Vintage genetic engineering

With the genome of the grapevine in hand, how likely are enologists and wine growers to resort to genetic engineering to tackle the problems facing viticulture? Laura DeFrancesco reports.

The complete genome sequences of two grapevine cultivars now grace the public genome databases, bringing the latest sequencing technologies to bear on one of the oldest uses of biotech—winemaking. Last December, a group of academic and industrial researchers published the sequence of a cultivated clone of the wine grape Pinot Noir (*Vitis vinifera*)[1], just four months after the completion of the genome of a highly inbred line of the variety by a consortium of French and Italian researchers[2]. The grapevine genome now joins the august group of completed plant genomes, being the first fleshy fruit crop and one of only four flowering plants to be sequenced.

Green technology. Transgenic Shiraz grapes display resistance to powdery mildew, an important disease of grapes. The grape on the right was transformed with a proprietary gene that was isolated from grape and reengineered before reinserting into grape. Source: Dennis Gray, University of Florida.

This accomplishment has been hailed by researchers around the globe as a boon to grape breeders and vintners, who must bathe their vineyards annually in pesticides and fungicides. (Grapes are among the most heavily sprayed of all crops, requiring an average of 12 applications in a season.) But the question remains whether modern technologies for improving wine grapes will be embraced by the industry, given the special place that the culture of winemaking holds among both growers and consumers, not to mention the resistance to anything genetically modified (GM) in Europe, the historic center of viticulture.

The view from the chromosomes

Grapes turned out to be an easy mark for sequencers, having a mere 487 megabases of DNA, as compared to other crop plants, like corn, that have five times that number. However, the genome is highly heterogeneous, owing to its outbreeding by wind and insect pollination. That led the Italian-French Consortium, coordinated by the French National Institute for Agriculture Research in Colmar, Genoscope, the French national sequencing facility in Evry, and the Italian Ministry of Agriculture's VIGNA-CRA initiative to choose a line obtained by a series of backcrosses that is near homogenous (designated PN40024). According to microsatellite analysis, PN40024 is 97% homogeneous, whereas the starting material has as much as 26% heterozygosity. The other group, which was led by Riccardo Velasco at the Istituto Agrario di San Michele all'Adige, Italy, chose rather to embrace the heterogeneity to create a detailed single nucleotide polymorphism (SNP) map—they located close to two million SNPs on the 19 grapevine chromosome pairs. The two groups also applied different sequencing technologies: the Genoscope group used basic Sanger sequencing, whereas the Adige group combined Sanger sequencing with the latest method for large-scale sequencing, sequencing by synthesis (SBS), pioneered by the US biotech company 454 of Branford, Connecticut, which participated in the study. This methodology was particularly useful because of the need for greater oversampling with the high degree of heterogeneity of the natural line, and represents the first application of the SBS technology to a complex genome, according to Brendon Hill, director of communications at 454.

The picture emerging from the genome from both groups should be encouraging to grape geneticists and breeders. Found amid the sequence were over 150 genes for aroma and flavor, three times the number found in other flowering plants. In addition, by carrying out a BLAST search of the sequence with known plant gene sequences for various pathogen-resistant genes, numerous grape homologs have been identified, which bodes well for either conventional or transgenic approaches to improving grape crops. Whereas most transgenic grapes with increased pathogen resistance in field trials have genes inserted from other species, researchers can, with the grape genome sequence in hand, more easily move to homologous genes, with the hopes of making GM wine more palatable to otherwise reluctant growers and consumers.

The creation of detailed genetic and SNP maps should be welcome new tools for geneticists using marker-assisted breeding and selection. Anne-Françoise Adam-Blondon, a principle investigator in the Genoscope group and coordinator of the French network on grapevine genomes and breeding, says the availability of molecular markers should speed up the identification of grapevine genes with commercial applications, like pathogen-resistant genes. To ensure this, her group continues to develop tools that will facilitate the exploitation of the available genetic resources, such as the alignment of the grapevine genome with the genome of other resistant species. "We can now put our energy that was once put into the development of markers into fine phenotyping for functional genomics studies and to study sequence variation and its effect on agronomic traits in natural resources," she says.

Adige's Velasco echoes these sentiments. "Our goals are on assisted breeding or more advanced breeding by design. Therefore, the huge amount of markers developed in the project and their link to genes, and consequently phenotypes, is fundamental." He adds, "The viticulture and enology world is not ready to accept GM grapes."

Transgenic grapes in trials

Nonetheless, field trials on transgenic grapes have been going on for over a decade (**Table 1**), with some of the earliest in France, until fear of a backlash from the public caused the French champagne producer, Moët & Chandon, an early adopter of the technology, to abandon its program in 1999 (ref. 3). The pace appears to have quickened in the past few years: 43 permits for field trials with transgenic grapes have been issued by US Animal and Plant Health Inspection (APHIS, Raleigh, North Carolina) since 1998, 11 in the past two years alone, according to APHIS's records (http://www.isb.vt.edu/CFDOCS/fieldtests1.cfm). This has caused some critics of genetic engineering, displaying a curious sort of pessimism (or optimism depending on your point of view), to gird their loins for a deluge of commercial GM wines hitting the market[4].

In addition to US field trials, transgenic grapes have undergone field testing in Italy (modified auxin production), Germany (fungal resistance), France (grapevine fanleaf virus resistance) and Australia (fruit quality and color modifications). Chilean researchers have been particularly productive with transgenic grapevines, having developed technology that allows them to produce literally thousands of lines. With the support from both the public and private sectors, researchers at La Platina Research Center of the Chilean Institute for Agricultural Research (Santiago) have created transgenic table grapes with enhanced fungal resistance. (Chile is a major exporter of table grapes, being the main supplier of grapes to the US during the winter.) More than 700 transgenic lines are in field trials, and after three seasons, the Chileans have obtained fruit from some of their transgenic lines, though the yields are highly variable. According to Patricio Hinrichsen, a leader of the group, they are targeting table grapes for the American market, sensing that wine would be a harder nut to crack. "Wine producers use tradition as a marketing tool," he says. With table grapes, "it's a completely different world…. There will be people who will not be worried about these issues."

Built-in pest resistance

Grapevine transformation systems have lagged behind other plants, in part because of the difficulty of establishing embryonic cultures, according to Dennis Gray of the University of Florida. Gray and colleagues have developed a system for culturing and transforming grape with *Agrobacterium* that they have applied to over 30 different varieties. Meanwhile, a group at the New York Agricultural Experimental Station of Cornell University in Geneva, New York, led by Bruce Reisch, have worked out a grapevine transformation technique using biolistics, a method for transforming cells by particle bombardment (which gets around the intellectual property issues surrounding the use of *Agrobacterium*).

Using his biolistic technology, Reisch, a convention grape breeder who has produced a number of commercial lines, has made a transgenic grapevine expressing the antifungal endochitinase gene from *Trichoderma harzianum*, which is active against fungi (powdery mildew and Botrytis bunch rot), and other lines expressing both naturally occurring antimicrobial peptides from *Xenopus laevis* and synthetic ones (magainins, which are 21–26-residue cationic peptides that disrupt microbial membranes). Results in the field so far are promising; transgenic vines expressing endochitinase have shown resistance to *Botrytis cinerea* in the field and displayed some reduction in symptoms of *Unicular necator*, the fungus that causes powdery mildew. With their lines expressing magainin, Reisch and his group observed a reduction in gall size from *Agrobacterium* infections.

University of Florida's Gray has introduced into grapes hybrid lytic peptides that confer resistance to the bacterium that causes Pierce's disease (*Xylella fastidiosa*). *X. fastidiosa,* which is endemic in parts of the US Southwest, lives in the xylem, and is spread from vine to vine by sap-eating insects. In the late 1990s, the disease nearly wiped out the grape industry in the Temecula Valley in southern California, when a new, more robust vector (the glassy-winged sharpshooter) arrived on the scene and destroyed vines in a single season—an unusual occurrence, as it typically takes several seasons before vines are completely destroyed by Pierce's disease. The wine industry poured money into the development of control measures, and the pest has been kept at bay for a few years with a multi-pronged approach, combining beneficial insects and insecticides. However, pests can develop field resistance to pesticides, and insecticides can sometimes be pulled off the market, according to Donald Hopkins, of the Mid-Florida Research and Education Center in Apopka, so efforts to create grapes resistant to Pierce's disease continue at the University of Florida and elsewhere. With a proprietary dual-expression vector technol-

Table 1 Selected field trials of transgenic grape from US APHIS database

Institution	Date	Organism	Gene/phenotype	Phenotype
Cornell University	11/07	Grape rootstock	Various viral coat proteins plus selective marker from *Escherichia coli*	Grape leafroll and fanleaf virus resistance
	3/00	Grape	Chitinase from *Trichoderma harzianum* plus selective marker from *E. coli*	Powdery mildew and *Botrytis* resistance
University of Florida	12/07	Grape	Lytic peptide gene plus *Aequorea victoria* green fluorescent protein (GFP) marker fused to *E. coli* neomycin-resistance gene	*X. fastidiosa* resistance
	12/07	Grape	Endogenous *Vitis vinifera* gene for resistance to fungus plus lytic bacterial resistance gene and GFP antibiotic-resistance fusion gene	Powdery mildew and *X. fastidiosa* resistance
State University of New York, Geneva	6/05	Grape	Lignin biosynthesis gene from pea *Pisum sativum* plus selective marker from *E. coli*	Powdery mildew resistance
University of California	7/04	Grape	Selective marker plus β-glucuronidase from *E. coli*	Visual marker
	7/04	Grape	Polygalacturonase inhibitor protein from pear (*Pyrus communis*) and selective marker	Visual marker
Anton Caratan and Sons, Delano, California	9/00	Grape	Selective marker	Improved quality
Universita degli Studenti Ancona, Italy	8/99	Grape	Synthesis of auxin Synthesis of tryptophan-2-monooxygenase	Fecundity
Bundesanstalt für Züchtungsforschung, Germany	8/00	Grapevine	Resistance to fungi Synthesis of chitinase Synthesis of glucanase Synthesis of ribosomal-inactivating protein	Improved quality
L'Institut National de le Recherché Agronomique, Colmar, France	1994, 1999, 2004	Grape	Viral proteins	Virus resistance

Source: http://www.isb.vt.edu/CFDOCS/fieldtests1_output.cfm ogy developed by Gray for controlling gene expression in grapes, which he used to create pathogen-resistant grapes, his university is seeking to organize a development company to commercialize the technology and expand its application.

In a clever twist, Gray's strategy to control Pierce's disease capitalizes on the bacteria's dissemination through the plant in xylem. Gray has engineered rootstock to produce lytic peptides, which can then, like the bacteria, travel throughout the plant in the xylem. This leaves the grape itself unmodified (allaying growers' fears of consumer rejection, perhaps). In addition, expression solely in rootstock would essentially eliminate any possibility of the transgene getting loose in the environment through seed or pollen.

The notion of using rootstock is taking root, so to speak. French researcher, Marc Fuchs, at Cornell since 2004 after working in viticulture for 20 years in France, is tackling a major problem for French vintners, the grapevine fanleaf virus, which affects over 60% of total grapevine acreage in France, causing losses of over $1 billion annually, according to Fuchs. Fuchs and others are trying to produce virus-resistant rootstock by introducing viral genes into the vine genome, a well-established technology used successfully to combat viruses in squash and papaya[5]. Although the precise mechanism protecting the plants remains unclear, recent evidence suggests that small interfering RNA induction is involved with this process in some of the transgenic lines.

Numerous rootstocks and varieties have been produced with resistance to several of the leading viral pests, and reports of viral resistance in experimental plots are accumulating, although the evidence is still preliminary and needs to be confirmed. One concern that Fuchs has put to rest is that recombinant viruses will be generated from the transgenes and natural virus genomes in the field. Detailed studies by Fuchs have failed to detect any recombinant viruses in test plots with transgenic grapes, although he did detect some in control plots. To his mind, that makes virus gene transfer a non-issue.

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> Marc Fuchs, Cornell University, Ithaca, NY

Working together

Although work on transgenic grapes is going full speed ahead in places like Florida and Chile, most people involved with the work still wonder when and how commercialization will ever happen. Support from the wine industry, though substantial and important, according to Cornell's Reisch, pales in comparison to the kind of support given to other crops by agribusiness. And the fear of a public outcry against GM grapes may continue to keep transgenesis of grapevines from taking hold. Some groups are hedging their bets by using both conventional and molecular breeding technologies. In fact, the two approaches are complementary rather than competitive.

But for some of the problems faced by vintners, conventional breeding programs have come up short—crossbreeding grapes used in wine production to grapes displaying natural pest resistance, for example, has been disappointing as the flavor was destroyed. And, there are no naturally occurring viral-resistance genes that might be targeted through selective breeding; transgenesis provides the only path to permanent viral resistance. Not to mention the fact that it takes upwards of 30 years to take a new variety from conception into the market. Genetic engineering may be just the ticket, but until a product is ready for commercialization—and the best estimates put that still at five to ten years away—it's impossible to know how the technology will fare in the marketplace.

Cornell's Fuchs is optimistic that the path to commercialization is clearing. Acknowledging grower resistance to GM wines, he notes, "The wine industry is not very receptive to any innovation." However, over the past five to ten years, he has detected a shift in thought among the people with whom he interacts. "Most growers are convinced that science should move forward in case scientists have an interesting plant to offer the industry. They could then make the choice to use it or not," he says.

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