc	1.53 b	99.30 ab	17.16 a	1.88 ab	4.93
P97(N6)-2-8-B	10.14 a	2.32 b	49.30 bc	32.35 a	1.13	1.23	2.30	1.23	0.604 bc	1.53 b	99.39 ab	17.12 a	1.89 ab	4.67
P39-7-9-B	9.96 a	2.13 c	49.04 bc	32.77 a	1.12	1.33	2.38	1.30	0.600 bc	1.50 b	99.97 a	16.88 a	1.94 a	4.78
P53-30-21-B	8.99 b	2.58 a	54.22 a	28.21 b	1.22	1.31	2.36	1.17	0.658 a	1.93 a	96.30 c	16.28 b	1.73 c	4.75
P99(N6)-4-14-B	10.05 a	2.29 b	50.38 b	31.43 a	1.14	1.24	2.25	1.20	0.616 b	1.61 b	98.75 b	16.94 a	1.86 b	4.75
P53-28-B-B	9.08 b	2.54 a	53.09 a	29.12 b	1.22	1.32	2.42	1.23	0.646 a	1.82 a	97.14 c	16.48 b	1.77 c	4.87
P39-8-B-B	9.95 a	2.25 bc	49.26 bc	32.50 a	1.14	1.27	2.37	1.21	0.603 bc	1.52 b	99.71 ab	16.93 a	1.92 ab	4.73
LSD	*0.34*	*0.16*	*1.58*	*1.41*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.018*	*0.12*	*1.18*	*0.29*	*0.08*	*n.s*
Wilson	9.42 b	2.69 a	54.53 ab	28.17 b-d	1.16	1.08	2.01	0.92	0.660 ab	1.94 ab	96.54 bc	16.21	1.74 bc	4.09
W14-10-2-B	9.90 a	2.34 c	52.92 b	29.47 b	1.09	1.22	2.14	0.96	0.643 b	1.80 bc	97.50 ab	16.40	1.80 ab	4.18
W59-8-2-B	9.95 a	2.46 bc	51.09 c	31.04 a	1.14	1.18	2.16	0.99	0.622 c	1.65 c	98.65 a	16.68	1.87 a	4.28
W171-17-15-B	9.34 bc	2.57 ab	53.82 ab	28.81 bc	1.18	1.21	2.10	0.98	0.652 ab	1.87 ab	97.13 b	16.18	1.78 b	4.25
W73-27-B-B	9.06 c	2.64 a	55.23 a	27.39 d	1.21	1.20	2.23	1.04	0.669 a	2.02 a	95.87 c	16.20	1.69 c	4.48
W171-17-B-B	9.21 bc	2.64 a	54.72 a	27.97 cd	1.21	1.15	2.12	1.02	0.662 a	1.96 a	96.41 bc	16.17	1.73 bc	4.32
LSD	*0.34*	*0.17*	*1.66*	*1.42*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.02*	*0.16*	*1.16*	*n.s.*	*0.08*	*n.s.*

[t]Oleic/Linoleic (O/L) ratio= % oleic / (sum of relative % of oleic + linoleic). [u]Oleic/Linoleic (O/L) ratio= % oleic / % linoleic. Higher O/L ratio indicates longer shelf life. [v]Iodine value= % oleic (0.8601) + % linoleic (1.7321) + % eicosenoic (0.7854). Lower iodine value indicates longer shelf life. [w]% total saturated = % palmitic + % stearic + % arachidic + % benhenic + % lignoceric. [x]Polyunsaturated/saturated (P/S) ratio= % polyunsaturated (linoleic)/% total saturated. [y] % total long chain saturated = % arachidic + % benhenic + % lignoceric. [z] Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by DE Partridge, J Hu and PM Phipps.

Table 37. Fatty acid composition, iodine values, oleic/linoleic (O/L) ratio, % total saturated, polyunsaturated/saturated (P/S) ratio, and % total long chain saturated of peanut kernels from non-transformed parent lines and T_5 transgenic lines treated with and without Omega fungicide for control of Sclerotinia blight in Virginia, 2007.

Parent and T_5 line	Fatty acid composition (% of total)								O/L ratio					
	Palmitic 16:0	Stearic 18:0	Oleic C9 18:1	Linoleic 18:2 (n-6)	Arach-idic 20:0	Eico-senoic 20:1	Benhenic 22:0	Ligno-ceric 24:0	Oleic+ Linoleic[t]	O/L ratio Linoleic[u]	Iodine Value (%)[v]	% total saturated[w]	P/S ratio[x]	Total long chain saturated[y]
Non-treated														
NC7	8.99	3.71	57.32	24.53	1.47	0.91 b	2.14	0.93	0.70	2.34	82.76 a	17.24 b	1.42 a	4.54
N70-8-24-B-B	9.44	3.49	57.20	24.20	1.46	1.01 a	2.27	0.94	0.70	2.36	82.40 b	17.60 a	1.38 b	4.67
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.06*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.31*	*0.31*	*0.02*	*n.s.*
Wilson	8.95	3.22	54.79	27.06	1.39	1.11	2.36	1.13	0.67	2.03	82.96	17.04	1.59	4.88
W73-27-B-B-B	8.72	3.14	55.24	26.79	1.39	1.17	2.40	1.16	0.67	2.06	83.19	16.81	1.59	4.94
LSD	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	10.04 a	2.72	50.40	30.98	1.26	1.10	2.32	1.19	0.62	1.63	82.47	17.53	1.77	4.77
P39-7-9-B-B	9.82 b	2.64	50.10	31.30	1.25	1.20	2.41	1.30	0.62	1.60	82.59	17.41	1.80	4.96
LSD	*0.19*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Treated with Omega 500														
NC7	9.16 b	3.52	56.05	25.62	1.47	0.95 b	2.26	0.97	0.69	2.19	82.63	17.37	1.47	4.69
N70-8-24-B-B	9.50 a	3.41	56.07	25.14	1.49	0.99 a	2.41	0.99	0.69	2.23	82.20	17.80	1.41	4.89
LSD	*0.33*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*0.03*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Wilson	8.99 a	3.10	54.61	27.23	1.38	1.11	2.41	1.17	0.67	2.01	82.96	17.04	1.60	4.95
W73-27-B-B-B	8.79 b	3.13	54.83	27.04	1.39	1.17	2.44	1.20	0.67	2.03	83.04	16.96	1.60	5.03
LSD	*0.17*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Perry	9.91	2.70 a	49.44	31.66	1.29 a	1.17 b	2.53	1.31 b	0.61	1.56	82.27 b	17.73 a	1.79	5.13
P39-7-9-B-B	9.84	2.51 b	49.76	31.51	1.24 b	1.22 a	2.52	1.39 a	0.61	1.58	82.49 a	17.51 b	1.80	5.16
LSD	*n.s.*	*0.17*	*n.s.*	*n.s.*	*0.04*	*0.04*	*n.s.*	*0.07*	*n.s.*	*n.s.*	*0.20*	*0.20*	*n.s.*	*n.s.*

[t] Oleic/Linoleic (O/L) ratio= % oleic / (sum of relative % of oleic + linoleic). [u]Oleic/Linoleic (O/L) ratio= % oleic / % linoleic. Higher O/L ratio indicates longer shelf life.
[v]Iodine value= % oleic (0.8601) + % linoleic (1.7321) + % eicosenoic (0.7854). Lower iodine value indicates longer shelf life.
[w] % total saturated = % palmitic + % stearic + % arachidic + % benhenic + % lingnoceric. [x]Polyunsaturated/saturated (P/S) ratio= % polyunsaturated (linoleic)/% total saturated.
[y]% total long chain saturated = % arachidic + % benhenic + % lignoceric. [z]Means followed by the same letter(s) in a parent group and column are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant. Data provided by J Hu and PM Phipps.

AFLATOXIN CONTENT

Aflatoxin can be produced during a post-harvest infection of peanut kernels by *Aspergillus flavus* Link or *A. fumigatus* Fresenius. Aflatoxin content was determined using an ELISA kit from Romer Labs with reliable detection limits between 1 and 20 ppb. Aflatoxin levels were assessed in naturally infested fields at the Tidewater AREC with a history of peanut production. Because many of the individual lines show aflatoxin levels near or below the detection limit of the kit, differences in lines are not likely to be biologically relevant (Table 38). From these tests, there is no indication that the expression of oxalate oxidase in peanut will cause an increase in aflatoxins, however, future inoculation studies should prove more conclusive.

Table 38. Evaluation of aflatoxin in peanut kernels from non-transformed parent lines and transgenic lines in 2005, 2006 and 2007 field trials.

	Aflatoxin (ppb)*			
	Virginia			North Carolina
Line	2005	2006	2007	2007
NC 7 (non-transformed)	1.754	1.969	0.321 a	0.479 b
N70-6-B-B-B.....	1.770	1.645	0.001 b	-
N70-8-B-B-B.....	2.140	1.886	0.000 b	0.925 a
LSD.....	*n.s.*	*n.s.*	*0.206*	*0.409*
Perry (non-transformed)	1.461	1.548	0.394 a	0.857 b
P39-7-9-B-B	1.483	1.737	0.000 b	1.199 a
P53-30-21-B-B	1.411	1.893	0.034 b	-
LSD.....	*n.s.*	*n.s.*	*0.047*	*0.320*
Wilson (non-transformed)	1.761 a	2.176 a	0.556 a	0.906
W171-17-15-B-B.....	1.999 a	-	-	-
W73-27-B-B-B	0.583 b	1.413 b	0.021 b	1.509
W14-10-2-B-B.....	-	0.716 c	0.010 b	-
LSD.....	*0.760*	*0.619*	*0.052*	*0.432*

**Means followed by the same letter(s) in a column and parent group are not significantly different according to Fisher's Protected LSD (*P*=0.05); n.s. denotes differences within a group are not significant; - denotes line not tested. All values were below the lower detection limit of assay..

ENVIRONMENTAL ASSESSMENT

CURRENT AGRONOMIC PRACTICES FOR VA-TYPE PEANUTS

PRODUCTION

U.S. peanuts were grown on 496 thousand hectares in the U.S. in 2007 compared to corn at 15,332 thousand hectares and soybeans at 10,427 thousand hectares. On average, VA-type peanuts constitute less than 15% of the total peanut acreage in this country and are considered a specialty crop (USDA, 2007). Traditionally, VA-type peanuts have been grown in southeastern Virginia, North Carolina, South Carolina, and parts of Texas and Oklahoma. In recent years, production has increased in South Carolina and parts of Texas (Phipps, personal communication).

PESTICIDE USAGE

VA-type peanuts are susceptible to several diseases for which chemical treatments are used as a control method. Fluazinam (Omega 500, Syngenta) and boscalid (Endura, BASF) are used for control of Sclerotinia blight in the U.S. Fluazinam can also suppress southern stem rot and Rhizoctonia pod rot while boscalid can be used for control of web blotch and suppression of leaf spot (Phipps, 2007).

CHANGES TO PEANUT FARMING PRACTICES

We would not expect transgenic peanuts with resistance to Sclerotinia blight to be grown outside of the VA-type peanut growing areas where buying stations for handling and storage facilities for processors of VA-types are located. VA-type peanuts thrive in cooler climates and do not do well outside of Virginia, the Carolina's and a few counties in Texas and Oklahoma.

The only expected change from normal peanut growing practices would be a reduction or elimination in usage of the fungicides fluazinam and boscalid, which are used almost exclusively for the control of *S. minor* in peanuts. An increase in the use of other pesticides after the introduction of N70 and W171 peanuts is not expected since the transgenic plants have not shown any tendency to reduce or increase occurrences of other diseases, pests or weeds over three years of testing in large field plots.

The elimination of costly chemical sprays of fluazinam and boscalid will improve the profitability of growing VA-type peanuts. We do not expect the use of transgenic peanuts with resistance to Sclerotinia Blight to result in any increase in the planted acreage of peanuts with the possible exception of Virginia where the acreage has been steadily decreasing because of yield and profit losses to Sclerotinia blight.

EFFECTS ON NON-TARGET ORGANISMS

Some studies have observed decreased herbivory from insects and decreased susceptibility to non-oxalic acid secreting pathogens on transgenic plants expressing Oxox. Reduced herbivory of European corn borer on transgenic corn expressing wheat germin has been observed (Ramputh et al., 2002). Transgenic potato expressing germin was found to have increased resistance to *Phytophthora infestans* and *Streptomyces reticuliscabiei* but not to *Erwinia carotovora* (Schneider et al., 2002). These instances of increased resistance have been attributed to the upregulation of plant defenses due to the increase of the signaling molecule hydrogen peroxide produced from the degradation of endogenous oxalic acid in the plant and not directly to the enzymatic activity of Oxox. These effects have not been observed in transgenic peanuts expressing Oxox as shown in Table 16 in the section “Effects on Other Diseases.”

OUTCROSSING

A preliminary outcrossing study was completed in 2006. Based on the results of that study, outcrossing studies were planned for 2007 and 2008. Plot design included a non-transgenic row bordered on each side by a transgenic row. A total of 24 rows of non-transgenic plants were planted beside each transgenic row to determine the percentage of outcrossing that occurs under field conditions. Rows were 3-m long and 0.9-m apart (Figure 21). Each transgenic cultivar was planted in a plot with the corresponding non-transgenic parent line. These plots were cultivated under normal growing conditions and the seeds from each row were harvested and bulked. Embryos of 95 (2006) to 285 (2007) seeds from each row were excised and tested for the presence of the oxalate oxidase enzyme. In 2006, our results showed zero or low outcrossing in most rows with the highest outcrossing being in the 5B row in the W59 line (5.3%). These outcrossing frequencies were in the expected range for Virginia-type peanuts. In 2006, plot design failed to isolate the most distant rows of the outcrossing block from production of transgenic peanuts in other plots. As a result, the outcrossing data were inconclusive. In subsequent years, outcrossing plots contained adequate buffer rows. In 2007 we found the highest outcrossing rates to be 1.1%, occurring in all three cultivars. We also observed that outcrossing occurs as far away as 19 rows (17.4 m) from the closest transgenic row. These results indicate that the likelihood of transgene escape is minimal.

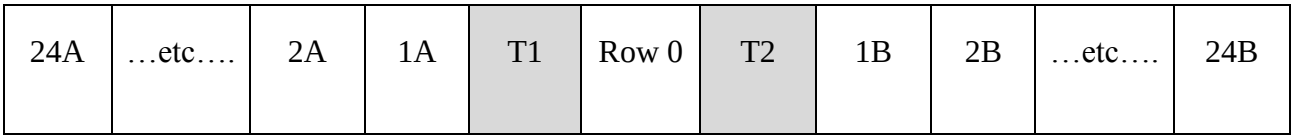

Figure 21. Field layout for outcrossing studies.
Gray indicates rows that were planted a transgenic line while white indicates rows that were planted to the corresponding non-transgenic parent cultivar.

Table 39. Outcrossing incidence based on oxalate oxidase expression by seedlings grown from seed harvested from non-transformed lines planted in rows near T_4 transgenic lines in Virginia, 2006.*

T_4 line	Row																
	7a	6a	5a	4a	3a	2a	1a	T-1	0	T-2	1b	2b	3b	4b	5b	6b	7b
W59-11-B-B	0 (0.0)	1 (1.1)	0 (0.0)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)	92 (96.8)	3 (3.2)	ND	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)	5 (5.3)	3 (3.2)	0 (0.0)
P39-7-B-B.....	0 (0.0)	0 (0.0)	1 (1.1)	0 (0.0)	1 (1.1)	0 (0.0)	0 (0.0)	78 (82.1)	1 (1.1)	ND	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)
N70-8-B-B	0 (0.0)	2 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	91 (95.8)	0 (0.0)	ND	1 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

*Number of embryos positive for the oxalate oxidase gene in a total of 95 seed tested per row with percentages for outcrossing in parentheses. Rows T-1 and T-2 were planted to a transgenic line and all other rows were planted to the corresponding non-transformed parent cultivar; Wilson, Perry or NC 7. Number in parenthesis represents the percentage of embryos testing positive. Rows were 15 ft long and spaced 36 in. apart. ND = not determined.

Table 40. Outcrossing incidence based on oxalate oxidase expression by embryos of seed harvested from non-transformed lines planted in rows near T_5 transgenic lines in Virginia, 2007*.

T_5 Line	Row																							
	23a	19a	15a	11a	7a	6a	5a	4a	3a	2a	1a	T-1	0	T-2	1b	2b	3b	4b	5b	6b	7b	11b	15b	19b	23b
W171-17-15-3-23	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.4)	0 (0.0)	284 (99.6)	3 (1.1)	282 (98.9)	2 (0.8)	1 (0.4)	2 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
P39-7-9-43-18	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.8)	1 (0.4)	0 (0.0)	285 (100.0)	3 (1.1)	285 (100.0)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
N70-8-24-5-2	0 (0.0)	1 (0.4)	0 (0.0)	1 (0.4)	1 (0.4)	0 (0.0)	0 (0.0)	1 (0.4)	0 (0.0)	1 (0.4)	0 (0.0)	277 (98.2)	1 (0.4)	279 (98.9)	3 (1.1)	4 (1.4)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

*Number of embryos positive for oxalate oxidase expression in a total of 285 seed tested per row with percentages positive for outcrossing in parentheses. Rows T-1 and T-2 were planted to a T_5 transgenic line and all other rows were planted to the corresponding non-transformed parent cultivar; Wilson, Perry or NC 7. Rows were 15 ft long and spaced 36 in. apart.

MATERIALS AND METHODS

ADVANCED FIELD TESTING OF TRANSGENIC LINES

GENERAL FIELD TESTING PROCEDURES

Field tests were completed by Darcy Partridge-Telenko, Jiahuai Hu and Pat Phipps. Permits for field testing each year were obtained from APHIS for 2004-2008 by Elizabeth Grabau. In 2004-2007, all trials were conducted at the Tidewater AREC Research Farm in Suffolk, VA. In 2007, trials were also conducted at the Upper Coastal Plain Experiment Station at Rocky Mount, NC in cooperation with NC State University. The field sites in Virginia were a Kenansville loamy sand and were rotated with corn, cotton and peanut. Peanuts were grown using standard production practices and irrigated during periods of drought stress. Plots were two rows 9.1 m long and 0.9 m apart. Transformed and non-transformed lines were replicated four times in a randomized complete block design.

SEED AND HAY ANALYSIS

Hay analysis was performed under the direction of Dr. Chris Teutsch at Southern Piedmont AREC at Blackstone. Mineral analysis of seeds was performed by Athena Tilley, Lab and Research Specialist II, in the Crops, Soils, and Environmental Sciences Department at Virginia Tech. Fatty acid analysis was performed under the direction of Dr. Ben Corl in the Dairy Science department at Virginia Tech.

OXALATE OXIDASE ASSAY

Oxox expression was assayed using a coupled reaction (Livingstone et al., 2005). This assay used horseradish peroxidase (HRP) to detect the hydrogen peroxide produced when Oxox

degrades OA. First, 200 µL of assay buffer (0.0017M OA in 2.5 mM succinic acid, pH 4) was added to each well of a 96-well plate containing peanut embryos or leaf disks. After incubation for 15 min at 37C, 100 µL of assay solution was removed to a new 96-well plate and 70 µL of developing solution (50 mL 0.1 M sodium phosphate buffer, pH 7.0, 15 µL N,N-dimethylaniline containing 3 µg of aminoantipyrene and 27 µL of 140 mg/mL HRP) was added. Reactions were allowed to continue at room temperature for 30 min. A purple color developed in the presence of Oxox and the absorbance was measured at a wavelength of 562 nm.

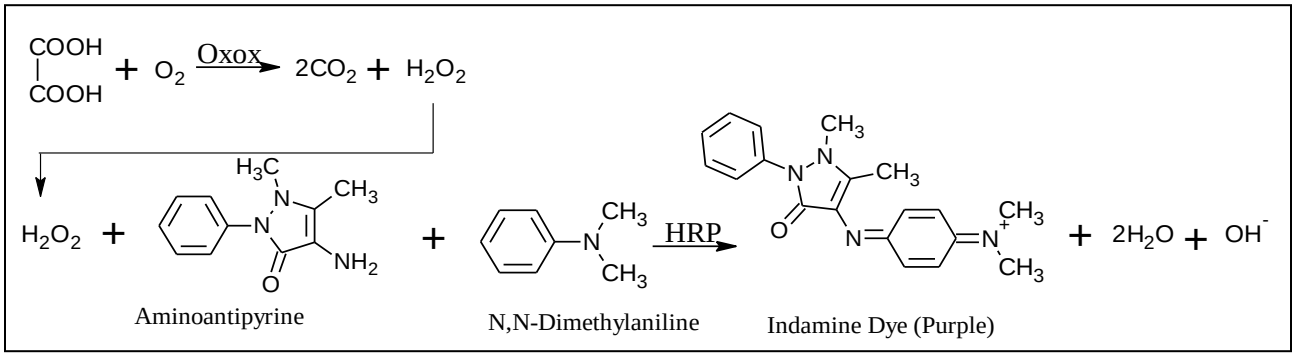

Figure 22. Coupled reaction for the detection of Oxox expression.
Figure made using ChemSketch by ACDLab. HRP, horseradish peroxidase.

MOLECULAR CHARACTERIZATION OF TRANSGENIC LINES

DNA EXTRACTION

DNA for Southern blots was extracted using either leaf tissue or hypocotyl tissue. Young, unfolded leaflets were ground in liquid nitrogen and DNA was extracted using a modification of the method described by Sharma et al. (2000). Fifteen mL of extraction buffer (100 mM Tris; 50 mM EDTA, pH 8.0; 200 mM sodium chloride; 10 mM ß-mercaptoethanol) was added to each gram of ground tissue followed by 1 mL 20% SDS and 5 µL RNase A (20 mg/mL). This mixture was incubated at 65°C for one hour and mixed by inversion every 15 minutes. SDS and proteins were precipitated by adding 5 mL 5M potassium acetate, pH 4.8 and

incubating on ice for 20 minutes followed by centrifugation at 18,000 x g for 20 minutes. DNA was precipitated from the supernatant with 10 volumes of isopropanol with at least 1 hour incubation at -20°C. The DNA was pelleted and resuspended in 50:10 Tris-EDTA (TE).

Fifteen grams of pre-swollen diethylaminoethylcellulose (DEAE-cellulose) was equilibrated first in 200 mL of elution buffer (2 M sodium chloride; 10 mM Tris, pH 7.4; 1 mM EDTA) while stirring. The DEAE-cellulose was allowed to settle and small particulates poured off. This was repeated twice in wash buffer (400 mM sodium chloride, 10 mM Tris pH 7.4, 1 mM EDTA). Finally, the DEAE-cellulose was suspended in 40 mL of wash buffer. Ten to fifteen mL was added to a 25 mL gravity drip column (Bio-Rad) to a final bed volume of 5 mL.

The resuspended DNA was then bound to the column and washed two to three times with wash buffer. DNA was eluted in 5 mL of elution buffer and precipitated with isopropanol for at least 1 hour at -20°C. The DNA was pelleted by centrifugation at 18,000 x g at 4°C for 20 minutes. The pellet was washed with 70% ethanol, allowed to air dry and resuspended in approximately 1 mL of 1:10 TE. DNA was extracted from hypocotyl tissue by grinding 2 g of tissue in 15 mL of extraction buffer. The same procedure was followed for leaf tissue through the first isopropanol precipitation and resuspension. The samples were then extracted with one volume of phenol-chloroform-IAA followed by one volume of chloroform-IAA. The DNA was then isopropanol precipitated, pelleted by centrifugation at 18,000 x g for 20 minutes at 4°C, washed with 70% ethanol and resuspended in 1 mL 1:10 TE. All chemicals were obtained from Sigma-Aldrich or Fisher Scientific.

DNA was quantified using the DYNA Quant 200 fluorometer from Hoefer. The DNA binds to a fluorescent dye, Hoechst 33258, which can be read by the fluorometer with an excitation at 365 nm and emission at 460 nm.

SOUTHERN BLOT ANALYSIS

Southern blot analysis was used to determine the number of plasmid inserts in the peanut genome and whether or not they are intact. This was accomplished using several different restriction enzymes (see Genetic Characterization section) and ^{32}P labeled probes. DNA probes were PCR amplified using Accuzyme (Bioline) and reaction products cleaned using a PCR clean-up kit (Qiagen). Probe (50 ng each) (Table 41) was radiolabeled with ^{32}P alpha dCTP (Perkin Elmer) using HighPrime (Roche), a mixture of random primers and Klenow enzyme. Sephadex columns (Roche) were used to separate labeled DNA from unincorporated dCTP. DNA from each sample (10 µg each) were digested with restriction enzymes (New England Biolabs) and separated on a 1% agarose gel in TAE (Tris-acetate, EDTA, pH 8) buffer overnight at 23 mAmps. Blotting and washing procedures were performed as described by Sambrook et al. (1989).

Table 41. Primers used to synthesize probes for Southern blots.
Primers were obtained from Invitrogen and Integrated DNA Technology (IDT).

Probe	Forward Primer Number	Forward Primer Sequence	Reverse Primer Number	Reverse Primer Sequence
Probe 1	391	ATGAAAAAGCCTGAACTCACCG	392	GTCACTGGATTTTGGTTTTAGGAA
Probe 3	388	AAGGAAGAGTATGAGTATTCAACATTTCC	389	TTACCAATGCTTAATCAGTGAGGC
Probe 4	386	TTGAGATCCTTTTTTTCTGCGC	387	TTCCATAGGCTCCGCCC
Probe 5	140	CCCTCTACAGGACTTCTGCG	141	CTGGCTGTTGAAGGAACACAA
Probe 7	430	GAATTCACTGGCCGT	431	TTTCAATATTATTGAAGCATTTAT
Probe 8	432	AAACGCCAGCAACGCGG	433	CGAAGACCTGCAGGCATGC

PROTEIN EXTRACTION

Protein extractions from seeds were carried out using one of two methods: TriReagent (Invitrogen) for total protein or phosphate buffered saline (PBS) extraction for soluble protein. The procedure supplied by Invitrogen was used for total protein extraction. Soluble protein was extracted by grinding the seeds, defatting with 20 volumes of acetone and incubating in 5 volumes of PBS at 65°C for one hour. Cell debris was removed by centrifugation at 18,000 x g for 15 minutes. Protein was quantified using Bradford assay reagents (Bio-Rad).

WESTERN BLOT ANALYSIS

Equal amounts of total protein (2 μg, peanuts; 4 μg barley) from each treatment were separated on a 10% tris-bis polyacrylimide gel (Invitrogen) and transferred to a nitrocellulose membrane. Dot blots were created by adding 2 μg of total protein in a volume of 2 μL directly to nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk (NFDM) in Tris buffered saline-Tween (TBST) for one hour. Primary antibody was applied for 1 hour in 2.5% NFDM followed by three 5 minute washes in TBS-tween and secondary antibody for 1 hour.

Polyclonal rabbit-anti-Oxox sera (1:10,000) was used as the primary antibody followed by a rabbit-anti-goat sera (1:10,000) conjugated to HRP as the secondary antibody (Lane et al., 1992). Protein marker from Bio-Rad was used. The blot was visualized using Immobilon Western chemiluminesent HRP substrates (Millipore).

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CHAPTER 3

FUTURE WORK

FUTURE STEPS TOWARDS DEREGULATION

U.S. DEPARTMENT OF AGRICULTURE

As illustrated in Chapter 2, the petition for the USDA is nearing completion. Upon submission, the agency has 180 days to respond. The response may be an approval, denial, or request for more data or clarification.

FOOD AND DRUG ADMINISTRATION

The requirements for the FDA are similar to those for the USDA. Parts of the USDA petition will be used to satisfy requirements by this agency. Because Oxox is a stable, glycosylated protein, there is some concern that it may be allergenic. There are several type of allergies, but Type I is of the most concern with this protein. Type I allergies are caused when a person's body mounts an immediate immune response to a generally non-harmful substance. IgE recognizes the allergen and causes the release of histamine, which causes an allergic response. Food allergens from plants are often seed storage proteins. The only members of the cupin superfamily to be identified as food allergens are the 7S and 11S globulins from peanut and soybean and a GLP from pepper (Mills et al., 2002). The allergen with the highest percent identity to Oxox is peanut arachin at 16.7% (Table 42). None of the available sequences of known allergens show significant similarity to barley Oxox. The sequence for the GLP from black pepper is not available in the public databases.

Table 42 Major allergens from cereals and the cupin superfamily of proteins.
Percent identity was determined using the Align (Myers and Miller, 1988) function of Biology Workbench (Subramaniam, 1998) to compare each allergen to barley Oxox (gi:2266668).

	Protein	Identity to Oxox (%)	NCBI reference
Known Cereal Allergens	Wheat gliadin	12.3	gi:73912496
	Wheat glutenin subunit y	13.0	gi:14329759
	Rye secalin omega	10.6	gi:2145025
	Barley y-3 hordein	14.0	gi:34329260
	Barley alpha-amylase	16.4	gi:166985
Known Allergens of the Cupin Superfamily	Soybean beta-conglycinin (7S globulin)	14.8	gi:21465631
	Peanut conarachin (7S globulin)	10.6	gi:52001225
	Peanut Ara h 1 (7S globulin)	13.7	gi:16612200
	Soybean glycinin (11S globulin)	12.5	gi:18641
	Peanut Arachin (11S globulin)	16.7	gi:72324
	Peanut Ara h 3 (11S globulin)	15.3	gi:3703107

The pepper GLP was discovered during an investigation of the mugwort-birch-celery-spice syndrome. The researchers found that IgE from patients with pollen allergies cross reacts with a 28kDa GLP found in black pepper (Leitner et al., 1998). To further investigate this finding, germin and a GLP from Arabidopsis were expressed in transgenic tobacco and IgE antibodies isolated from patients with wheat and pollen allergies were tested for their ability to bind to the two proteins. It was found that IgE from 29% of the patients tested was able to bind to germin and 22% could bind Arabidopsis GLP (Jensen-Jarolim et al., 2002). The study also showed that IgE would only bind to the proteins when they were glycosylated. Skin prick tests of patients with positive IgE binding were also positive. However, skin prick tests and IgE binding have less than a 50% correlation with actual food allergy (Sampson, 2003). The only way to diagnose a food allergy is to have people eat suspected allergens and look for a reaction. No germins have been identified as major allergens.

We plan to address any questions concerning allergenicity by demonstrating that Oxox is found in common foods. Preliminary findings showed the presence of Oxox in cereal, Malt-O-

Meal, Ovaltine and barley malt (Figure 23). We have also observed that when peanuts are cooked at 350°F, most of the Oxox is no longer detectable by the anti-germin antibody that we obtained from Drs. Byron Lane and Andrew Cumming (Figure 24 and Figure 25). This may mean that the majority of the protein is degraded and thus not available to cause an allergic reaction. The next step in this testing will be to test human antibodies to see if they are able to bind to Oxox in a cooked peanut sample.

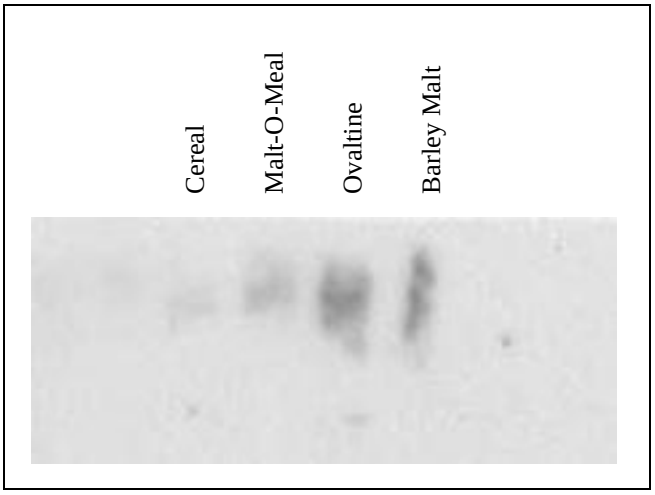

Figure 23. Western blot showing Oxox in cereal, Malt-O-Meal, Ovaltine and barley malt.

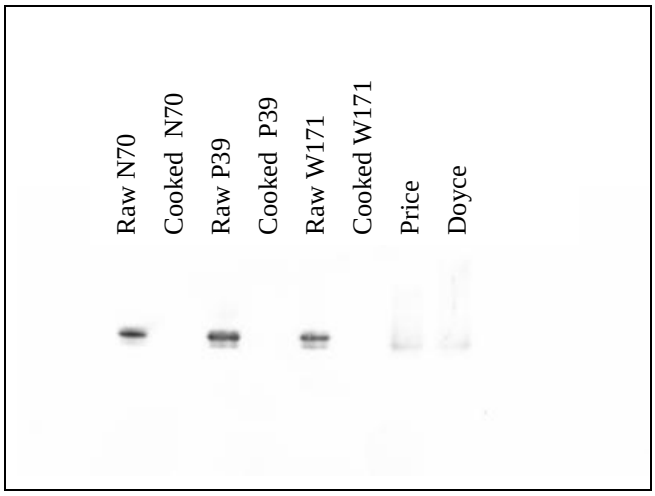

Figure 24. Western blot showing the presence of Oxox in raw and roasted peanuts as well as the barley cultivars 'Price' and 'Doyce'.

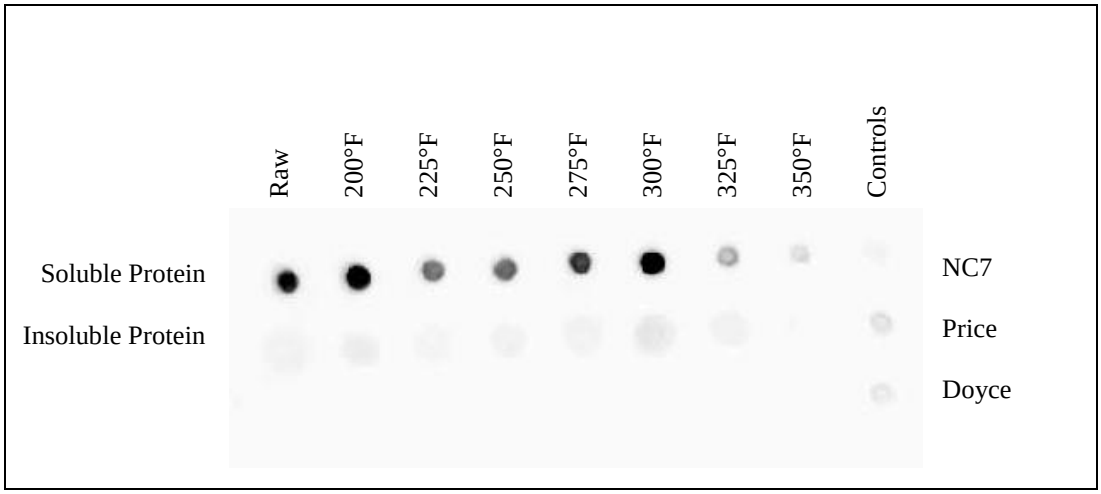

Figure 25. Protein dot blot showing Oxox in N70 peanuts cooked at a temperature range from 200°F to 350°F.

Environmental Protection Agency

The EPA's data requirements for registering transgenic plants are the same as those for microbial pesticides. Much of the data required by the EPA is not applicable to Oxox as expressed in peanuts. We will seek waivers for this data under 40 CFR section 158.45.

Table 43. Data required by the EPA for which we plan to request a waiver under 40 CFR section 158.45.

Requirement
Persistence in Soils
Acute Oral Toxicity
Acute Dermal Toxicity
Acute Inhalation Toxicity
Avian Oral Toxicity
Avian Injection Toxicity
Freshwater Fish Toxicity
Freshwater Invertebrate Toxicity
Nontarget Plant Toxicity
Nontarget Insect Toxicity
Honey Bee Toxicity

Final Steps Toward Marketing

Intellectual Property Rights

According to a study conducted by Graff et al. (2003), 75% of U.S. patents that apply to agricultural biotechnology were filed by and granted to the private sector. Of the 25% assigned to the public sector, many have been sold or licensed to the private sector, severely limiting their use and causing many products developed at universities to be unmarketable. Table 44 lists all of the technologies used in making Sclerotinia-resistant peanut varieties that are under patents by the public sector.

Table 44. Patents on technologies used in making transgenic peanuts expressing Oxox.

Technology	Patent Number	Year Filed	Year Granted	Year Expires	Assigned To	Owned By
Gene Gun	4945050	1984	1990	2004	Cornell	DuPont
	5371015	1992	1993	2012	Cornell	DuPont
Hygromycin Resistance	4727028	1983	1988	2005	Eli Lilly	Novartis (Syngenta)
	4960704	1988	1990	2007	Eli Lilly	Novartis (Syngenta)
	5668298	1995	1997	2014	Eli Lilly	Novartis (Syngenta)
	6048730	1990	2000	2020	Eli Lilly	Novartis (Syngenta)
	6365799	2000	2002	2020	Syngenta	Syngenta
Oxalate Oxidase	5547870	1994	1996	2014	Zeneca Limited	Syngenta
35S Promoter	5352605	1993	1994	2011	Monsanto	Monsanto
	5164316	1989	1992	2009	Monsanto	Monsanto
	5196525	1991	1993	2010	Monsanto	Monsanto
	5322938	1992	1994	2011	Monsanto	Monsanto

A commercial license is readily available from Bio-Rad for the use of the PDS/He1000 system in making transgenic plants. However, the Oxox and Hyg genes and the 35S promoters are patented by the major agricultural biotechnology firms Monsanto and Syngenta. Syngenta may be open to licensing because they have already made some of their technologies available for licensing. The status of licensing from Monsanto is unknown. One way to deal with this is to partner with one of the companies. In this case, Syngenta would be the logical choice because the company holds patents on the two genes used as well as a license from Monsanto for the use of the 35S CaMV promoter. The other option is to wait for the patents to expire. That approach may be risky because companies can renew patents by adding additional claims.

FURTHER STEPS

The transgenic plants must be approved by germplasm committees in order to be released as new cultivars. The transgenic plants are also likely to be evaluated prior to release by each state where the peanuts will be grown.

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