Opinion TRENDS in Biotechnology Vol.22 No.4 April 2004

Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability

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The vast majority of wine proteins have recently been identified as pathogenesis-related (PR) proteins. During the growing season, these proteins are expressed in developmentally dependent and inducible manners in grapevine leaves and grape berries, in which they are believed to play an important role in protection against fungal pathogens and possibly other stresses. Because of their inherent resistance to proteolytic attack and to the low pH values characteristic of wines, vinification can be seen as a 'purification strategy' for grape PR proteins. The inevitable consequent accumulation of these proteins in wines becomes a technological nuisance because they adversely affect the clarity and stability of wines. Genetically modified vines underexpressing PR proteins would certainly lead to stable wines but would increase the plant susceptibility to fungal attack, and the actual trend seems to be in the opposite direction, that is overexpressing these proteins to obtain plants with enhanced resistance to pathogens – a trend that will probably augment problems associated with protein instability in the resulting wines.

Grapevine (*Vitis* species) is economically the most important fruit species globally because of the numerous uses of its fruit in producing wine, juice, table grapes, dried fruit and organic compounds [1]. *Vitis vinifera* is currently the major species cultivated because of its high quality for use in wine production. However, *V. vinifera* is susceptible to an array of diseases. Fungal pathogens are a major problem in the cultivation of grapevine around the world (Table 1). The most threatening fungal diseases are the powdery and downy mildews, which were introduced into Europe during the 19th century along with accessions of the American wild *Vitis* species in which they were endemic [2]. In general, fungal infection decreases yield and berry and wine quality through a reduction in plant vitality and productivity or by direct infection of the berries. Control is generally achieved by widespread application of fungicides. The economic costs and negative environmental impact associated with these applications has led to a recent search for alternative strategies, involving manipulation of host defense mechanisms.

Pathogenesis-related proteins in grapevines

Pathogenesis-related (PR) proteins are typically acidic, of low molecular mass and highly resistant to proteolytic degradation and to low pH values. They encompass 14 families of structurally and functionally unrelated proteins [3], some of which have been detected in grapevine (Table 2). Members of several of these families were shown to have damaging actions on the structures of the parasite, thus exhibiting antifungal activity in *in vitro* bioassays and supporting a possible role for these proteins in plant defense [4,5]. These include the PR-5 proteins (thaumatin-like proteins and osmotins), which are thought to create transmembrane pores and have therefore been termed permatins; the PR-2 proteins (β-1,3-glucanases) and the PR-3 and 4 proteins (chitinases), enzymes that hydrolyse β-1,3-glucans and chitin, respectively. β-1,3-glucans and chitin are also structural components of cell walls in most higher fungi.

Several studies have now been published on the induction of PR proteins in vine plants and on their inevitable accumulation in grapes during the growing season [6]. This occurs in healthy grape berries in a developmentally dependent manner as a normal part of the ripening process, with véraison (the French term used by viticulturalists to denote the inception of ripening) apparently being the trigger for gene expression. Expression of genes coding for several PR proteins increases dramatically in grapes during ripening. There is a significant increase in total grape protein content [per berry and per gram (fresh weight)] after véraison, with only a small number of proteins being synthesized in significant amounts during ripening [7]. The two most prominent soluble proteins accumulated in grapes during ripening have been identified as chitinase and a thaumatin-like protein [8]. Chitinase alone has been reported to account for half of the soluble protein in ripe grapes [9]. PR proteins can also be induced in leaves and pre-véraison berries, as part of an induced defense against the classical PR protein

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 0167-7799/$ - see front matter doi:10.1016/j.tibtech.2004.02.001

Table 1. The major widespread and economically important pathogens affecting grapevines worldwide[a]

Causal agent	Properties of pathogen	Disease	Specific characteristics of disease
Uncinula necator Schwein.[b] Burrill[c] (anamorph *Oidium tuckeri* Berk.)[d]	Obligate biotrophic fungus	Powdery mildew	The most economically important disease of *Vitis vinifera* worldwide
Plasmopara viticola Berk. and M. A. Curtis[b] Berl. and De Toni in Sacc.[c]	Obligate biotrophic oomycete	Downy mildew	Affects *V. vinifera* worldwide
Botrytis cinerea Pers.:Fr.	Necrotrophic fungus	Grey mould rot	One of the most common and widely distributed grapevine diseases
Elsinoe ampelina (de Bary)[b] Shear[c]	Non-obligate fungus	Anthracnose	Affects *V. vinifera* and its hybrids in tropical and subtropical regions
Phomopsis viticola (Sacc.)[b] Sacc.[c]	Non-obligate fungus	Phomopsis cane blight and leaf spot	A wood disease
Botryosphaeria dothidea (anamorph *Fusicoccum aesculi*)[d] (Moug:Fr) Ces and De Not	Non-obligate fungus	Excoriosis	A wood disease
Eutypa lata Pers.:Fr. Tul.[c]	Ascomycete fungus	Eutypa dieback	A major grapevine disease in many countries that infects the vine stock. A wood disease

[a]See [46] for authority nomenclature.
[b]Abbreviation or name of the author who first classified the fungus.
[c]Abbreviation or name of author who later reclassified the fungus.
[d]Anamorph of fungus and author who classified it.

gene-inducers (wounding, chemical elicitors, pathogen attack or abiotic stress) by the expression of specific PR genes [8,10]. Together, these processes modulate the levels and proportions of the PR proteins in grapes, in a way that seems to depend on the cultivar, region, climate and agricultural practices. Thus, the actual pattern of proteins present in mature grapes could depend on the precise environmental and pathological conditions that occurred during vegetative growth. Indeed, a careful inspection of the published data reveals inconsistencies as to which proteins accumulate in mature grapes, and a recent work showed that the environmental conditions prevailing during vegetative growth determine the pattern of major polypeptides that accumulate in mature grapes [11].

PR proteins in wine

Wines, like many other natural food products, contain varying amounts of different nitrogenous substances – the most important of which are proteins. These polymers do not contribute significantly to the nutritive value of wines because their concentration is relatively low, varying from 15 to 230 mg per liter [12]. However, they have considerable technological and economic importance because they affect the clarity and stability of the wine greatly. Instability of proteins in white wines is one of the most common non-microbial defects of commercial wines. Coagulation of proteins in wines can result from unfavourable storage conditions, leading to their aggregation. The denatured protein can subsequently precipitate to form an amorphous sediment or deposit, or flocculate and thus produce a suspended and unattractive haze in the bottled wine, which reduces its commercial value, making it unacceptable for sale [6]. Translucency is of vital importance to wine quality because this property makes the first impression on the consumer, who will reject wines containing hazes or cloudy precipitates, regardless of how the wine tastes. It is therefore imperative that wines stay stable and clear, regardless of the conditions of storage.

Although they exhibit great diversity [13], the vast majority of the wine proteins are structurally related and have recently been identified as PR proteins, regardless of the grape variety, region, year or winemaking conditions [13,14,15]. More specifically, one study showed the major haze-forming proteins to be chitinases (PR-3 family) and thaumatin-like proteins (PR-5 family), with a minor 13 kDa component of the haze-forming protein complement belonging to the PR-4 family of plant defense proteins [16]. Another study showed that wines contain a large number (tens and possibly hundreds) of distinct polypeptides, exhibiting similar molecular masses but

Table 2. Families of pathogenesis-related (PR) proteins reported in grapevine. Induction and expression of PR genes[a] and PR protein detection[b] in grapevine leaves, berries and wines

Family[c]	Type member[c]	Properties[c]	Leaves (Refs)	Berries (Refs)	Wines (Refs)
PR-2	Tobacco PR-2	β-1,3-glucanase	[12][a,b], [39][a,b]	[12][a,b], [41][b], [42][a]	
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	[12][a,b], [40][a]	[12][a,b], [41][b], [43][a], [44][b]	[18][b], [19][b], [43][b]
PR-4	Tobacco R	Chitinase type I, II	[12][a,b]	[12][a], [19][b]	[19][b]
PR-5	Tobacco S	Thaumatin-like	[40][a]	[9][a,b], [12][a], [44][b], [45][b]	[16][b], [18][b], [19][b]

[a]Induction and expression of PR genes.
[b]PR protein detection.
[c]Adapted with permission from [3].

different electrical charges. Structural similarity was observed among most of the wine polypeptides, which exhibited a high degree of homology to PR proteins, notably osmotin and thaumatin-like protein [13]. All grape cultivars synthesize a set of PR proteins, identical to those forming the haze in wine [7,15,17]. The wine proteins are derived from the grape pulp [14] and survive the vinification process simply because they are inherently highly resistant to proteolysis and to the low pH characteristic of musts and wines. For these reasons, vinification can be seen as a 'purification strategy' for grape PR proteins [12]. The majority of the other grape proteins either precipitate in the grape juice or are degraded because of proteolytic activity. Paradoxically, the grape PR proteins are very stable proteins in the short to medium term (in grape juices and during the winemaking process) but can become unstable in the long term (in wines) [16]. Therefore the unavoidable accumulation of PR proteins in wines becomes a technological challenge, which is rather difficult to overcome.

Bentonite fining (the deliberate addition of an adsorptive compound that is followed by the settling or precipitation of partially soluble components from the wine) is currently and commonly used in the wine industry as a clarifying method to remove proteins that are a potential source of haze in wines. As a cation exchanger, bentonite is not specific for proteins, resulting in the removal of important wine aroma and flavour compounds, which has prompted researchers to look for alternative strategies. However, the development of alternative methodologies for specifically removing the proteins from wines has been hampered by several factors [12].

Enzymatic degradation of wine proteins into small peptides and/or their component amino acids has been suggested as an alternative to bentonite fining. Indeed, at temperatures >35°C, juices and wines treated with commercial proteolytic enzyme preparations exhibit reduced amounts of protein [6]. Unfortunately however, under normal winemaking and wine storage conditions, the haze-forming proteins present in grape juices and wines are known to be highly resistant to proteolytic attack by grape- or yeast-derived proteases and by proteases of non-grape origin. Commercial proteolytic enzyme preparations are active on exogenous proteins (e.g. bovine serum albumin) added to juice and wine under winemaking conditions, indicating the absence of enzyme inhibitors or other components that could protect proteins from hydrolysis in wine. In addition, the remarkable resistance of wine proteins to proteolytic enzyme preparations is observed in the presence or absence of all other wine components, demonstrating that this resistance is an inherent property of these molecules [16]. Nevertheless, proteolysis is detected in must and wine proteins at temperatures >35°C and/or high pH or after ethanol (50% v/v) precipitation of the proteins, suggesting that proteolytic resistance is conferred by the native conformation of the PR proteins. Given the known proteolytic resistance of PR proteins from other plants, this phenomenon is not surprising.

Removal of the PR proteins with specific, immobilized antibodies has to take into account the low pH values typical of wines, incompatible with antigen–antibody interactions. Indeed, the conformation of immunoglobulins is reversibly altered at pH 2.5 to 3.0 in a way that abolishes their interaction with antigens.

The other techniques available all have deleterious effects. Ultrafiltration techniques remove most proteins but lead to great losses in important organoleptic compounds involved in flavour, aroma and colour, leave residual proteins in the filtrate and have high set-up costs [6]. The capacity of immobilized grape proanthocyanidins (a specialized group of bioflavonoids) to bind proteins from wine results in protein-stable wines. However, their use is limited by reduction in protein-binding capacity after a small number of regeneration cycles [12]. Flash pasteurisation, a method of heat pasteurizing (15 to 30 s at 71.5 to 74°C) beverages before filling into containers for the purposes of killing spoilage microorganisms, has a significant detrimental effect on wine quality [6]. Thus the absence of suitable methods for the specific removal of the wine proteins prompted researchers to look for alternative strategies.

Genetic engineering of grapevine

Wild-type varieties of *V. vinifera* are characteristically sensitive to fungal attack, although the degree of susceptibility varies with the cultivar, the climate conditions and possibly with all other factors that affect the synthesis and accumulation of the PR proteins and other natural defense mechanisms. The resulting grapes produce wines with varying amounts of PR proteins and, consequently, with varying tendencies to form hazes. Fungicides might successfully control fungal diseases in grapevine. Indeed, fungicide application is a well-established anti-fungal treatment. However, the general use of fungicides has high economical costs and destructive environmental consequences. The transgene approach involves the use of alien genes expressed in plant genomes that code for proteins with anti-fungal activity, enabling the plant to protect itself against fungal attack [18]. This has been successfully achieved for grapevine [18,19,20]. The most attractive candidates for the genetic manipulation approach are genes encoding chitinases or β-1,3-glucanases. The first report of anti-fungal tolerance to grapevine involved transformation of *V. vinifera* varieties with a chitinolytic enzyme (an endochitinase) from *Trichoderma* [21]. Subsequently, the grapevine rootstock 41B was transformed with the pathogen inducible promoter sequence (cloned from alfalfa) and the stilbene synthase gene (cloned from grapevine) as a strategy for improving plant tolerance to fungal disease, particularly *Botrytis cinerea* and *Eutypa lata* [22]. A rice chitinase gene was recently introduced into the somatic embryos of grapevine by *Agrobacterium* infection. Some of the transformants obtained displayed enhanced opposition against powdery mildew caused by *Uncinula necator* [20].

The trend towards the overexpression of PR proteins to obtain plants with enhanced resistance to fungal attack is not devoid of serious drawbacks. Indeed, the genetically modified vines overexpressing PR proteins would certainly augment the problems associated with protein instability

in the resulting wines, which at this time is overcome only at the expense of wine quality [12].

In an era in which the applications of molecular biology are expanding at a rapid rate, the problems associated with wine turbidity could be tackled by genetically modifying the patterns of gene expression in the grapes. Thus, lowering the expression of the genes coding for PR proteins would probably decrease or eliminate the problems associated with wine protein instability. However, given the physiological role attributed to PR proteins, the genetically modified vines underexpressing PR proteins would certainly become more susceptible to the attack of fungal pathogens. In other words, the technology available today allows (i) the production of vines resistant to fungal attack (with the economical and environmental benefits that go with them), capable of producing high quality grapes but wines with increased instability problems; or (ii) vines oversensitive to fungal pathogens that lead to stabilized wines but either do not produce grapes at all or produce only at the expense of intensive applications of chemical fungicides.

Concluding remarks

The solution for stabilizing wines – involving the development of tactics to minimize accumulation of PR proteins in grapes without compromising the natural defenses of the plants and the quality of both grapes and wines – currently relies on a delicate balance among viticultural practices, oenological practices and fungicide applications. Cultural practices and crop phenology (e.g. practices such as pruning) can be used to develop alternative disease-management programs. Thus, appropriate removal of basal leaves after bloom, shoot positioning and trellising, as well as training systems and pruning practices of grapevines are known to reduce the severity of fungal infection and improve fruit composition by altering the canopy microclimate [23,24]. Post-harvesting processing and winemaking conditions are also important in determining the concentration of PR proteins in the finished wine. For example, mechanical harvesting coupled with prolonged transport of the wounded fruit results in higher PR protein levels in the resulting juices and wines, an effect that has been attributed to extraction of the protein from the skins rather than increased protein synthesis [6]. This procedure was found to double the amount of bentonite required for stabilization when compared to fruit harvested manually and transported from the same vineyard [25]. Disease control is generally achieved by the widespread application of fungicides. However, multiple applications of fungicides per growing season to control fungal pathogens in many regions of the world have resulted in the selection of resistant fungal populations, limiting the effectiveness of these sprays in controlling the disease. In addition, the costs to the grower, the environmental impact and the public concern over the use of pesticides on food and beverage crops calls for alternative methods of disease control. Unlike traditional chemical control or traditional pest and disease control, in which the main objective is the elimination of the pest and disease (at least 90% effectiveness) integrated pest- and disease-management aims at maintaining a population balance below the tolerance threshold, intervening only when the population density exceeds an action threshold [26]. Also, grapes acquire ontogenic resistance (acquired in a developmentally dependent manner, being constitutive rather than inducible) against *U. necator* rapidly after fruit set, which marks the beginning of grape development after sexual reproduction, so that refocusing of disease management on the crucial periods of high fruit susceptibility should greatly improve the efficacy of fungicide applications [27].

The actual technological difficulty in the specific removal of proteins from wines demands a search for new methodologies. Examples of potential alternative methods are:

(i) The search for exotic enzymes or enzyme systems capable of hydrolysing the PR proteins in the wines. In this respect, it was recently claimed that *B. cinerea* infection of *V. vinifera* berries results in lower levels of protein in musts because of proteolytic activity of proteins secreted by the fungus [28].

(ii) Subtle adjustments in the winemaking procedures that induce PR protein denaturation can render these molecules susceptible to the action of proteases; for example, it was reported that a short period of heating to 90°C does not adversely affect the sensory characteristics of white wines [29].

(iii) The use of yeast-derived mannoproteins and other glycoproteins, termed haze-protective factors (HPF), which exhibit haze-protective activity, not by preventing the proteins in wines from aggregating, but by decreasing the particle size of the haze, making it barely detectable to the naked eye [6].

(iv) The expression in grapevines of foreign proteins with antifungal properties but which are sensitive to low pH and/or susceptible to proteolytic enzymes.

(v) The exploitation of different antifungal defense mechanisms other than PR proteins in grapevine (e.g. the overexpression of phytoalexins in grapevines). Besides PR proteins, the accumulation of phytoalexins, such as stilbenes, is the other major defense mechanism frequently observed and well-characterized in grapevines [30]. Their production is controlled by a key enzyme, stilbene synthase, which produces *trans*-resveratrol, the major phytoalexin in this plant. This diphenol is subsequently metabolized into the other main phytoalexins of grapevine. Resveratrol plays an important role in resistance to colonization by fungi and exhibits outstanding biological properties in human health [30]. It is selectively accumulated in vine leaves and grape skins in response to various fungal infections, UV radiation or chemicals, and is present in wines in concentrations that depend on viticultural and oenological practices. It exhibits a rather unspecific antifungal character at physiological concentrations, enhancing the resistance of vine plants to *B. cinerea*, *Plasmopara viticola* and *Phomopsis viticola* [31,32]. Because of its antioxidant properties, resveratrol improves grape conservation during storage [32]. This phytoalexin can be considered a fungicide of natural origin, with potential uses that range from endogenous enhancement to exogenous applications. Most interest has now centred upon genetically engineering grapevine stilbene synthase gene to increase plant tolerance to pathogenic microorganisms and to improve the nutritional

quality of food products [30]. A chimeric gene combining a fungal inducible promotor (an alfalfa PR-10 promotor) with a defense gene (*Vst* 1, the *Vitis* stilbene synthase 1 gene) was introduced into the genome of 41B rootstock (*V. vinifera* cv. Chasselas × *V. berlandieri*). The leaves of some of the transgenic plants infected with *B. cinerea* accumulated resveratrol to levels 5- to 100-fold above the control and exhibited highly reduced symptoms [33].

(vi) The expression of detoxifying genes in grapevine. Eutypine, a toxin produced by the fungus *Eutypa lata*, is an important virulence factor involved in symptom development of the disease eutypa dieback. A gene, named *Vr-ERE*, has been cloned in *Vigna radiata* and encodes an NADPH-dependent aldehyde reductase, an enzyme exhibiting a high affinity towards eutypine and capable of reducing it to eutypinol. Because eutypinol is not toxic for grapevine tissues, this detoxification mechanism might play a role in defence. Overexpression of the *Vr-ERE* gene in grapevine rootstock 110 Richter *V. berlandieri* × *V. rupestris* increased the plant detoxification capacity. Indeed, the growth and development of the transgenic plants were not affected by the presence of the toxin, whereas those of the untransformed plants were highly inhibited [34].

(vii) The development of new fungicides or other compounds of natural origin – or even microorganisms – that are environmentally friendly. For example, endopolygalacturonase 1, a glycoprotein from *B. cinerea*, has recently been shown to activate defense reactions in grapevine [35]. Also, there is great potential for the use of bacteria as an alternative to fungicides in plant disease-management. Treatments with selected beneficial bacteria, known as plant growth-promoting rhizobacteria (PGRR), can induce systemic resistance against a broad spectrum of diseases caused by viruses, bacteria, fungi and even against plant insects [36,37]. A plant growth-promoting, biotic and abiotic stress resistance endophyte bacterium, a non-fluorescent *Pseudomonas* sp. strain PsJN, was used to enhance growth, facilitate development and induce resistance of grapevines to *B. cinerea* [38].

In the next few years, researchers around the world will focus on solving two of the major problems encountered today in grape production and winemaking: control over the level of fungal attack on grapevine leaves and berries and reduction of the risks associated with protein-haze formation in wines. Current solutions are inadequate and the search for alternative methodologies is becoming imperative. Theoretically, both problems can be tackled by genetic transformation of grapevine. However, a straightforward application of these techniques will lead to a biotechnological conundrum, in the sense that increasing the grapevine resistance to fungal pathogens by overexpressing PR proteins will lead to augmented risks in wine turbidity, whereas attempting to remove the PR proteins from wines by silencing their expression in grapes will surely boost plant susceptibility to parasites.

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