Opportunities for New Methods of Genetic Improvement of Grapevines

Alan Wei, Ph.D. and James A. Stamp, Ph.D.

FOR THOSE TASKED WITH securing clean stock for the establishment of (healthy) virus-test negative vineyards, it is frustrating to complete a new spring planting while vines in adjacent established vineyards show fall reddening. In some cases entire neighboring vineyards may turn red, or it may be single vines located through the edge rows of a particular block. The chances are that these vines are reddening due to the presence of either Grapevine Leafroll Virus type 3 (GLRaV-3) or Grapevine Red Blotch-associated Virus (GRBaV). Other viruses, especially other leafroll types and Corky Bark virus (GVB), may be involved in the development of reddened foliage, but GLRaV-3 and GRBaV are most likely responsible because they are so rapidly and effectively transmitted by, respectively, mealybug and treehopper insect vectors. Of course, the likelihood is that white varietals are similarly infected with leafroll and Red Blotch, but their disease status is hidden because they do not possess the chemical pathways to synthesize red anthocyanin pigments.

Identification of clean "virus test negative" grapevine stock is something that can be accomplished by careful examination and testing of nursery stock mother blocks. Clean stock can be identified by examining and testing CDFA-certified nursery Increase Blocks (www.cdfa.ca.gov/plant/pe/nsc/docs/regs/ccr_3024_grapevine.pdf) in October/November of the year prior to grafting. Late fall is the time when the Increase Block (IB) scion vines are most likely to show symptoms of stress (virus, mechanical, insect, nutritional, etc.), and it is a good time to eliminate vines, rows and blocks that do not appear healthy. Appropriate testing of apparently healthy vines will permit identification of clean stock that can be used for propagation. When selecting non-certified scion materials for propagation, the chances of finding infected vines are greater. However, careful observation and rejection of questionable stock can result in the identification of clean, virus test negative material. Historically, CDFA-certified stock has proven to be contaminated with economically important viruses (Stamp, 2010; Stamp and Wei, 2013, 2014).

If clean material of a particular selection cannot be identified, submission of dormant cuttings to the virus elimination program at Foundation Plant Services at UC Davis will result in the production of virus test negative plants in approximately three years.

Grapevine species are highly heterozygous and readily cross with close and distant genetic relatives, thus resulting in the varieties that are cultivated worldwide today. The genetic variability inherent in grapevine stock is the underlying reason why grapevines are propagated vegetatively: to maintain the unique genotype of fruiting varietals.

Dr. James A. Stamp is a Sebastopol, California scientist who specializes in the critical evaluation of vineyard performance issues and grapevine nursery plant material quality and propagation. He has more than 25 years of viticulture experience and established Stamp Associates after founding Novavine grapevine nursery, working in the plant biotech industry and completing a post-doctorate at UC Davis. Stamp Associates advises growers and winemakers in the U.S. and overseas in the establishment and management of high-quality. pathogen-tested vineyards, Contact Dr. Stamp at james@jamesstamp.net.

Dr. Alan Wei is the general manager of Agri-Analysis LLC, located in West Sacramento. He has more than 25 years' industry experience in developing microbial detection technologies, ranging from high sensitivity ELISA, PCR, qPCR, high throughput screening and in-field methods. He has more than 20 issued United States' Patents in this and related areas. Dr. Wei enjoys interacting and working with growers to learn from them and help identify and solve problems for them. Agri-Anlysis is a laboratory that tests for Red Blotch and other grapevine viruses to help growers protect their investments. Contact Dr. Wei at apwei@agri-analysis.com.

Targets for Non-traditional Improvements in Grapevine

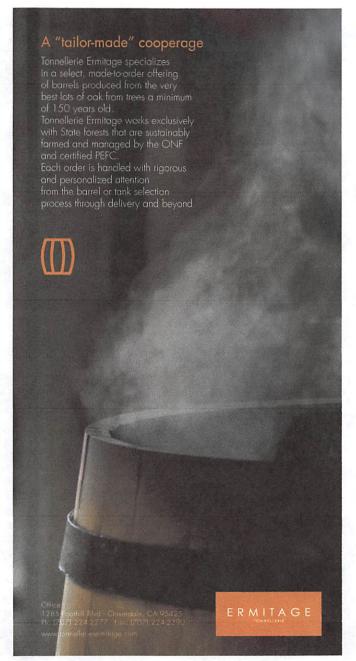
Foodstuffs containing the products derived from genetically modified organisms (GMO's) abound in U.S. chain supermarkets (*Time* magazine 2015), and many fast food restaurants typically serve portions containing some GMO food derivative. So what's all the fuss? Why is there this seemingly insurmountable kickback against bringing useful and popular technology to one of the, arguably, most technologically primitive plant species cultivated? Technologically primitive? Not all grape products are equal. Considered unacceptable in wine varieties, variability and unique genetics are desired in table grapes where novelty is key and large seedless berries of distinctive flavor or off-season harvest readiness are highly sought after.

The techniques of winegrape propagation are virtually unchanged since Roman times when varieties such as Shiraz (Syrah, Hermitage) were already distinguished. The Romans and their forebears understood that maintenance of distinct varietal properties was only possible by vegetative propagation. Striking of cuttings from favored vines occurs readily—and with little improvement this is still the basis of 21st century grapevine propagation.

The technology for winegrape improvement by using non-traditional techniques has existed since the late 1980s, and it is the perceived public rejection of GMOs that has prevented the commercialization of this technology in grapevines. Desirable winegrape improvement targets are many, and the methods that would be used have been proven in a wide range of crops, including potato, soybean and papaya. Perhaps one of the simplest targets for crop improvement is the development of resistance to important viruses, and of course, this would also be one of the top targets for grapevines.

The use of planting materials from virus-tested certified nursery stock is currently the primary virus control option. These certified stocks are derived from materials originated from foundation vineyards maintained by Foundation Plant Services at UC Davis. In vineyards where infected vines are present, management strategies rely on the elimination or rogueing of virus-infected vines and the reduction of insect vector populations (mealybugs and tree hoppers) through the application of systemic insecticides. The level of mealybug control required to limit virus spread is not known although encouraging results were recently reported (Wallingford et al, 2015). Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance (Oliver and Fuchs, 2011).

Very recently and disturbingly, leafroll virus 3 and Red Blotch have been detected in CDFA Protocol 2010-certified Foundation vines at FPS's Foundation Block west of UC Davis and in Protocol 2010 rootstock Increase Block vines under cultivation at a California nursery.



New Plant Breeding Technologies

Although "traditional" genetic engineering techniques developed in the 1980s were revolutionary at the time and resulted in products such as "Roundup-ready" crops, the public backlash against these approaches has soured the opportunities for a new generation of techniques—collectively referred to as New Plant Breeding Technologies, or "green biotechnology" (Costa et al, 2017). These technologies do not rely on the introduction of foreign genes into the target organism but rather the clever control of expression of native genes that theoretically could have been introduced through traditional breeding. These methods, called cisgenesis or gene editing, are ideal for improvement of highly heterozygous crops with long juvenile phases, such as grapevines, and result in the targeted expression of native genes far more rapidly than achievable through standard breeding.

Pierce's Disease Resistance

Pierce's disease (PD), caused by the bacterium *Xylella fastidiosa* (Xf), is an important disease of grapevines in California and many winegrowing regions. In the vineyard, Xf is spread by sharpshooters: insect vectors that feed on infected vegetation and then inject the bacterium into the sap of healthy grapevines. The disease is also graft transmissible if the scion and/ or rootstock materials are infected with the bacteria. The bacterium multiplies in the xylem, eventually reducing the movement of water throughout the vine, resulting in disease and/or death.

Conventional breeding methods can be accelerated by modern molecular tools such as PCR-based marker-assisted selection (MAS), for the rapid identification and selection of new varieties. Using this technique, Dr. Andy Walker's group at UC Davis has developed several new Pierce's Disease (PD) resistant varietals (Riaz et al, 2009). By scouring North America for wild and weedy Vitis species Walker identified candidate grapevine germplasm possessing natural resistance genes against Xf. The Mexican grape species Vitis arizonica was found to be the most promising candidate, possessing a single dominant PD-resistant gene. By using the molecular techniques of MAS, Walker was able to backcross wild species of grapevine with popular varieties, such as Cabernet Sauvignon, Chardonnay and Pinot Noir. By repeated backcrossing and by testing and selection of seedlings for PD-resistant traits, Walker was able to create PD-resistant grapevine plants that were up to 97 percent genetically identical to the backcrossed varietal parent. These plants are now undergoing trials, and wines derived from them have been favorably reviewed. Because PD is such a serious problem in many premium wine regions of California there is strong demand for these resistant plants, especially for replanting diseased vineyard rows adjacent to riparian habitat. The hope is to establish PD-resistant border rows to prevent the movement of the disease from these rows inward to non-resistant traditional varieties

Biocontrol of Pierce Disease

Bacteriophage, also known informally as phage, is a virus that infects and replicates within a bacterium to eventually kill the bacteria. Phage therapy has been used to treat or prevent pathogenic bacterial infections in human medicine, food and agricultural applications. For example, the USDA and FDA have approved the use of several lytic phages specifically for bacterial pathogens, such as *Escherichia coli* O157:H7, *Salmonella spp.* and *Listeria* monocytogenes in foods, produce and on food processing surfaces (Sharma, 2013). It is quite likely that the fresh salads at the local market have been treated by a bacteriophage product. Since *Xylella fastidiosa*, the casual agent of Pierce's Disease is a bacterium, why not treat PD with bacteriophages?

Researchers at Texas A&M University evaluated the therapeutic and prophylactic efficacy of a phage cocktail composed of four virulent (lytic) phages for control of PD. Xf levels in grapevines were significantly reduced in therapeutically or prophylactically treated grapevines. PD symptoms ceased to progress one-week post-therapeutic treatment and symptoms were not observed in prophylactically treated grapevines. Cocktail phage levels increased in grapevines in the presence of the host. No phage-resistant Xf isolates were obtained, and Xf mutants selected for phage resistance in vitro did not cause PD symptoms. These results show that phages have great potential for biocontrol of PD in grapevine and other economically important diseases caused by *Xylella fastidiosa* (Das et al 2015).

In what appears to be a promising research track, Dr. **Don Hopkins'** research group at the **University of Florida** is studying the protection or "inoculation" of uninfected grapevines from PD by injecting them with a benign strain of *Xylella fastidiosa* (Hopkins, 2005). Field trials are currently underway in California and in southwestern USA.

Powdery Mildew Resistance

Grapevine powdery mildew (PM) caused by the fungal pathogen Erysiphe necator, is a major fungal disease in California and all grape-growing regions of the world. At present, fungicide applications are the main control method for powdery mildew but are costly, labor intensive and environmentally unsustainable. Agri-Analysis, Inc. has frequently found fungicide-resistant strains of E. necator in client samples: resistant to demethylation inhibitors (DMI) and quinone outside inhibitors (QOI). Dr. Walt Mahaffe's group at USDA Agriculture Research Service surveyed Napa Vineyards for QOI-resistant PM and found over 85 percent of samples contain QOI resistant E. necator and more than 62 percent were also resistant to at least some DMIs (e.g. mycobutinol and tebuconezol). Needless to say E. necator is a rapidly evolving fungal pathogen with significant economic impact in the wine industry. Dr. Andy Walker's group at UC Davis has investigated powdery mildew resistance in multiple accessions of the Chinese grapevine species Vitis piasezkii. Chinese Vitis species have attracted attention from grape breeders because of their strong resistance to powdery mildew and their lack of negative fruit quality attributes that are often present in resistant North American species. Walker discovered two distinct powdery mildew R loci designated Ren6 and Ren7 in multiple accessions of this Chinese grape species. Their location on different chromosomes to previously reported powdery mildew resistance R loci offers the potential for grape breeders to combine these R genes with existing powdery mildew R loci to produce grape germplasm with more durable resistance against powdery mildew (Pap et al, 2016). However, there may not be a single magic bullet against PM.

"Even with plant resistance you will still need to use fungicides to protect the investment in the resistant varieties. *E. necator* is constantly evolving and has circumvented everything we have thrown at it to date. I doubt this will change in the future. Look at any other crop that deploys plant resistance traits—it is an arms race. The pathogen gets around the trait, and breeders develop another. The difference is we can't afford to replant very often," said Dr. Walt Mahaffee.

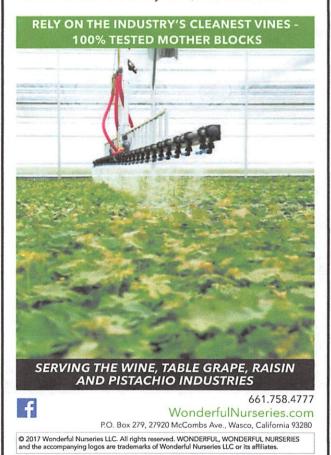
Innovative "green" biotechnologies to breed virus-resistant new crops can complement traditional breeding and genetic engineering methods and address their inherent limitations. Resistance can be achieved by a number of methods, including interference (RNAi), CRISPR and anti-viral antibodies. Some examples of the most recent developments in plant improvement are described below. These examples are not exhaustive, and interested readers should read more comprehensive reviews by Costa et al, (2017).

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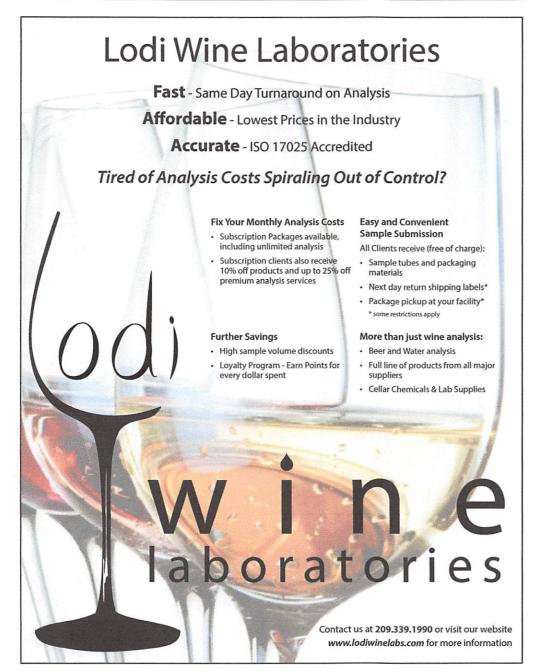
Fanleaf Virus Resistance

The human immune system consists of two fundamental pillars—the innate, general defense and the acquired, specialized defense. Both systems work closely together and take on different tasks to defend against foreign substances, such as viruses and toxins. Antibodies are a vital component of both pillars, especially in acquired defense. Plants, on the other hand, do not possess antibodies to eliminate viral infection although they have an innate defense system. Advances in recombinant antibodies have made it possible to introduce crop resistance by the expression of pathogen-specific antibodies that can bind and neutralize the virus and therefore hinder its ability to propagate inside plant cells.

Although traditional antibody approaches have been attempted for plant protection with limited success, the research community has been reinvigorated by a new class of antibodies called nanobodies derived from the camelid (Llama) family. These are single-domain antigen-binding fragments of heavy-chain only antibodies. Since their discovery in 1994, there has been intense interest in their use as therapeutic drugs for various human diseases, including cancers and infectious diseases. Recently, French researchers identified a set of nanobodies specific to grapevine fanleaf virus (GFLV) that confer strong resistance to GFLV upon stable expression in the model plant *Nicotiana benthamiana* and also in grapevine rootstock 41B. They showed that resistance was effective against a broad range of GFLV isolates

but not against any isolates of its close relative *Arabis* mosaic virus. They also demonstrated that virus neutralization occurs at an early step in the virus life cycle, prior to cell-to-cell movement (Hemmer C. 2018).

"Nanobodies are exciting in that they are one-tenth of the size of a traditional antibody. This means they can be more readily expressed in plants. Despite their small molecular size, they are resistant to organic solvent and heat. Some nanobodies have been shown to maintain activity even after exposure to 100° C heat treatment. These properties make nanobody-mediated resistance to plant viruses an attractive antiviral strategy," said Distinguished Professor Bruce D. Hammock who runs a nanobody research program aimed to develop next generation diagnostic and therapeutic tools in the Department of Entomology at UC Davis. Funded by a USDA SBIR grant, Agri-Analysis has developed a set of nanobodies for the viral coat protein of GLRaV-3. Work is in progress to use these nanobodies for the possible protection of grapevines from the GLRaV-3 virus.



Resistance to Leafrollassociated Virus 3

RNA interference (RNAi) is a biological process in which RNA molecules interfere with gene expression by neutralizing targeted mRNA molecules. Plants use RNAi to regulate gene expression and defend themselves against infection by viruses. Researchers have engineered RNAi constructs in plants to specifically down-regulate the expression of target viral genes. Papaya and plum are two successful examples of RNAi-mediated plant protection against viruses. Gonsalves et al. developed papaya resistant to papaya ringspot virus (PRSV). The research to obtain PRSV-resistant papaya involved isolating and characterization of the coat protein (CP) gene of PRSV, transforming embryogenic calli of a Hawaiian papaya cultivar with a PRSV CP construct, producing transgenic papaya plants expressing the PRSV CP construct and screening transgenic plants for resistance to PRSV (Gonsalves 2007). Following extensive research and close interactions with growers and consumers, PRSV-resistant papaya was commercially released. It was the first transgenic fruit crop to ever be released. PRSV-resistant papaya cultivars have been grown in Hawaii for the past 20 years. Similarly, "HoneySweet" is a transgenic plum cultivar that is resistant to plum pox virus (PPV) based on RNAi. After extensive studies under greenhouse and field conditions, HoneySweet plum was cleared for cultivation in the U.S. (Scorza et al 2016).

Resistance to the Grape Mealybug

RNAi can also be applied to engineer resistance against insect pests or insect vectors of pathogens by targeting specific insect genes. For example, RNAi constructs directed to down regulate key insect genes can be expressed in a plant, ingested by insects upon feeding and affect their viability (Galdeano et al 2017). Specifically, RNAi targeting osmoregulation genes of phloem-feeding insects such as aphids, whiteflies and psyllids have shown to significantly increase mortality rates of these insect pests by depriving them of water (Scott et al, 2013).

"RNAi represents a paradigm shift for grapevine protection against viruses and their insect vectors," said Dr. Marc Fuchs, Cornell University. Funded by a grant from American Vineyard Foundation (AVF), Dr. Fuchs and his colleagues are working on a RNAi approach against grapevine leafroll-associated virus 3 (GLRaV-3) and grape mealybugs (GM) simultaneously. Given Cornell's record of success in research and development of the world's first virus-resistant transgenic fruit crop, we expect Fuchs' interdisciplinary team to be successful in providing grapevines with effective and durable resistance against GLRaV-3 and GM.

Red Blotch Resistance

CRISPR-Cas9 is a gene-editing tool that allows researchers to precisely alter DNA sequences and modify gene function. Its potential applications include correcting genetic defects, diagnosing and treating diseases and improving traits of crops. This genetic tool can be programmed to target certain sequences of an organism's DNA, snip them out and even replace them with desirable DNA sequences. Researchers have used the CRISPR-Cas9 gene-editing tool to eliminate HIV from infected cells (Kaminski et al 2016). When HIV is not actively spreading throughout the body, the virus lays dormant inside cells where it can escape detection and is hard to get rid of. Grapevine red blotch virus (GRBV) may share similar properties in that when the virus

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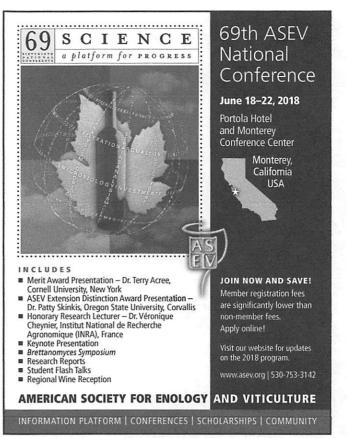
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is latent it lays low inside cells, especially during dormant season. If CRISPR can remove HIV from a cell's genome, it might help tear GRBV from its hideout as well if properly designed. One approach is to make a transgenic plant to express CRISPR-Cas9 as a transgene to edit the incoming virus. Another approach is to use CRISPR-Cas9 to modify essential host factors that are required for virus replication and movement. These approaches have been used to inhibit virus accumulation and introduce mutations in beet severe curly top virus (BSCTV) which a geminivirus the same genera as red blotch virus. Transgenic Arabidopsis plants overexpressing sgRNA-Cas9 were shown to be highly resistant to BSCTV infection (Ji et al 2015). For GRBV and leafroll viruses, no such work has been demonstrated yet. Research work is needed in this front. "CRISPR is a powerful tool due to its high specificity, efficiency and versatility. Its potential in cell and molecular biology research is far beyond DNA cleavage, and its usefulness in crop improvement will likely be limited only by our imagination," said Dr. Tristan Eifler, Ph.D., Research Associate at Agri-Analysis.

Summary

Driving through the Napa or Sonoma valleys in fall is reason enough to consider technology to provide resistance to the reddening diseases of Red Blotch and Leafroll viruses. Although clean stock can be sourced for planting, it is to be expected that these vines will be planted adjacent to virus-infected vineyards. It is not unusual to see Red Blotch and Leafroll symptoms in the second or third leaf of planted clean stock located adjacent to historically

virus-infected blocks. Leafroll-3 and Red Blotch are rapidly transmitted by their respective vectors, and it is understood that, for example, movement by wind or flight of the Three Cornered Alfalfa Tree Hopper into a clean block from an adjacent contaminated block can result in individual Red Blotch contamination of vines wherever the hopper alighted and momentarily fed. Similarly, it is known that contaminated Grapevine Mealybug can transmit LR3 with no more than 20 minutes' feeding on clean stock.

Fortunately, winemakers like to shift varietals, try new clones or rootstocks, switch vine densities or row orientations and so there is always the opportunity to plant more clean stock in contaminated locations. This is never truer than with Fanleaf virus where there is only one rootstock—VR039-16—that offers good resistance to the nematode *Xiphinema* index, the virus vector. This rootstock is planted in a virtual monoculture in locations such as Oakville and Rutherford in the Napa Valley and parts of Geyserville, Sonoma County. There is currently no good alternative to VR039-16, with the most promising alternative fanleaf virus-resistance rootstock, GRN1, being virtually impossible to propagate.

The utility of Leafroll- and/or Red Blotch-resistant vines is without question. Winegrowers seem content to continue to replant on a 10-15-20 year basis, and the arguments for revised viticultural specifications are real. However, what if vines were resistant to virus? This would represent a huge savings to the grower—not only if just considering that it takes up to five years for new plantings to reach expected production parameters.

The argument most frequently raised against genetically modified vines is that introduction of foreign DNA/RNA, protein will alter the vital



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characteristics of the target vine. How could a Cabernet Sauvignon vine expressing the coat protein gene of GLRaV-3 produce wine that could be called Cabernet Sauvignon? However, it is worth considering that like all complex organisms grapevines are awash with foreign genetic and proteinaceous materials embedded deep within the mechanisms of cell division and vine replication-either vegetatively or via sexual propagation. This is especially true when the vine is infected with viruses, fungi or bacteria, pathogenic or not, where multiple foreign genes are be expressed simultaneously by the plant. Grapevines lacking foreign DNA would most likely be impossible—because the natural biosphere associated with both the external vine and the internal vine tissues are essential in providing a balanced plant that can withstand various external threat factors. In fact, "microbiome" is an exciting emerging field of research to study the vast population of microbes in humans and plants and their function in protecting hosts against germs, breaking down food to release energy and producing vitamins etc. (Schlaeppi et al. 2015)

Industry Comments:

During the most recent survey of winegrape growers by the American Vineyard Foundation (AVF), 75 percent of respondents support the academic community pursuing biotechnology/gene modification research, 57 percent favor using genetically enhanced materials/products, and 77 percent support AVF-funded biotechnology research. "Growers affirmative

responses to our biotechnology research survey questions point to their increased awareness of biotechnology as an important tool for the future of our industry to enhance sustainability, disease management, productivity and profitability," said **Tony Stephen** AVF chairman and chief planning officer of **Scheid Vineyards**.

"NVG's mission is to preserve and promote Napa Valley's world-class vineyards. To this end, we think biotechnology as an important tool in sustainability, productivity and disease management. We echo the AVF comments above and would like to see more research done in this area to let our growers have more options and choices when it comes to disease resistance," said Jennifer Putnam, executive director of Napa Valley Grapegrowers Association.

Dan Martinez, Martinez Orchards: "In my experience, when GMO is mentioned as a potential research topic, a representative from any company with international ties/sales may want to see the research but doesn't actually want a product. We lack a consensus as to whether gene-editing is viewed the same as traditional GMOs. Even if it is accepted then there is the discussion as to whether Cabernet Sauvignon is still Cabernet Sauvignon if you have edited the genes and then what do you put on a label, I personally would like to see more research done to prove the concepts as I think it is currently the only long term solution with potential. At the end of the day, the people selling wine need to figure out if their consumers will buy wine made with grapes from a GM plant, rootstock or scion."

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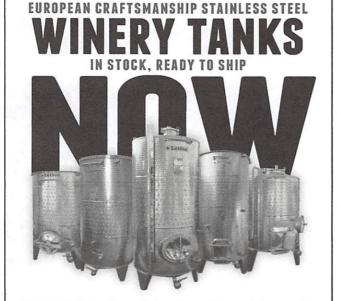
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François Guillaume, Guillaume Grapevine Nursery: "In Guillaume Nursery group, we don't make any study on GMO. The public opinion is really scared in Europe due to some real risk and some disinformation. People agree that GMOs can be a good way to care human health but they don't think that it is something safe to do it on plants. The Monsanto firm made a terrible publicity with their corn resistant to Roundup. They create varieties resistant to pesticide and now people are confounding resistance to pesticide and resistance to disease. In the agricultural sector, people are too divided. Some are saying 'We use this technique to care for people, why could we not do it with the plants?' All the studies in Europe were stopped a few years ago because in my own opinion, most of the researches were based on resistance to pesticide and not to disease resistance. As a lot of pesticides are going to be prohibited in Europe, they will have to move this way if to stay competitive but things are moving very slowly to protect the public. There is not a lot of information on the current studies and people who are working on it don't like to talk about their researches. They don't want to be considered as a bad scientist trying to make money on the health of people. In my opinion, GMO can be one of the future solution to lower the impact of disease control treatments on the environment.

We know that some vine varieties are naturally resistant to mildew. Using these genes and incorporating them into other vine variety would be a possible. These studies need to be very controlled and have to be done ethically. Creating a Cabernet Sauvignon resistant to mildew would be for sure a great improvement in viticulture but two things need to be studied carefully:

- · Are we going, after few years to create another type of mildew more resistant to the current pesticide if it mutates? This is what happened with the Roundup.
- · Is it going to be safe for the consumer and will the consumer buy the wine knowing this?"

John Duarte, president, Duarte Nursery, Inc. "Grape growers face numerous challenges that can be addressed through GMO technologies. AS far back as the mid 1990s, there were grape fanleaf virus-resistant grape rootstocks produced through GMO technologies. Virus resistance in other crops has been accomplished using GMO very successfully. Fanleaf virus is a devastating problem to specific farms where the soil has become infected with virused nursery stock or through local movement. Today, new potent viruses GVLR3 and GVRBaV, are spreading broadly through winegrowing areas due to established insect vectors. In recent years the spread and economic impact have accelerated greatly. GMO grapevines can very likely be used to address these newly problematic viruses. Pierce's Disease, fungal diseases and insect resistance are additional feasible targets.

"Most biotechnology successes have been in annual crops where the large acreages and annual replanting are business advantages-companies can offer their innovations to 100 percent of the market each year. Major crops, such as corn and soy are produced more abundantly and sustainably than ever before in history. Orchards and vineyards are typically replanted on a 20-25 year cycle. That means 4 to 5 percent of the industry is available for sale of new genetics each year. Vines and trees also have multiyear preproductive periods before the first crop revenues are collected. Financial returns for innovation investments in tree and vine crops are inherently challenging.

"GMO plants now feed the world. The agricultural biotechnology industry delivered the safest technological revolution in the history of the world. Global demand for fruit and nut crops is expanding with global economic development. A rich and diverse diet for all humans is more possible every decade. This will be best accomplished with an objective regard for those technologies and market systems that can deliver variety and abundance.

"All people deserve an honest political system and a pro human ecology policy. The technological and market potentials of GMO food has been hampered by business realities that include vague public acceptance controversies. Subjugating a large portion of the world's people to a less abundant and less diverse diet for sentimental or vague causes is disingenuous and antihuman. It is now time for those who would slow the further applications this technology to clearly argue their issues." WBM

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