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Green Genetic Engineering Technology: The Use of Endogenous Genes to Create Fungal Disease-Resistant Grapevines

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ABSTRACT.

Use of genetic engineering technology to add disease resistance to otherwise desirable varieties is an attractive approach to establishing a *Vitis vinifera* L.-based agriculture in the Caribbean. Endogenous genes isolated directly from grapevine were engineered for enhanced expression and transformed into *V. vinifera* 'Merlot', 'Shiraz' and 'Thompson Seedless', plus *Vitis* hybrid 'Seyval Blanc'. Transgenic plant lines were selected via greenhouse screening based on resistance to powdery mildew. Outstanding lines were vegetatively propagated and established, along with non-transgenic controls, into USDA APHIS-approved field test sites at UVI St. Croix and UF/IFAS Florida in 2007. Vines grew rapidly and began to fruit in 2008. Based on preliminary results, certain transgenic lines exhibited significant resistance to black rot and anthracnose diseases compared to non-transgenic control lines. Because these are three of the most serious tropical/sub-tropical fungal diseases of *V. vinifera*, availability of resistant lines will provide new opportunities for production in the region. Our adaptation of endogenous genes to modulate disease resistance is a first step in creating "green transgenic plants" that contain only genetic elements from grapevine, thus eliminating concerns about incorporation of foreign genes in GMOs.

KEYWORDS: Grape, genetic engineering, disease resistance, *Vitis vinifera*

INTRODUCTION

Although established in the 1500s in the Caribbean region, *Vitis vinifera* L. grape has not become a major crop due to its susceptibility to tropical and sub-tropical diseases. However, consumption of grape is high. The US Virgin Islands imported 102,686 kg of fresh grapes worth $236,000 in 2004. Florida is the 2nd largest consumer of grape products in the US but imports nearly all of this commodity because the market prefers varieties of *V. vinifera.*

Grape is grown in the Caribbean region both as a small farm crop and in commercial plantings. Accurate data for acreages planted are not available because some production occurs in small farm settings that are not surveyed. Grape production in Florida is estimated to be about 2,000 acres, although the extent of many small farm plantings and U-picks have not been evaluated. Both Florida, located roughly between

25° and 30° N latitude, and the Greater Antilles at nearly 20° N latitude share similar growing conditions for grape production (Watlington, 1990).

Grape is a high-value crop, prized for its multiple uses as a fresh fruit and processed food products (jelly, juice, raisins & wine). A host of anti-oxidants and vitamins found in grape and its products help prevent heart disease and cancer. Physicians now recommend drinking a glass of wine each day as a health supplement. The health attributes of grape have become increasingly well documented. For example, grape is an excellent source of soluble fiber. These attributes have contributed to make grape the world's most important fruit crop, including a major crop in the United States (FAOSTAT, 2002). In particular, wine represents the single most valuable product produced from a fruit crop, where as little as a pound of fruit can be converted into a bottle of wine.

In Florida, the average price for a bottle of locally-produced wine is $9.00. There have never been "wine gluts" of locally-produced product and our existing market is so large as to accommodate significant growth. Along with the over-demand for existing local varieties, there is continual interest expressed by vintners and consumers in local production of conventional *V. vinifera* varieties to take advantage of the Florida market.

There currently exists an immense potential for increased cultivation of conventional grape products in the Caribbean region if improved varieties suitable for the region are developed. For example, Florida's wine market has an annual turnover of $1.5 billion and the state is typically ranked *second* to California in wine consumption (Business Wire, 2003). Other than the very small amount of local production (well under 1% of total consumption), all grape products sold in Florida and the rest of the Caribbean must be imported. This is due to the severe obstacle to local cultivation and production imposed by diseases (see below). In the Caribbean region, wine consumption by locals and tourists is high with most of the wine and other grape products imported from the United States and Europe. In the Virgin Islands, with a population of 100,000, fresh grape imports have been steadily increasing. Agriculture and tourism are the backbone of many economies in the Caribbean region and there exists a need for the expansion of high income agri-industrial crops such as grape, which grows well under permanent minimal tillage cultivation, even on degraded hilly terrain (Olmo, 1979). In addition to providing impetus for increase in grape-related industries, expansion of grape cultivation could provide supplementary income for many families that subsist in marginal rural areas and/or depend on irregular low wage jobs in urban-centered economies (Lewis and Thiele, 1979).

Although several thousand grape varieties are known, only about 50 comprise the majority of plants cultivated for production (Winkler, 1962). With few exceptions, these varieties originated in antiquity; they are highly prized for specific varietal qualities (i.e., genetically-fixed phenotypic traits), whether it be for fresh fruit (e.g., 'Thompson Seedless' is responsible for 40% of US grape acreage) or wine (e.g., numerous varieties like 'Cabernet Sauvignon', 'Chardonnay', 'Merlot'). Improvement via genetic transformation offers the possibility of adding only desirable traits to existing varieties without disturbing phenotypic attributes. It is not possible to utilize breeding to introgress single traits into elite varieties.

Genetic transformation allows transfer of single or a few genes into existing varieties without otherwise altering the genetic milieu of the plant and is particularly

useful in grape due to the aforementioned genetic constraints and needs. Transfer of genes across phylogenetically divergent organisms has resulted in creation of transgenic crops with high agronomic value but has also raised concerns because of their ecological and evolutionary novelty including the following: 1) Movement of transgenes to unwanted species and landraces and their impact on native ecology, 2) Potential health issues stemming from use of antibiotic resistance markers, and 3) Possible unforeseen allergenic qualities of transgenic protein.

Certain concerns about implementation of GMO technology might be allayed if endogenous genes from the grape genome itself were used for genetic improvement through over-expression strategies. Recent advances in plant molecular biology have greatly facilitated efforts to isolate plant genes associated with agronomic traits. Such native endogenous genes already exist in a plant's genome and can be recovered and analyzed. The re-engineered genes then are placed in a construct with a promoter chosen to provide desired expression characteristics and the construct is re-inserted into the desired plant. Such genes often modify metabolism in a manner similar to natural or induced mutations without causing genetic contamination (Strauss, 2003). The improvements achieved should be similar qualitatively to those obtained via traditional breeding approaches, but significantly better quantitatively, due to the ability to over-express desired proteins, both temporally and spatially. The present report provides progress to date of inserting such endogenous genes into grape and testing the resulting GM grapevines for disease resistance in field plots.

MATERIALS AND METHODS

Genetic engineering of grapevine was accomplished as previously described (Dhekney et al. 2008; Li et al. 2006, 2008). Two different endogenous genes were inserted into into *V. vinifera* 'Merlot', 'Shiraz' and 'Thompson Seedless', plus *Vitis* hybrid 'Seyval Blanc'. Gene *vvtl-1* is the *Vitis vinifera* thaumatin-like protein gene 1, and *eg*-2 is a proprietary endogenous gene, both cloned from grapevine via standard techniques.

All genes were placed into a proprietary bi-directional duplex promoter (BDDP) system (Li et al. 2004) that also contained an enhanced green fluorescent protein gene (*egfp*) for visual detection of transformed cells, tissues and plants. The *egfp* gene was fused to a neomycin phosphotransferase gene (*nptII*), which confers resistance to the antibiotic kanamycin for selection of transgenic cells. The resulting *egfp/nptII* fusion gene was previously described by Li *et al.,* (2001).

Transformed plants were recovered and independently transformed lines were evaluated in a greenhouse for resistance to powdery mildew disease. Plants that were most resistant to disease were selected, cloned to produce replicates and established in field plots in Florida and the US Virgin Islands.

RESULTS AND DISCUSSION

Transgenic grapevines and non-transgenic controls were placed into USDA APHIS approved field plots at the University of the Virgin Islands (UVI), St. Croix campus in January 2007 and at the UF/IFAS Mid-Florida Research and Education Center (MREC) at Apopka Florida in April 2007. All plants grew rapidly. Minimal fungal disease control was administered. In particular, Ridomil^{TM} was applied as needed to

control downy mildew, an oomycetous fungal disease that the subject genes would not control. At the UVI site, a significant problem encountered in larger vines was chlorosis, which was inferred to be cause by pH imbalance due to a limestone under layer. In 2008, certain vines at both sites produced fruit, which was somewhat unexpected because grapevine typically requires 2 – 3 years of growth for maturation to fruiting stages. At the MREC site, resistance to black rot and anthracnose diseases was observed in 2 out of 5 lines tested, when compared to controls. During this time, the vines had received only Ridomil™ control. However, as summer progressed, resistance under conditions of no fungal disease control began to break down. In August 2008, we instituted a typical broad spectrum disease control regime and will continue to evaluate resistance for the remainder of the 2008 season. Several seasons will be required to properly evaluate the plants over time. Unfortunately, TSTAR funding was curtailed for non-invasive pest research in Florida, such that renewed support will come from other sources.

It is important to note that the genes used in this study to control fungal diseases are native to grapevine. For example, VVTL-1 belongs to a group commonly known as PR (pathogenesis related) protein genes, several of which have been cloned and expressed in plants. They have been grouped (PR 1 to PR 5) based on their structure and mode of action (Punja 2001). A number of PR proteins exhibit antifungal properties, which variously cause inhibition of fungal cell wall synthesis and/or a disruption in cell wall structure leading to cell lysis (Seliterennikoff 2001). PR proteins are classified into different groups including cysteine rich antimicrobial peptides (Broekaert et al. 1992; Cammue et al.1995; Epple et al.1997; Thevissen et al. 2000), glucanases and chitinases (Nielsen et al.1997), chitin binding proteins (Van Damme et al.1999) and thaumatin like (TL) proteins (Selitremmikoff 2001). The PR proteins have been cloned from a number of commercial crop plants like rice (Agrawal et al. 2000), wheat (Rauscher et al.1999), barley (Byrngelsson et al.1994), tobacco (Ponstein et al. 1994), tomato (Van Damme et al.1999) and other plants.

The PR 5, or thaumatin like (TL) proteins, share significant amino acid homology to thaumatin (Selitremmikoff 2001). TL proteins inhibit a wide range of plant pathogens *in vitro* (Selitremmikoff 2001) and have been cloned from several plant species (Huynh et al. 1992; Hu and Reddy 1997; Cheong et al. 1997; Koiwa et al. 1998). TL proteins are known to be differentially expressed in reproductive tissues such as pistils and ripening fruits (Neale et al. 1990; Vu and Huynh 1994; Fils-Lycaon et al. 1996; Barre et al. 2000). The UF/IFAS grape biotechnology laboratory cloned *V. vinifera* thaumatin-like protein (VVTL-1) from grapevines derived from embryogenic cultures that were subjected to *in vitro* selection with the culture filtrate of *Elsinoe ampelina*, the causal agent of grapevine anthracnose (Jayasankar et al. 2003). Protein produced from VVTL-1 significantly inhibited *E. ampelina* spore germination and hyphal growth *in vitro*. Plants regenerated from *in vitro* selected cultures similarly inhibited fungal growth in leaf assays. Similar results have been obtained with VVTL-2 cloned from grape, which was expressed in leaves and ripening berries in response to powdery mildew infection (Jacobs et al.1999; Tattersall et al. 1997) and exhibited antifungal properties (Salzman et al. 1998).

Gene *eg-2* is another endogenous gene from grape that produces a naturally-occurring protein. We recorded resistance to powdery mildew in greenhouse screening studies of plants transformed with vvtl-*1* or eg-*2*, which led to the current field trials.

Both VVTL-1 and EG-2 proteins occur naturally in ripened berries of grape. As such, there is no reason to believe that there are any human health-related toxicological issues associated with its expression in transgenic grapevines.

As techniques of molecular biology have developed and become refined, so has our knowledge of genomics (Li, 2005). In particular, our understanding of how genes function and regulate plant growth and development has increased immensely. This has led to our ability to identify the endogenous genes that render disease resistance to plants. Our use of endogenous genes in this manner is novel for grape, but not for other plants. However, their over-expression in a bi-directional duplex promoter system does constitute a new approach to re-engineering plants that contain only native DNA and proteins. This "green" approach should alleviate many concerns expressed by producers and consumers regarding the use of plants modified with molecular techniques vs. traditional plant breeding.

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