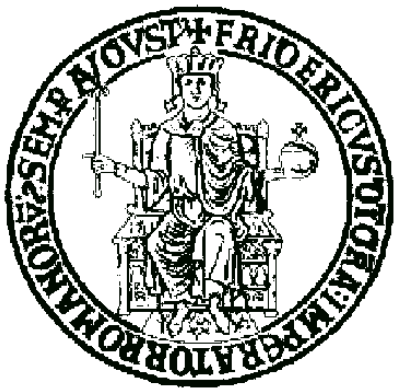

University of Naples “Federico II”

Department of Agriculture

PhD in Agrobiology and Agrochemistry

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# Discovery and genotyping of DNA sequence variations in grape.

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie (1867 - 1934)

# Table of contents

**Chapter 3**

**Investigation of grapevine basal immunity**

# Abstract

The common grapevine, *Vitis vinifera* subsp. *sativa* L. (2n=6x=38) is one of the major fruit crops worldwide in terms of economic value and cultivated area. In the panorama of Italian viticulture and oenology, Campania region is characterized by a rich biodiversity, which include a distinctive number of varieties including very ancient ones (more than one hundred years old). This biodiversity need to be preserved to protect producers and consumers from food frauds and on the counterpart is an essential genetic resource for breeding and grapevine exploitation. The recent complete sequencing of grape genome represents an essential step forward for structural and functional genomics studies. In this contest, one major objective was pursued, in the frame of the project SALVE (Safeguarding of the plant biodiversity of Campania) funded by Campania region, to investigate the molecular basis of Campania grape germplasm. Microsatellite and retrotransposon-based markers have been used in a large collection of 62 grapevines and homonymies and synonymies were found, reinforcing the knowledge that molecular evaluations can provide further insights into genetic structure and differentiation of *Vitis* germplasm accumulated during centuries of cultivation and selection. Indeed, Procidana and Coglionara are grapes cultivated and sampled on the Ischia Island and identified as synonymous in this study. In our effort, we were able to identify 19 grape-specific alleles, which represent useful tools for many purposes such as traceability in germplasm, typicity preservation and varietal identification. Within the collection analyzed, Aglianico del Taburno is a model for astringency and the strong resveratrol content confer high positive nutriceutical properties to their grapes and wines. For this reason, a transcriptomic analysis of key genes of the phenyl-propanoid pathway was carried out in different tissues of the berry during the fruit maturation. This study was correlated with the chemical analysis of total phenols, flavans, anthocyanins and tannins detected in skin and berry using a spectrophotometric assay. Anthocyanin analysis revealed the highest amount of these molecules in ripening berry skin and the lowest in seed. Correlating the transcriptomic and chemical data, the key-genes able to control production of poly-phenolic compounds in berry tissues have been determined. Poly-phenolic compounds are key-molecules of grapevine defense and in particular of PTI (PAMP-Triggered Immunity). Indeed, they are produced as defense compounds after pathogen recognition. Nowadays, much has been written about defense mechanisms and chitin perception in *Arabidopsis*, but little is known in grapevine. Considering powdery mildew as the most destructive disease of grapevines world-wide, an intense structural and functional study of PTI-involved grape genes has been

conducted. Grapevines are highly resistant to many the powdery mildew of other species, called non-adapted (e.g. *E. cichoracearum*). Using *Arabidopsis* as model species, we identified and functionally studied the grape genes involved in the perception of chitin in the epidermal cells apoplast, as LysM-RLK, and in the intracellular signal transduction. Our findings suggest that three candidates, *VvLYK1*, *VvLYK2* and *VvLYK3,* can play a different role in chitin signaling in *V. vinifera*. We demonstrated that a total resistance to powdery mildew was re-established in the *A. thaliana cerk1* mutant only by *VvLYK*1, but not by *VvLYK*2 and *VvLYK*3. Further experiments also suggested an inactive *VvLYK*2 gene and an active but unknown function of *VvLYK*3. Regarding the signal transduction pathways, we found *VvMAPK*3 and *VvMAPK*6 to be not involved in biotic response, since they both did not show expression differences in powdery mildew infection and chitin treatment time courses. The transcriptomic data generated in this study allowed us to identify *VvWRKY*24 as the positive regulation factor of grape defense to powdery mildew, but excluded *VvWRKY*16. Indeed, the *VvWRKY*24 had the strongest up-regulation in response to powdery mildew infection and chitin treatment. To better understand the possible interactions between two or more genes and regulatory mechanisms, an over-expression and silencing transformation in Shiraz have been conducted. The transformations conducted gave rise to few positive embryos after ten months from co-cultivation with *Agrobacterium*. This result is strictly correlated with the known recalcitrance of grapevine to transformation. As far as we know, this represents the first complete structural and functional genomics study in grapevine aimed to the identification of the major genes involved in chitin perception and signal transduction.

# 1. General introduction

## 1.1 Origin and evolution of the cultivated grape, *Vitis vinifera*

Grapevine (*Vitis vinifera* L.) represents one of the major crop species on a world-wide scale, with a world production approaching 67 million of tons (46.3% in Europe) and a harvested area of over 6.9 million hectars (Faostat, 2013). The cultivated grapevine belongs to the genus *Vitis* (2n = 38 chromosomes) or *Muscadinia* (2n = 40 chromosomes), within the family *Vitaceae*, which contains approximately 1000 species assigned to 17 genera. The etymology of the name *Vitaceae* comes from Latin (*viere* =to attach) and is referred to grapevine behavior as woody lianas able to climb with their leaf-opposed tendrils. The genus *Vitis* occurs predominantly in the temperate and subtropical climate zones of the Northern Hemisphere (Mullins *et al.,* 1992; Wan *et al.,* 2008). This genus probably comprises 60-70 species (plus up to 30 fossil species and 15 doubtful species) spread mostly throughout Asia (~ 40 species) and North America (~ 20 species) (Alleweldt and Possingham, 1988; Wan *et al.,* 2008). The genus is often divided into two major groups: the American group and the Eurasian group. The American group contains between 8 and 34 species, of which several have become economically important as wine or juice grapes, as *Vitis labrusca, V. aestivalis, V. riparia, V. rupestris* and *V. berlandieri*. The Eurasian group hold approximately 40 known species originated from different areas, like *V. vinifera* (origin in southern Caucasus), *V. sylvestris* (central Asia and Mediterranean region), *V. amurensis* (north-eastern China and Russian Siberia) and *V. coignetiae* (Japan). The Eurasian specie *V. vinifera* L. has become the world's leading fruit crop grown in almost 90 countries for wine and juice production or as fresh table grapes or dried grapes (raisins) giving rise to the overwhelming majority of cultivated grape varieties. The origin of viticulture should be temporarily located along with the origin of agriculture, however the capacity of humans in transforming grape in wine, oenological sciences, have been proved recently with the discover of the oldest winery in the world (6,100-year-old), the Areni-1 winery. This archeological site was unearthed in 2007 in the Areni-1 cave complex in the village of Areni in the Vayots Dzor province of the Republic of Armenia by a team of Armenian and Irish archaeologists (Figure 1). This winery is geographically closed to the southern Caucasus, the site of origin of domesticated grapevines 2000 years before.

Figure 1. Jars for fermentation found in the Areni-1 winery in 2007.

After Caucasus and Georgia, other signs of the viti-viniculture itinerary have been discovered in Egypt, like seeds, jars and fermentation traces stored in the grave of the king Scorpion the First (3150 a.C). Other traces were the viticulture and winemaking scenes depicted on the walls of the private tombs from the Old Kingdom (2575-2150 BC) until the Graeco-Roman Period (332 BC-395), the scenes showing that the elaboration process was very similar to the traditional European method (Guasch-Jané, 2008). Figure 2 represents a scene of grape harvesting and wine making found in the tomb of Nakht at Sheikh Adb'l-Qurna, Western Thebes (The Irep en kemet project).

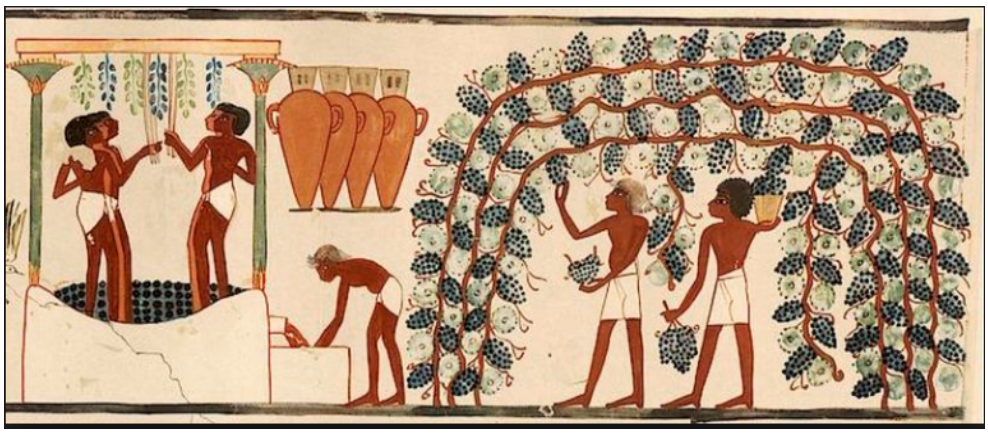

Figure 2. Viticulture and winemaking scene discovered in the tomb of Nakht at Sheikh Adb'l-Qurna, Western Thebes.

Egyptians already had the habit of storing small jars for one or less liter of wine, labeled with the production region, year and winemaker. Historically, the Greeks have even been involved in the first appearance of wine in ancient Egypt and embraced the production aspect as a way

to expand and create economic growth throughout the region. Greeks improved their knowledge about winemaking science and modified the oenological process increasing sugars (raisins) or decreasing acidity (anticipated harvesting). Greek wine was widely known and exported throughout the Mediterranean using amphorae with Greek styling and art. An immense impact on the development of viticulture and oenology was also given by the Roman Empire. In that time, wine was an integral part of the Roman diet and winemaking became a precise business. Essentially all of the major wine-producing regions of Western Europe today were established during the Roman Imperial era.

The use of this crop from more than 8000 years until today generates a high biodiversity. To better understand and make order in grape diffusion and differentiation various terms have been developed, as varieties, cultivars, accessions, clones, biotypes and autochthonous varieties. Cultivated grapevines of sufficiently similar vegetative and reproductive appearance are usually called "grape varieties" by growers and "cultivars" by botanists. On the counterpart wild grapes are achieved in American and French catalogues, thought assignment of a unique identifier, called accession number; it means that wild species can be also called accessions. Because grapevines are heterozygous across a large number of chromosome positions or locus in their genomes, each seed may give rise to a cultivar with distinct characteristics (Thomas and Scott, 1993). For cultivar characterization, ampelography (Greek *ampelos* = vine, *graphos* = description), namely the study of the botanical description, identification, and classification of plants belonging to the genus *Vitis*, have been used for long time and is still essential for viticulturists. The advent of DNA fingerprinting has led to its adoption for the identification of selections in grape collections and is increasingly being used to uncover the historical origins and genetic relationships of grapevines. Today, an estimated 10,000 grape cultivars are being grown commercially, although DNA fingerprinting suggests that a more accurate figure may be approximately 5000 (This *et al.,* 2006). Many cultivated grapes are closely related to one another, and many are known by several synonyms (different names for the same cultivar) or homonyms (identical name for different cultivars). Most current cultivars are not products of deliberate breeding efforts but are the results of continuous selection over many centuries of groups of grapevines that were spontaneously generated by mutation and intra-specific crosses via sexual reproduction and via somatic hybridization. It has been demonstrated that some genetic mutations affect important grapevine traits, as growth habit, leaf shape, disease resistance, cluster architecture, berry color, and other quality attributes (Bessis, 2007). For instance, dark-skinned (i.e., anthocyanin-accumulating) fruit is the "default" version in the *Vitaceae*, and it appears that

virtually all *V. vinifera* cultivars with green-yellow fruit (so called white cultivars) have a single common ancestor that arose from mutations of two neighboring genes of an original dark-fruited grapevine (Cadle-Davidson and Owens, 2008; This *et al.,* 2007; Walker *et al.,* 2007). Due to vegetative propagation, some traits arise from somatic mutations rather than during sexual reproduction and may be propagated both vegetatively and by seeds and result in individual plants of the same cultivar having slightly different genotypes and sometimes phenotypes (Franks *et al.,* 2002; This *et al.,* 2006). This genotypic diversity, which accumulates over time, is termed clonal variation (Mullins *et al.,* 1992; Riaz *et al.,* 2002). Today, clones, also called biotypes, exist for most major grape cultivars, and their success is based on the ability of some clones to perform differently in diverse environments (terroir).

The privileged position of the Italian territory in the Mediterranean area contributes to a various environment spread all over the peninsula. This Italian characteristic is the origin of a high range of vines and wines from table grapes to dessert wines which put Italy in pole position for grape production worldwide. In 2013 have been registered a production of 8.1 millions of tons, an increasing of 2 millions of tons then 2012 (Faostat, 2013). The Italian viticulture is also characterized by the highest number of DOP (Protected Designation of Origin) and IGP (Protected Geographical Indication) varieties in Europe (Figure 3). Those acronyms were introduced in Italy in 1963 to protect the naming of wines' original locations and classify wine quality. According to the EU definition, DOP products are "produced, processed and prepared in a given geographical area, using recognized know-how". Their quality and properties are significantly or exclusively determined by their environment, in both natural and human factors. In Italy, the DOP wines can be sub-classified in DOC (Denominazione di Origine Controllata) and DOCG (Denominazione di Origine Controllata e Garantita). The EU definition of an IGP product is one closely linked to the geographical area in which it is produced, processed or prepared, and which has specific qualities attributable to that geographical area. For example, according to the regulating decree of 30 November 2011 (Gazzetta del 20 dicembre 2011 nº 295), wines produced with Aglianico del Taburno grapes cultivated in one of the twelve areas of Benevento reported in the decree (Apollosa, Bonea, Campoli del Monte Taburno, Castelpoto, Foglianise, Montesarchio, Paupisi, Torrecuso, Ponte, Cautano, Vitulano and Tocco Caudio) can be sell as DOCG wines, on the counterparts if the same genotypes is grown in different Campania areas must be used only to make IGP wines.

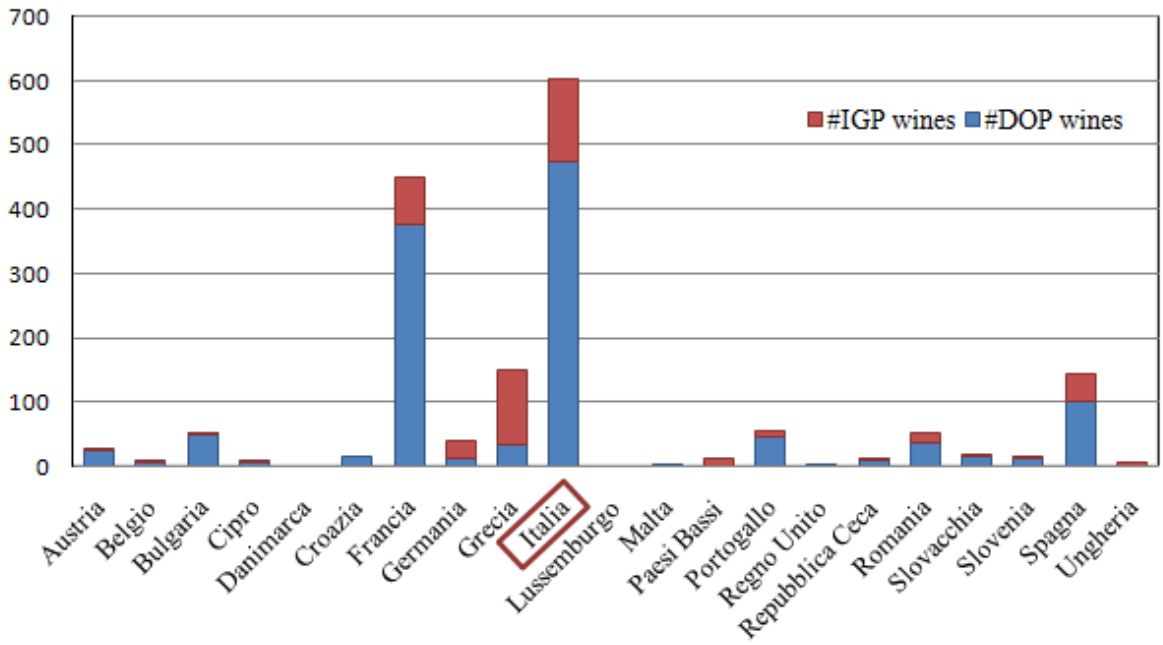


Figure 3. Numbers of DOP and IGP wines registered in European countries. (Source: E-Bacchus).

Each Italian region shows a variable collection of autochthonous cultivars. This term refers to grape varieties that are almost exclusively the result of natural cross-breeding in a particular growing area, and have a long history in that area; their best quality is given only in the local pedoclimactic conditions. Nowadays, autochthonous grape varieties are increasingly appreciated as true resources of a region and as the basis for unique wines reflecting their incomparable terroir. A map of peculiar DOC wines produced in Italy is reported in Figure 4. Most of them are entirely produced with regional autochthonous varieties. The only problematic correlated with Italian grape biodiversity is the correct classification and so preservation of these genetic resources. The scientific community is working hard to solve this problem using the available ampelographic and molecular tools and collecting the results obtained in free-access websites, as the Italian *Vitis* Data Base (http://www.Vitisdb.it/), the European *Vitis* Database (http://www.eu-Vitis.de/index.php) and the *Vitis* International Variety Catalogue (www.vivc.de/).

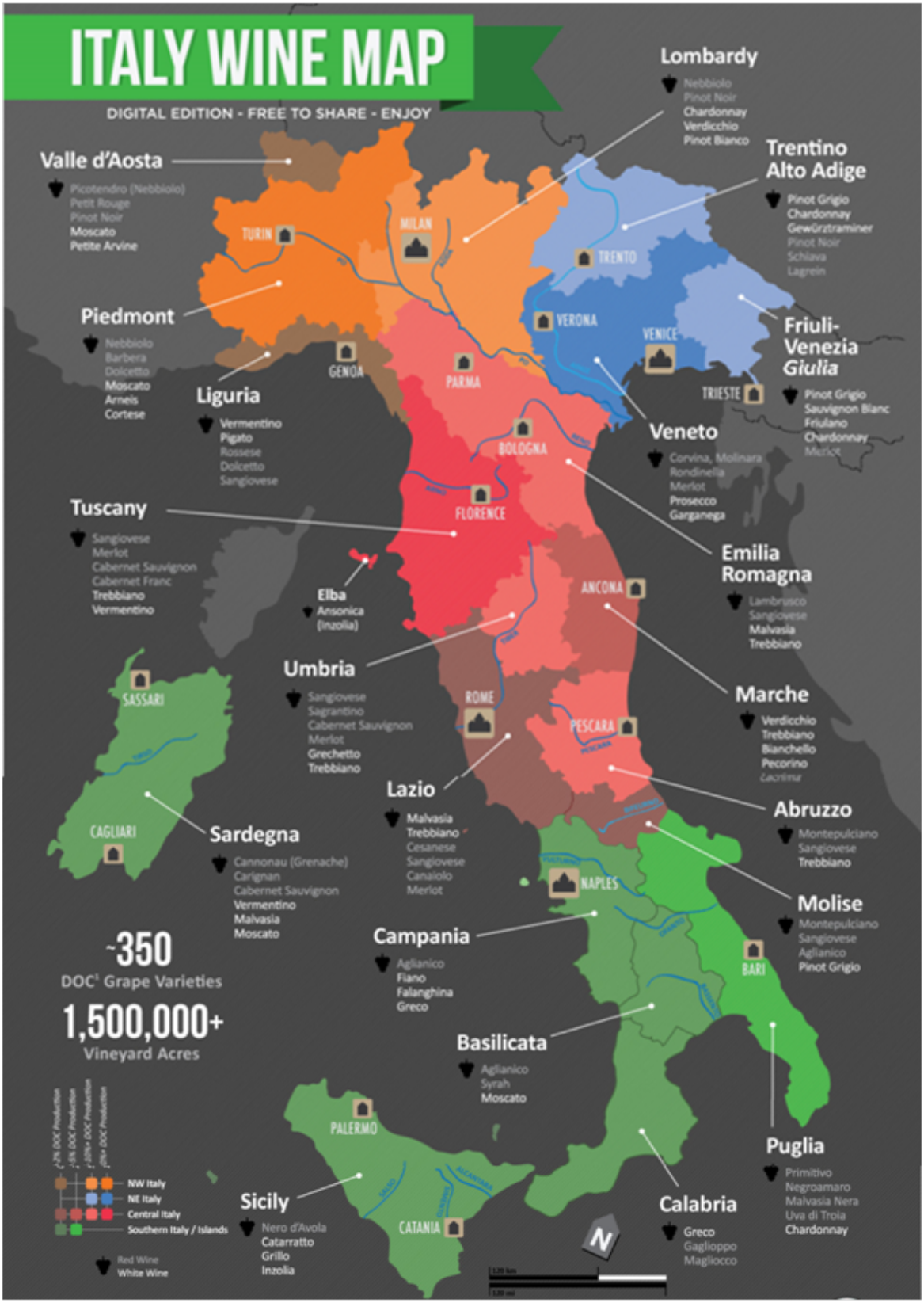


Figure 4. Italian wine map: per each region the name and berry color of DOC grape varieties is shown. A different region color indicates wine production (See Legend). Source: Wine Folly.

## 1.2 *V. vinifera* reproductive biology

Grapevines grown from seeds, like other woody perennials, have a vegetative phase of several years before they reach their reproductive phase and become able to produce fruit. By contrast, vines grown from cuttings are able to fruit in the first growing season under optimal conditions. Perennial woody species require two consecutive growing seasons for flower and fruit production because buds formed in the first year give rise to shoots carrying fruit in the second year. Reproductive growth is very similar in the different *Vitis* species and begins with flower formation, which can be divided in inflorescence induction, flower initiation and flower differentiation. During this stage, the formation of inflorescence and flower meristems are seasonally separated, respectively summer-fall-winter and spring. Approximately two weeks after the formation of ovules and five to ten weeks after bud-break, anthesis marks the beginning of bloom, exposing the male and female floral organs for pollination. In contrast to their wild relatives and the few cultivars with female flowers, cultivated grapevines are typically self-pollinated, whereby pollen originates from the flower's own anthers. The pollen grains absorb water from the moist stigma, germinate and form a pollen tube, which carries two fused sperm cells. Those cells penetrate the stigma and deliver its package of chromosomes into the egg inside the ovule for double fertilization. The resulting diploid embryo and (initially) triploid endosperm together form the daughter generation, whereas all other berry tissues are part of the maternal plant. After pollination, the fruit start development from berry set stage until maturation. The harvested fruits are mature berries showing a heterogeneous color in the fruit cluster, usually white, pink or black. For a visualization of the phenological stages described below bud swelling, bud opening, inflorescence, flower and fruit development of a white berry variety are represented in Figure 5.

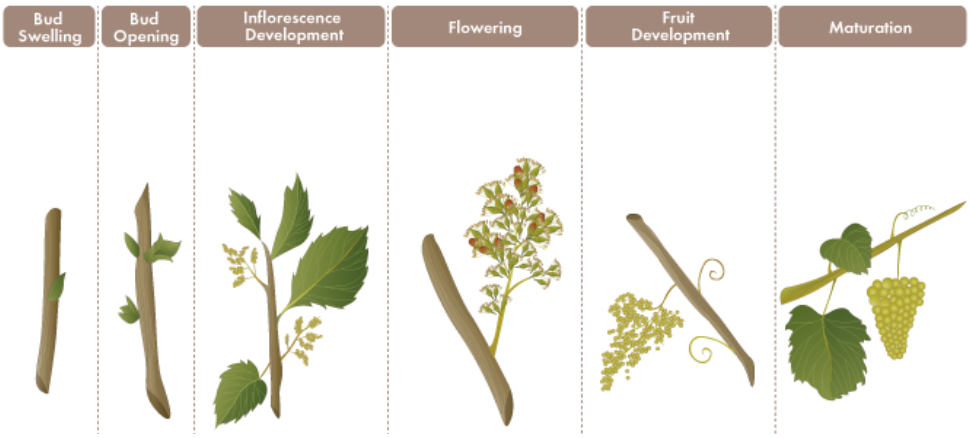


Figure 5. Grapevine phenological stages. (Source: the SQM Company)

These biological bases of grape breeding are still under investigations to speed up the acquisition of new biological information and improve experimental approaches. As genetic system, grapevine is not easy as *Arabidopsis* or rice; it requires large growing facilities and experimental fields and their generation time varies between two and five years depending on genotype and growing conditions. In addition, grapevine genotypes are highly heterozygous and the relevance of near-homozygous lines was not considered till recently because of the need to generate a high quality reference sequence (Jaillon *et al.,* 2007) as well as more efficient genetic systems (Boss and Thomas, 2002).

## 1.3 Grapevine breeding and management in the –omics era

The today -omics sciences' knowledge about *Vitis sp.* can incredibly speed up grape breeding programs, which have been started along with the origin of viticulture. Indeed, the first form of breeding put down roots with the natural and human selection of wild species and their domestication, and evolved in breeding programs focused on development of a new variety. The breeder's ideotype can be different, but in sensu largo in grape is a clone, a grapevine or a rootstock carrying desirable traits, as better quality, higher yield or greater resistance to biotic or abiotic stress. *V. vinifera* is a dioecious allogamous obligate specie highly eterozygous. The self-fertilization is not contemplated due to inbreeding depression inducing sterile hybrids in the F2 or F3 generation. The best breeding program in grape is the backcross with a parent to maintain a high level of heterozygosity. In the past, different strategies have been tested on grape especially in the post-phylloxera era (after 1860) to obtain a resistant hybrid able to produce a good quality wine, but breeders are still working on it.

The vegetative breeding is based on clonal or mass selection (either positive or negative) of clones carrying genic or chromosome mutations. In this case the mutations are generated artificially or naturally in buds apical cells and can structurally be classified in global or chimerical. The global mutations occur in all dividing cells, while chimerical ones occur in one or few cells of the apical meristems giving rise to a mutant sector. Chimeras are of several types, mericlinal, sectorial or periclinal, but the last one is of some interest in viticulture. In a periclinal chimera, mutation is restricted to derivatives of the L1 histogenic layer and such plants can show mutations in different tissues. An example of periclinal mutations in grape is seen in the cultivars Pinot meunier, Pinot blanc and Pinot gris (Figure 6). They are all mutant of Pinot noir, the Pinot blanc and Pinot gris show variations in berry colors, while Pinot

meunier is distinguishable in having tomentose (densely covered with trichomes) shoot tips and expanding leaves (Skene *et al.,* 1983).


Figure 6. Leaves and clusters of three periclinal mutants of Pinot noir: Pinot Meunier, Pinot Gris and Pinot Blanc.

Other examples can be reported in Malvasia Rosa, the pink berry colored Malvasia di Candia aromatica, in Concord apirena, the seedless version of Concord and Early Cardinal, the Cardinal grape with an earlier berry ripening. These kinds of mutations are correlated to single gene or multiple genes, also called chromosomal mutations. Classic examples of polygenic mutations are Muscat Cannon Hall and Leopold III, which are tetraploid genotypes of Zibibbo (Moscato di Alessandria) and Alphonse Lavallée, respectively.

Cross-breeding is the most common and longer used breeding method in plants. In grapevine, various intraspecific hybridization programs (cross between varieties of the same species) have been carried out worldwide getting new genotypes still used in viticulture. Few examples of genotypes obtained using different cross strategies are reports:

~ Simple cross (A x B):

- Italia obtained from Bicane x Moscato d'Amburgo (table grape, Alberto Pirovano, 1911);
- Ruby Cabernet obtained from Carignane x Cabernet Sauvignon (wine grape, Dr Harold Olmo, 1936);
- Cardinal is a cross of the Flame Seedless (or Flame Tokay) and Ribier (table grape, Snyder and Harmon, 1939);
- Incrocio Manzoni 6.0.13 or Manzoni bianco obtained from Riesling nano x Pinot blanc (wine grape, Luigi Manzoni, 1930).

~ Complex cross (A x B) x C:
    - Carnelian and Centurion cross of (Carignan x Cabernet Sauvignon) x Grenache (wine grape, Dr Harold Olmo, 1972 and 1975);
    - Gloria cross-developed at the Geilweilerhof Institute, Germany between Silvaner x Müller-Thurgau.

~ Complex cross (A x B) x (C x D):
    - Bacchus cross between (Sylvaner x Riesling renano) x Müller-Thurgau (wine grape, Peter Mori, 1933);
    - Matilde cross between Italia and Cardinal (table grape, Paolo Manzo, 1962).

~ Backcross (A x B) x A or (A x B) x B:
    - Mariensteiner obtained from (Silvaner x Riesling) x Riesling (wine grape Wurtzberg Institute, Germany, 1971).

The cross-breeding was intensively increased by French scientists, nurserymen and winegrowers in the last half of the 19th century when phylloxera and American fungal diseases began to decimate the vineyards of France and later those of all grape growing European countries. Governmental programs were initiated in France to import germplasm from America and to initiate breeding programs to combine fungus- and phylloxera resistance with the high wine quality of *V. vinifera* (Alleweldt and Possingham, 1987). These efforts proved to be partially successful as dying vineyards were gradually replanted with hybrids of French and American *Vitis* species and with *V. vinifera* cultivars grafted on to phylloxera-tolerant rootstocks. Today, the original own-rooted grape vineyards have largely been replaced by grafted vines Therefore, at the beginning of XX century, grafted vines were more expensive and needed more fields work. For this reason various interspecific breeding programs were developed. The first products were the 'direct producers' hybrids (DPH), plants coming from the inter-specific cross between phylloxera-resistant American plants, and the European one, being able to directly produce grapes for juice (Galet, 1998). Othello is a hybrid obtained from Clinton (*V. labrusca* x *V. riparia*) x Black Hamburg, that was used in post-phylloxera fields. The general condemnation of interspecific hybrids was due to sensitiveness to European limy soil, inadequate resistance to phylloxera and primarily undesirable flavor compounds introduced from American *Vitis* species. The DPH wines were unfamiliar and were not widely accepted, although these hybrids were cultivated on more than 400 000 h, due to their high productivity. In 1934 and in 1955 all these hybrids were

prohibited in France. This led to a drastic change of strategy in France resulting in the use of *Muscadinia* since 1975 (Bouquet, 1980; Merdinoglu *et al.,* 2003).

In the last decades, biotechnologies and genetic engineering offer scientists an incredible amount of information and new techniques useful for breeding improvement. Plant protoplasts (naked plant cells lacking cell walls) are valuable tools for somatic hybridization by protoplast fusion, and genetic transformation allowing the introduction of DNA or viruses into grapevine plant cells by either electroporation (Valat *et al.,* 2006) or polyethylene glycol (PEG) treatment (Jardak-Jamoussi *et al.,* 2002). Compared to other plants, grapevine protoplasts are recalcitrant to propagation and regeneration possibly due to special features of oxidative metabolism (Papadakis *et al.,* 2005). Transient transformation of grapevine protoplasts was reported by Jardak-Jamoussi *et al.* (2002) who incubated protoplasts in the presence of PEG and the plasmid pB1426 carrying the *uidA* and *npt*II genes, but no transformed tissues or organ were regenerated. Plants regeneration from grapevine protoplasts has been reported only once using embryogenic cultures as donor tissue (Reustle *et al.,* 1995; Bouquet *et al.,* 2008). Another suitable tissue for genetic transformations approaches is given by undifferentiated cell suspensions (Torregrosa *et al.,* 2002). However, stable transformation can be difficult to induce in both tissues due to regeneration recalcitrance. Direct organ transient transformation could be an alternative technique to avoid protoplasts or cell suspensions. The most promising methods of plant transient expression use Virus-induced Gene Silencing (VIGS) for repressing a gene (Robertson, 2004) or over expressing a gene using agro infiltration (Kampila *et al.,* 1997). These methods have been investigated in grapevine, but not any protocols have been published.

Genetic engineering of grapevine is based on two limiting factors: the complexity of the grapevine transformation procedure and the low transformation efficiency. Therefore, detailed protocols have been published by Kikkert *et al.* (2005) for biolistic transformation and by Bouquet *et al.* (2006) for *Agrobacterium* mediated transformation. This protocol have been also used from Chaïb and collaborators (2010) to check the efficiency of a new breeding system based on two grape *Vvgai1* mutants, the microvines with reduced stature (*VvGAI1/Vvgai1*) and picovines of very small stature (*Vvgai1/Vvgai1*) (Figure 7).

Figure 7. From left to right, phenotypes of picovine (*Vvgai1/Vvgai1*), microvine (*VvGAI1/Vvgai1*) and wild-type (*VvGAI1/VvGAI1*) young plants.

The *Vvgai1* mutant allele confers a dwarf stature, short generation cycles and continuous flowering. In a hypothetical breeding programm, the picovine genotype can be crossed with a *Vitis* spp. accelerating the strategy from two years to six months. Furthermore, 50% of F1 generation is immediately selectable due to them small size. The microvine is a step forward in molecular breeding providing a model system for rapid genetic studies of grapevine by changing the perennial long life cycle of the plant to one with features and advantages similar to an annual plant.

The molecular approaches are also essential for internationally accepted grapevine identification and to investigate the genetic inter- and intra-varietal variability (Meneghetti *et al.,* 2012). Different molecular markers have been used on *V. vinifera* in several studies to distinguish among cultivars and clones, such as random amplified polymorphic DNA (Herrera *et al.,* 2002; Karatas *et al.,* 2010), inter-microsatellites (ISSR) (Tamhankar *et al.,* 2008), single nucleotide polymorphism (SNP) (Owens *et al.,* 2003, Troggio *et al.,* 2007, 2008), specific sequence amplified polymorphism (S-SAP) (Labra *et al.,* 2004), inter-retrotransposon amplified polymorphism (IRAP) and retrotransposone microsatellite amplified polymorphism (Pelsey *et al.,* 2002; D'Onofrio *et al.,* 2009), methylsensitive amplified length polymorphism (M-SAP) (Imazio *et al.,* 2002), chloroplast DNA polymorphisms (Arroy-Garcia *et al.,* 2006; Hunt *et al.* 2010), S-SAP (Wegscheider *et al.,* 2009), single-strand conformation polymorphism (SSCP) (Degirmenci Karatab *et al.,* 2010). Among all the marker used, microsatellites or Simple Sequence Repeat (SSR) markers, have become molecular markers

of choice, since they offer some advantages over the other molecular markers, including codominant inheritance, hypervariability, and high cross-species transferability. Once they have been developed, they are easy to use and the data can be readily compared among laboratories. Additionally, since the pioneering work of Thomas *et al.* (1994), many studies based on the inheritance of highly polymorphic nuclear microsatellite (nSSR) markers have been performed to identify misnaming and clarify the parentage of several cultivars (Sefc *et al.,* 2009; Maul *et al.,* 2012). SSR markers are useful to detect cultivar pedigree. A peculiar example of incorrect pedigree documentation is the case of Müller-Thurgau, also called Riesling-Sylvaner. The cultivar was believed to originate from a cross, which Prof. Hermann Müller, born in the Thurgau province in Switzerland, carried out around 1890 between Riesling and Sylvaner. However, SSR studies confirmed Rielsing as a parent, but showed that Sylvaner is not related to Müller-Thurgau (Sefc *et al.,* 1998; Thomas *et al.,* 1994). Dettweiler and collaborators (2000) identified Madeleine Royal as the real parent of this hybrid. Another useful class of markers is single nucleotide polymorphisms (SNP). Myles *et al.* (2011) demonstrated that SNPs are powerful tools for confirmation or invalidation of some breeders' data and uncovering parentages for traditional cultivars.

As previously introduced, large grapevine collections are available especially in Europe (http://www.bioversityinternational.org/). The correct administration of these collections is the starting point to exploit grape genetic resources and to identify certain genotypes conferring interesting traits for breeding. The difficulties in management of large size collections (M-core) imply the identification of genetic core collections (G-cores) able to represent with a minimum of repetitiveness, the genetic diversity of a crop species and its wild relatives (Frankel, 1984). In grapevine various G-cores have been developed using molecular markers (Cabezas *et al.,* 2011; Emanuelli *et al.,* 2013; Le Cunff *et al.* 2008). Le Cunff *et al.* (2008) analyzed 2.262 unique genotypes with 20 SSR markers to construct genetic core collections. Subsets of 92 cultivars represent 100% of total SSR diversity and most single nucleotide polymorphism (SNP) diversity in three reference genes. Thus, the approach showed clearly that core collections can effectively be used for SNP discovery and are representative of the whole collection (Le Cunff *et al.,* 2008). Another demonstration of usefulness of G-cores was carried out from Emanuelli and his collaborators (2013), who investigated 22 SSR loci and 384 SNPs in 2273 in 2273 accessions of domesticated grapevine *V. vinifera* ssp. *sativa*, its wild relative *V. vinifera* ssp. *sylvestris*, inter-specific hybrid cultivars and rootstocks. G-cores with 58 (362 alleles) and 110 (plus 52 rare alleles) individuals were developed using the M-method not the random sampling. These results

showed that only a small number of accessions are needed to retain the most frequent alleles as well as the whole allelic diversity (8% and 15% of cultivated grapevines in G-58 and G-110, respectively). The high level of heterozygosity in grapevine leads the capture of all the genetic diversity with such a small number of individuals. Nowadays, SNP genotyping platforms based on BeadArray technology (Illumina, San Diego, CA, USA), GeneChip (Affymetrix, Santa Clara, CA, USA) and SNPlex™ genotyping system (Applied Biosystems, Foster City, CA, USA) are available resulting flexible systems that allow for wide combinations of numbers of SNPs to be simultaneously interrogated (from 48 to hundreds of thousands) (Di Gaspero and Cattonaro, 2010). Those types of platforms and the molecular marker technologies represent a peculiar step forward in high-throughput genotyping (HT-genotyping), which is generally coupled with high-throughput phenotyping (HT-phenotyping) in breeding programs. Recent advances in next-generation sequencing (NGS) and HT-genotyping promise a substantial acceleration of the breeding process. In Figure 8 is reported a schematic representation of a grapevine breeding program to introduce disease resistances using traditional and Marker-assisted (MAS) selection. The first year after the breeding, the susceptible genotypes are removed and the selected plants are transferred in vineyards. The development of a new cultivar can be carried out in 25-30 years with classical breeding and in 10-15 years with MAS selection. A reduction of the breeding time of up to 10 years is conceivable using MAS, which can be used to make decisions earlier and perform targeted backcross breeding programs by marker-assisted backcrossing (MABC).

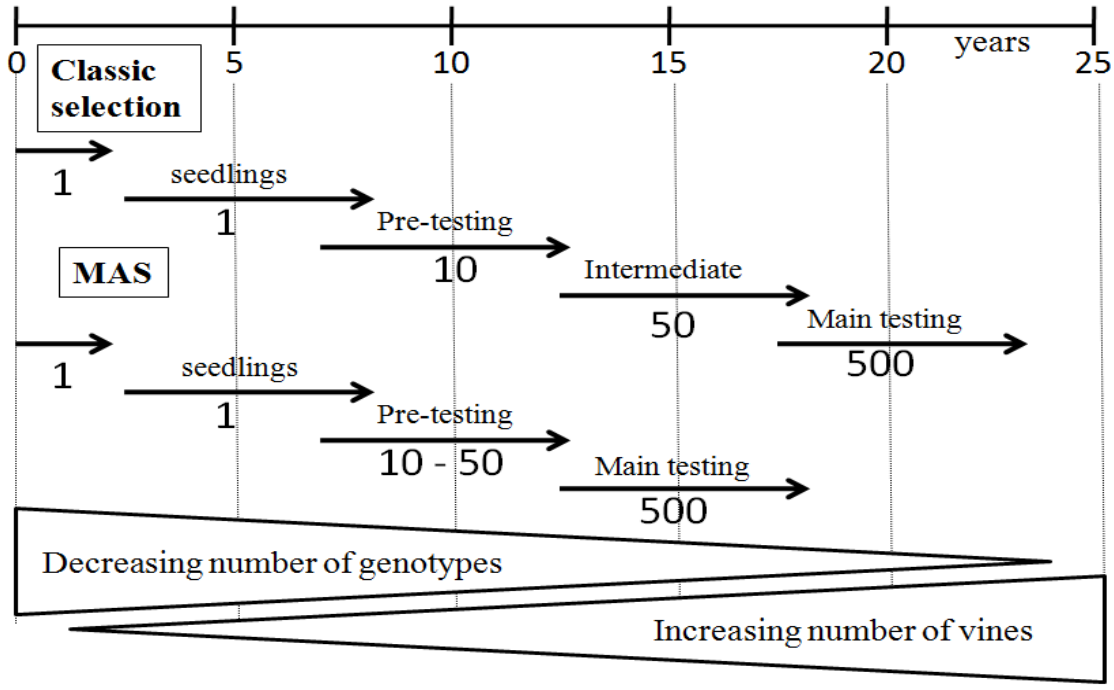


Figure 8. Comparison of the schedule of selection steps in a breeding program using classical breeding and MAS. The number of plants generated is reported under the black arrows.

Those breeding procedures can be facilitated and revolutionized through whole-genome strategies, which utilize full genome sequencing and genome-wide molecular markers to effectively address various genomic factors through a representative or complete set of genetic resources. Breeding will greatly benefit from the growing amount of information that genomics, physiology and biochemistry will convey to grapevine. Modern biotechnology has advanced to the point where the development of highly reproducible genetic engineering protocols now allows the identification and screening of grapevine-derived genes for introducing desirable traits, particularly disease resistance.

## 1.4 Grapevine and its genome

The availability of the complete genome offered new possibilities for grape cultivars characterization through assessment of molecular markers, which can help answering questions about grape diversity, evolution and domestication events. Understanding the genetic and molecular basis of existent natural genetic variation within the genus *Vitis* will provide the information and tools for the genetic improvement required to cope with new threats (e.g. new pathogens or pests) while maintaining specific berry composition (Martinez-Zapatero *et al.,* 2010). Fortunately, this challenge has currently been met by a set of new opportunities that derive from the rapid development of molecular biology technologies and their application to grapevine and other interacting organisms. As a consequence, in the last decade, there has been a rapid increase in the genomic resources that are available for grapevine research (This *et al.,* 2006; Troggio *et al.* 2007). On August, 26th 2006 a great step forward in grapevine biology was achieved through the publication of the first draft of the grapevine genome by the French-Italian Consortium (Jaillon *et al.,* 2007), followed a few months later, by the publication of a second genome draft by the Institute of S. Michele all' Adige (Velasco *et al.,* 2007). The sequencing of the grapevine genome represented the fourth genome of the flowering plants, the second one among wood plants and the first one concerning fruit producing plants. The French-Italian sequencing was obtained by the selection of the PN40024 line, a particular clone characterised by a high degree of homozygosity (approximately 84%) and obtained through multiple auto-fecundation cycles in order to by-pass the high heterozygosis that characterise grapevine. The 475 Mb genome is organized in 19 chromosomes and 26.346 gene predictions have been identified. The genome sequence obtained by the ENTAV 115 clone, which is the Pinot noir line used by IASMA for their sequencing, is slightly larger, with a size for the haploid genome estimated at 504.6 Mb

and a total number of 29.585 gene predictions (http://genomics.research.iasma.it/gb2/gbrowse/grape).The genome available on line has been updated recently from an 8X to a 12X coverage of the genome sequence and a 12X assembly (www.genoscope.cns.fr/esterne/GenomeBrowser/*Vitis*). The gene structure predictions of 8X and 12X assemblies contain different types of subsequence predictions, including genes, mRNAs, UTRs, introns, exons, and inter-genic sequences. For the 8X genomic sequence the methods for gene prediction were edited using the GAZE software (Jaillon *et al.,* 2007). For the 12X prediction two versions are available the 12Xv0 version, performed with the GAZE software by the Genoscope in Evry, France, and the 12Xv1 version, which is the result of the union of v0 and a gene prediction performed with JIGSAW software at the CRIBI in Padova, Italy (Forcato C., 2010). The v1 prediction is available at http://genomes.cribi.unipd.it/ and was used to design the latest available gene expression microarray for grapevine, based on NimbleGen technology, which is the whole-genome array for grapevine. 40% of grape genome composition is made up of transposable elements (TEs), mostly localized within intronic spaces. Looking at proteome, grapevine shows an expansion of gene families with aromatic features. In particular, stilbene synthase and terpene synthase families show an higher gene copy number than in other species. Those gene families are involved in synthesis of resveratrol, known for the beneficial effects on human health and terpenes, components of resins and aromas, respectively. Furthermore, a high number of resistance genes have been identified. The resulting proteins contain a nucleotide binding site (NBS) and a leucine-rich repeat (LRR) responsible for recognition specificity. They are organized in clusters and their heterogeneity seems to function in genome evolution as the basic material for the generation of new resistance specificities (Velasco *et al.,* 2007).

## 1.5 Resistance and susceptible traits to European grapevine disease

One of most important challenges in plant breeding is improving resistance to diseases that threaten grapevine worldwide. The ever-growing world population, changing pathogen populations and fungicide resistance issues have increased the urgency of this task. The severity of diseases depends on weather conditions (it can varies from year to year), on the presence of *inoculum* (history of the disease) and on the susceptibility of the vines. This means that a disease can be devastating one year and insignificant the next. Grapes are a crop susceptible to many diseases; however, the degree of susceptibility varies depending on the variety and on the disease. The major grape diseases in Europe are downy mildew

(*Plasmopara viticola* Berk.), powdery mildew (*E. necator*), grey mold (*Botrytis cinerea* Pers.), anthracnose (*Elsinoe ampelina*), fruit rot (*Rhizopus stolonifer* Ehrenb. Fr. Lind) and crown gall (*Agrobacterium tumefaciens*). Downy mildew, powdery mildew, grey mould, fruit rot and anthracnose are caused by fungi that attack the berries, reducing yield and quality. Crown gall is caused by a bacterium and can kill the plant. Also insects can be very dangerous for grape, an example is the grape phylloxera (*Daktulosphaira vitifoliae*) incurable in Europe. For an effective pest management program the first step is the correct identification of the disease. One of the most important fungal diseases in viticulture worldwide is powdery mildew, caused by the ascomycete *Uncinula necator* (syn. *E. necator*), an obligate biotrophic parasite. Symptoms appear as greyish powdery or dusty patches of fungus growth on the upper side of the leaves and on other green parts of the vines, leading to a decrease in photosynthetic activity. In infected clusters, berries turn hard, brown, are smaller than uninfected ones, and may split open. Besides direct loss of yield, infected berries fail to properly mature and significantly alter wine quality (Calonnec *et al.* 2004). Almost no *V. vinifera* cultivar is immune to *U. necator*, but other grapevine species such as *V. labrusca*, *V. aestivalis* or *V. berlandieri* as well as *Muscadinia rotundifolia* possess various levels of resistance (Mullins *et al.* 1992). In order to introduce resistances into the gene pool of V. *vinifera* various resistance traits have been investigated. Breeders generated F1-plants, the 'direct producers' hybrids (DPH), by inter-specific crosses, but the hybrids resulted with poor and unacceptable wine quality. The increasing knowledge about molecular markers and the availability of grape genome enable the marker-assisted selection (MAS), introgression and pyramiding of resistance loci throughout the breeding process. The starting points is the positional cloning based on available genetic maps with tightly linked markers for important trait loci, mostly resistance genes. Nowadays, a wide range of relevant traits have been identified and mapped along the 19 chromosomes. In table 1 all the resistance loci discovered in grape cultivars are reported together with associated markers, chromosomal localization and references (Adam-Blondon *et al.,* 2011).

Table 1. List of the resistance loci discovered in grape cultivars. Per each locus, name, resistance trait, associated marker name and position in the genome and reference are reported.

| Name | Resistance trait | Associated marker | Genome Position [Chr/Mb] | Reference |
|---|---|---|---|---|
| *PdR1* | Pierce's disease | VMCNg3h8; VVln64; UDV095 | 14/25.3; 14/26.6; 14/26.1 | Riaz *et al.,* 2006; 2008 |
| *Rdv1* | *D. vitifoliae* | Gf139; VMC8e6 | 13/21.9; 13/22.5 | Zhang *et al.,* 2009 |
| *Rpv1* | *P. viticola* | VMC72; VVIb32 | 12/-; 12/10.3 | Merdinoglu *et al.,* 2003 |
| *Rpv3* | *P. viticola* | UDV112; VVIn16 | 18/-; 18/23.4 | Welter *et al.,* 2007 |
| *Rpv4* | *P. viticola* | VMC7h3; VMCNg2e2.1 | 4/4.7; 4/5.2 | Welter *et al.,* 2007 |
| *Ren1* | *E. necator* | UDV-020; VMC9h4-2; VMCNg4e10-1 | 13/-; 13/18.4; 13/18.4 | Hoffman *et al.,* 2008 |
| *Ren3* | *E. necator* | UDV15b; Vvlv67 | 15/7.1; 15/10.9 | Welter *et al.,* 2007 |
| *Run1* | *E. necator* | VMC8g9; VMC4f3.1 | 12/20.4; 12/13.1 | Barker *et al,* 2005 |
| *sdI* | Seedlessness | VMC7f2; VMC6f11 | 18/26.9; 18/23.2 | Cabezas *et al.,* 2006 |

To date, there are few examples of single dominant *R* genes that have proven to be durable in the field, one of them is the *Run1* locus. Indeed, *Run1* seems to be effective against all powdery mildew isolates in France and Australia. However, this may be caused by the combined action of more than one R gene located at the *Run1* locus, that was obtained from *M. rotundifolia* cultivar G52, a cross between the two *M. rotundifolia* cultivars Thomas and Hope (Detjen, 1919). If both cultivars have contributed different R genes to the *Run1* locus, it remains to be determined whether they would provide resistance to different powdery mildew isolates. On the other hand, if the powdery mildew resistance at the *Run1* locus is only conferred by a *single R* gene then this has important implications for the deployment of this resistance gene within the vineyard (Dry *et al.,* 2010).

An alternative strategy to pathogens resistance is the re-establishing of the basal immunity as in the case of non-adapted species unable to suppress basal immune responses failing to enter the epidermal cell. An example is given in the case of *V. vinifera* cv. Cabernet Sauvignon, in which inoculations with the adapted powdery mildew species *E. necator* results in rates of successful penetration (as determined by the presence of haustoria in epidermal cells) of over 90% within 48 h, whereas inoculation with the non-adapted cucurbit powdery mildew species *E. cichoracearum* under the same conditions, results in rates of successful penetration of < 15% (Feechan *et al.,* 2013). In modern resistance breeding, effectors are emerging as tools to accelerate and improve the identification, functional characterization, and deployment of resistance genes. Since genome-wide catalogues of effectors have become available for

various pathogens. Effector-assisted breeding has been shown to be successful for various crops and the "effectoromics" has contributed to classical resistance breeding as well as for genetically modified approaches.

Another class of gene involved in powdery mildew resistance have been considered after the first tests in barley, the susceptible genes, in particular the MILDEW RESISTANCE LOCUS O (*mlo*) locus. The Mlo proteins mediate powdery mildew susceptibility in the model plant species *Arabidopsis* and the crop plants barley and tomato. This *mlo* locus is recessive and differs from race-specific incompatibility to single pathogen strains because it confers a broad spectrum resistance to almost all known isolates of the fungal pathogen, the *mlo* resistance alleles have been obtained by mutagen treatment of any tested barley susceptible wild-type (*Mlo*) variety, and the resistance is apparently durable in the field despite extensive cultivation in Europe (Büschges *et al.,* 1997). In grapevine, three members of the Mlo family (*VvMLO3*, *VvMLO4* and *VvMLO17*), which are Arabidopsis orthologous were found to be specifically induced at the transcriptional level during infection by grapevine powdery mildew (Feechan *et al.,* 2008), suggesting that these VvMLO proteins may have a role in modulating antifungal defense responses in grapevine. However, the mechanism by which *mlo* suppresses PTI to facilitate penetration of adapted powdery mildew species on selected plant hosts is still not understood (Feechan *et al.,* 2013).

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# Chapter 2

# Grapevine biodiversity in Campania region: its investigation through molecular and transcriptomic markers

## 2.1 Introduction

The common grapevine, *Vitis vinifera* subsp. *sativa* L. (2n=6x=38) is one of the major fruit crops worldwide in terms of economic value and cultivated area. Literal, archaeological and paleo-botanical resources have been essential tools to understand the spreading of viticulture in Europe and in particular in Italy and France, starting from the Caucasian area (Ergul *et al.,* 2011). Nowadays, more than 450 varieties are registered in Italy (Mipaaf, 2012), with some grapevines being cultivated only in Campania region (Southern Italy). In the panorama of Italian viticulture and oenology, Campania region is characterized by a distinctive number of ecotypes and ancient grapes (more than one hundred years old). This peculiar condition is the consequence of historical, social, geographical and cultural elements, such as the region orography, the fragmentation of land tenures, the soil volcanic origin and the traditional cutting asexual propagation coupled with cultivation of different varieties in a single vineyard. These aspects make the Campania grapes some of the world's finest wines, characterized by a particularly heterogeneous collection of varieties, some of which have been growing here since the first half of the 19$^{th}$ century. This biodiversity includes registered varieties, ancient grapes and autochthonous biotypes carefully preserved from genetic erosion and particularly useful as genetic resources in breeding programs. Using the National Register of Varieties (Mipaaf, 2012), the varieties registered in Campania have been reported in Table 1 along with their berry color, synonymous and number of registered clones.

Table 1. Grapevine varieties registered in Campania region. For each grapevine name, berry color, synonymous and number of clones registered are reported.

| Variety | Berry color | Synonymous | # registered clones |
|---|---|---|---|
| Aglianico | Black | Glianica, Glianico, Ellenica, Ellenico | 19 |
| Aglianicone | Black | - | 0 |
| Asprinio | White | - | 0 |
| Bellone | White | - | 0 |
| Biancolella | White | Janculillo, Janculella | 0 |

| | | | |
|---|---|---|---|
| Bombino Nero | Black | Bombino, Bonvino | 1 |
| Bombino Bianco | White | Ottenese, Bombino, Bonvino | 3 |
| Casavecchia | Black | - | 0 |
| Catalanesca | White | - | 0 |
| Cesanese | Black | Cesanese Comune | 1 |
| Ciliegiolo | Black | Morettone | 4 |
| Coda di Volpe | White | - | 0 |
| Falanghina | White | - | 3 |
| Fiano | White | - | 6 |
| Forastera | White | Forestiera, Furastiera | 0 |
| Ginestra | White | - | 0 |
| Greco | White | - | 6 |
| Greco Nero | Black | Greco, Maglioccone, Gregu Nieddu | 4 |
| Guarnaccia | White | - | 0 |
| Malvasia | Black | Malvasier, Roter Malvasier | 0 |
| Malvasia | White | Verdana, Iuvarella, Malvasia | 1 |
| Montonico | White | Muontonico, Mantonico | 1 |
| Olivella nera | Black | - | 0 |
| Pallagrello bianco | White | - | 0 |
| Pallagrello nero | Black | - | 0 |
| Pepella | White | - | 0 |
| Piedirosso | Black | Piede di Colombo, Piede di palumbo, Per E palummo, palombina | 4 |
| Procanico | White | | |
| S.Lunardo | White | Don Lunardo | 0 |
| Sciascinoso | Black | - | 0 |
| Verdeca | White | Pampanuto | 3 |

In a large panorama of genotypes, an efficient characterization system is important to avoid cases of synonymy (identical genotypes but different names) and homonymy (same names but different genotypes), to definite population structures, trace plant products and protect breeders' rights. The oldest grape discrimination technique is the ampelography (Αμπελος, "vine" and γραφος, "writing"), which is the science of phenotypically distinguishing grapevines. The authoritative ampelographic reference is the *Précis d'Ampélographie Pratique* (Galet, 1952), translated into English as *A Practical Ampelography: Grapevine Identification* (Galet, 1979), which features leaves, fruits and other traits for most domesticated and many wild vines. This technique can result elaborate and time-consuming. Moreover, it has been reported that morphological characterization is often inaccurate for the discrimination of closely related cultivars because of confounding environmental and developmental factors and control by epistatic and pleiotropic gene effects (Dodig *et al.,*

2010; López *et al.,* 2009) In contrast, DNA based molecular markers can overcome many of the limitations of phenotypic-based diversity analysis and provide a more direct measure of genetic variability (Gutpa *et al.,* 1999). Among the most interesting markers used for this purpose, the microsatellites (simple sequence repeats, SSR) are popular for their reproducibility, co-dominant nature, polymorphism, hyper-variability and high cross-species transferability (This *et al.,* 2004). They have been adopted as the standard markers for germplasm management in many plant species (Varshney *et al.,* 2005). Also in grape they have become markers of choice for compilation, standardization and exchange of information concerning germplasm collections (This *et al.,* 2004; Ibáñez *et al.,* 2009; Cipriani *et al.,* 2010; Emanuelli *et al.,* 2013). In this contest, Laucou and collaborators (2011), analyzing 4.370 grape accessions, defined a minimal set of nine SSR primers proved to be discriminatory with a reduced probability of false identity. These primers have been already successfully employed by Bacilieri *et al.* (2013) to genotype several *V. vinifera* accessions, supporting archeological and historical data on grapevine domestication in Europe and Asia. Bergamini *et al.* (2012), amplifying a selected number of microsatellite loci in over 2000 accessions of the Italian grapevine "Sangiovese", demonstrated the presence of an ancient variety ('Negrodolce', believed to have been lost) in its pedigree. More precise information can be provided combining SSR with other molecular markers. In this regard, retrotransposon-based markers can be particularly interesting in that their use, combined with SSR, have been proved to allow the coverage of both inter- and intra-genic DNA (Laucou *et al.,* 2011; Ziarovska *et al.,* 2012; Carcamo *et al.,* 2010). Retrotransposons are characterized by their capability to translocate and change their genomic location, whereby they generate transposition polymorphism. They consist in elements, hundreds to thousands of nucleotides long. The long terminal repeats (LTRs) that bind to a complete retrotransposon contain ends that are highly conserved in a given family of elements (Moisy *et al.,* 2008). In grape, retrotransposons seem suitable additional markers also for their abundance in the genome (Shimamura *et al.,* 1997), their presence in many copies (Varshney *et al.,* 2005) and their ability to track an insertion event and its subsequent vertical radiation through either pedigree or phylogeny analysis (Kalendar *et al.,* 2011). REMAP (REtrotransposon-Microsatellite Amplified Polymorphism) and IRAP (Inter-Retrotransposon Amplified Polymorphism) markers are cheap to establish and assay, easy to perform and reproduce (Kobayashi *et al.,* 2004). In *V. vinifera* three retro-elements are known, *Gret1*, *Vine-1* and *Tvv1* (Verriés *et al.,* 2000; This *et al.,* 2007). Currently, only *Gret1* has been fully sequenced and it is known to be associated with mutations causing most white-fruited *V. vinifera* genotypes due to its insertion

into the promoter of *VvMybA1*, the transcription factor controlling the final step in anthocyanin biosynthesis during ripening (This *et al.,* 2007). Pereira *et al.* (2005) demonstrated that *Gret1* is useful as molecular marker and it may play an important role in the expression of phenotypes that characterize a cultivar. REMAP have been used for genetic diversity assessment of various crops such as rice (Branco *et al.,* 2007), wheat (Georgi *et al.,* 2010) and banana (Teo *et al.,* 2005; Nair *et al.,* 2005). IRAP have been employed in germplasm studies in barley (Bradley *et al.,* 2010), tobacco (Xiao *et al.,* 2006) and grape (D'Onofrio *et al.,* 2010).

Among all the Campania varieties, Aglianico shows the highest number of clones; it is probably due to his oenological importance in the wine market worldwide (Figure 1). Indeed, recently (6$^{th}$ June 2013) Eric Asimov, the wine writer for the *New York Times,* described Aglianico as one of Americans favourite red Italian grapes, going on to say that he finds wines made from the grape "delicious, structured, and age-worthy."

Figure 1. Grape clusters of Aglianico del Taburno.

Aglianico is a later-maturing grape and is characterized by a high content of total flavonols and anthocyanins, showing a notable presence of quercetin-3-O-glucoside, malvidin-3-O-glucoside and petunidin-3-O-glucoside. The strong antioxidant activity and the high resveratrol content confer high positive nutriceutical properties to these grapes and wines (De Nisco *et al.,* 2013). Moreover, the high content of polyphenols mainly tannins, may confer to the wine a harsh and astringent character. Rinaldi *et al.* (2014) carried out a preliminary characterization of proanthocyanidins of seeds and skins of Aglianico grape cultivar and compared it with international cultivar as Merlot and Cabernet Sauvignon; the results showed that Aglianico is a model for astringency. As the knowledge of the phenolic composition at each grape time involved in winemaking is essential to choose the optimal harvest time and to perform successful oenological practices, likewise, the understanding of the genetic control of

phenylpropanoids by synthesis during ripening is crucial to establish the quality of berry and the consequent structure of wine. An exhaustive overview of the phenyl-propanoid pathway in grapevine is represented in Figure 2, in which each compound formed starting by chalcone, intermediate molecules and each key genes are reported (Dixon *et al.,* 2013). This pathway involves a high number of enzymes in the various branches; them activities are highly regulated from transcription factors and/or in response to different developmental and environmental triggers. There is also evidence that the enzymes involved in flavonoid metabolism might be acting as membrane-associated multi-enzyme complexes, which have implications on overall efficiency, specificity, and regulation of the pathway (Jaakola *et al.,* 2002). The amino acid phenylalanine is produced via the shikimate pathway, converted and enzymatically coupled with the carboxylated acetyl-CoA (malonyl-CoA) to be the starting molecule for all phenolic compounds synthesize, as proanthocyanidins, anthocyanins and flavonoids. The phenylalanine ammonia-lyase (*PAL*) catalyzes the first step in phenylpropanoid metabolism in which phenylalanine undergoes deamination to yield trans-cinnamic acid and ammonia. The cinnamic acid is hydroxylated to 4-coumarate (also known as p-coumarate) from a cytochrome P450-dependent mono-oxygenase, the cinnamate 4-hydroxylase (*C4H*). At this point a tyrosine molecule is used from the 4-coumarate:CoA ligase (*4CL*) to catalyze the ATP-dependent formation of the CoA thioester 4-coumaroyl CoA, which brings to formation of chalcones by chalcone synthase (*CHS*) activities. This is the starting point for the formation of the majority of flavonoids, of which the basic molecular structure is represented in a box of the Figure 2. The chalcone isomerase (*CHI*) catalyzes the synthesis of flavanones, the flavanone 3-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*) and flavonoid 3'-5'-hydroxylase (*F3'5'H*) induce the formation of flavonols and the dihydroflavonol reductase (*DFR*) brings to production of leucoanthocyanidins. Afterwards the activity of different enzymes as anthocyanidin reductase (*ANR*) and anthocyanidin synthase (*ANS*) joined with modifications as glycosylation, methylation, acylation, polymerization and oxidation, caused the development of anthocyadinins, anthocyanins and proanthocyanidins (condensed tannins). All of these compounds are commonly detected in grapes and wine, in particular proanthocyanidins are responsible of the astringency in red wines, the anthocyanins influence the berry skin color and the flavonoids protect berries from UV-B light exposition (Koes *et al.,* 1994).

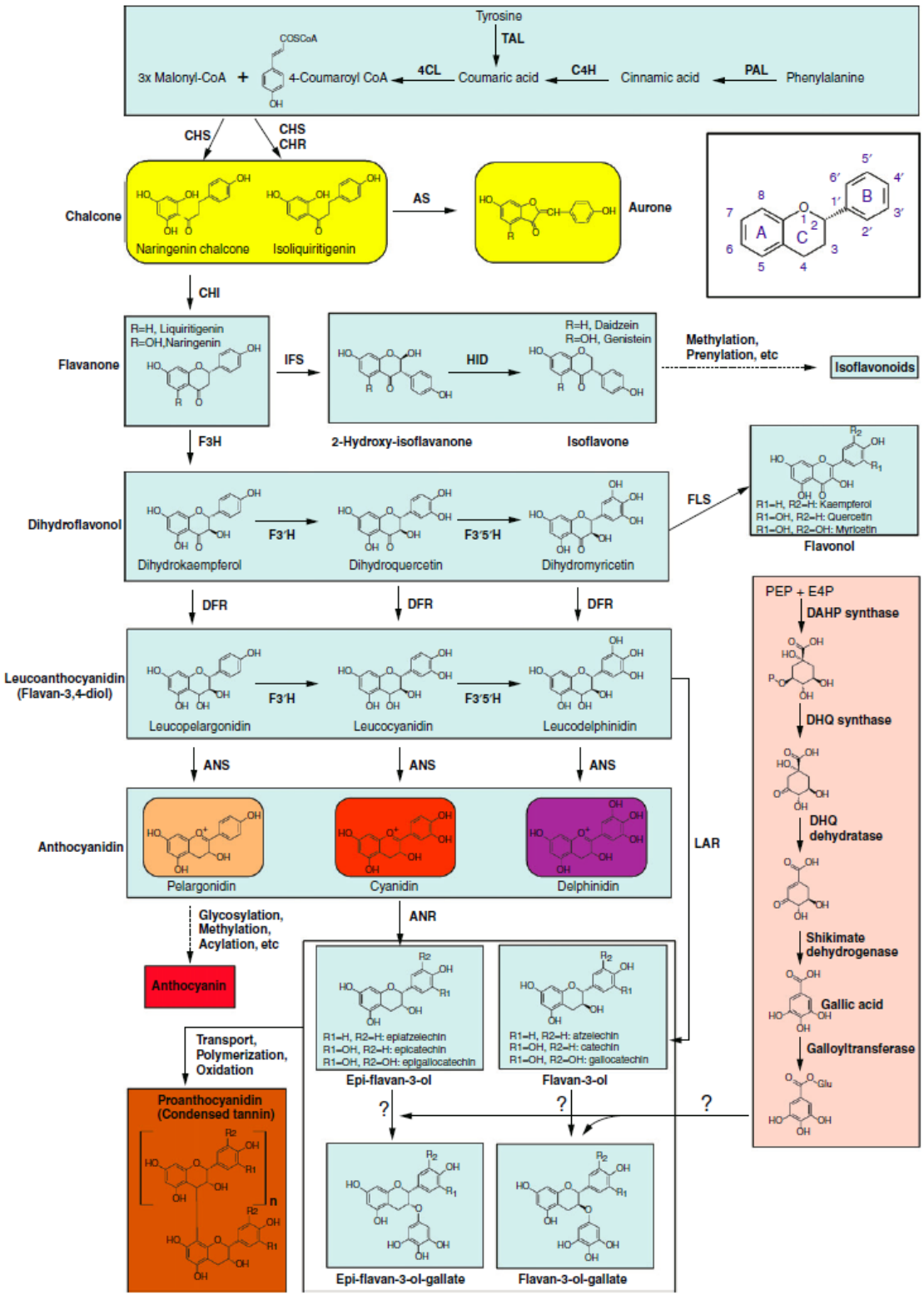


Figure 2. Scheme for the biosynthesis of flavonoids, with emphasis on anthocyanins and condensed tannins. The numbering scheme for the majority of flavonoids described in the present article is shown in the box, second line right. Abbreviations for enzymes are: *ANR*, anthocyanidin reductase; *ANS*, anthocyanidin synthase; *AS*, aurone synthase; *C4H*, cinnamate 4-hydroxylase; *CHI*, chalcone isomerase; *4CL*, 4-coumarate coenzyme A ligase; *CHR*, chalcone reductase; *CHS*, chalcone synthase; *DFR*, dihydroflavonol reductase; *F3H*, flavanone 3-hydroxylase; *F3'H*, flavonoid 3'-hydroxylase; *F3'5'H*, flavonoid 3'-5'-hydroxylase; *FLS*, flavonol synthase; *HID*, 2-hydroxyisoflavanone

dehydratase; *IFS*, 2- hydroxyisoflavanone synthase; *LAR*, leucoanthocyanidin reductase; *PAL*, L-phenylalanine ammonia-lyase; *TAL*, L-tyrosine ammonia-lyase.

The outline of the anthocyanin biosynthesis pathway in Figure 2 describes a linear pathway that begins with phenylalanine and malonyl- CoA and results in the production of stilbenes, flavoinoids and glycosylated anthocyanins. These molecules are a group of naturally occurring phenolic compounds that are responsible for the coloration of fruits, vegetables, and flowers. They have also many beneficial effects for humans including the reduction in the incidence of coronary heart disease, enhancement of visual acuity, maintenance of normal vascular activity, as well as anti-carcinogenic, anti-mutagenic, anti-inflammatory, and anti-oxidative properties (Gosh *et al.,* 2007). In plant, anthocyanin accumulation includes a complex phyto-chemical cocktail, usually correlated to tissue color and plant immunity. The toxicity, and so antibiotic activity, of anthocyanin is activated only as a consequence of a mechanical damage, as pathogen penetration or insect chewing, resulting in their breakdown and aglycons liberation. This mechanism is a plant defense adopted to avoid damage to own vitally active cells. Wegener and Jansen (2013) demonstrated the correlation between antioxidants, such as anthocyanin, and abiotic stress in potato. After wounding, the control tubers of three breeding clones revealed an increase in peroxidase enzyme (POD) activity in two years; in particular a purple clone displayed a clear rise in enzyme activity after wounding, despite a relatively high POD level in its fresh tissue. This fact may underline the role of POD, involved in resistance expression, within wound stress responses.

### 2.1.1 Aim of the research

Italy is one of the biggest grapevine producers with more than 450 varieties registered, most of them used in winemaking. In the panorama of Italian viticulture and oenology, Campania region is characterized by a vigorous biodiversity, which include a distinctive number of ecotypes and various ancient grapes (more than one hundred years old). This biodiversity reflect the ancient heritage of Campania vineyards and a great respect for traditions. While substantial genetic diversity has been maintained since domestication of *Vitis vinifera,* there has been very limited exploitation of this diversities especially about the phenolic compounds that influence the wine structure and their regulation in response to abiotic and biotic stress, as pathogens. In this contest have been developed the main purposes of these research focusing the attention on the exploitation of the cited biodiversity and using

it to increase the genetic basis knowledge of phenolic compounds production. The main goals are the following: (a) fingerprinting of grapevines grown in Campania region using codominant and dominant markers and search of confidential alleles useful in traceability studies; (b) investigation of the expression profile of 15 key-genes of phenyl-propanoid pathway in the most commercialized wine grape of Campania region, Aglianico del Taburno, across five developmental stages and three different berry tissues and chemical quantitative estimation of anthocyanin, tannin and total polyphenols in berry skin and seeds; (c) study of the effect of wounding stress on anthocyanin accumulation in Aglianico del Taburno leaves.

## 2.2 Materials and methods

### 2.2.1 Plant material

SR and retrotransposone-based analysis were carried out on sixty-two grape cultivars sampled from even producing areas of Campania region. The list showing all analyzed cultivars and respective growing areas is given in Table 2.

Table 2. Local and reference grapevines analyzed with molecular markers. For each of them the code used in population structure analysis, local name, berry color, use, origin and sampling area are reported.

| Code | Grapevine | Berry color | Geographic origin, province, state | Sampling area, city, province | Use |
|---|---|---|---|---|---|
| 1 | Abbuoto | Black | Lazio | Latina | Wine |
| 2 | Aglianico bianco | White | Campania | Paternopoli , Avellino | Wine |
| 3 | Aglianico del Vulture | Black | Campania | Pietradefusi , Avellino | Wine |
| 4 | Aglianico del Taburno | Black | Campania | Pietradefusi , Avellino | Wine |
| 5 | Aglianico Taurasi | Black | Campania | Pietradefusi , Avellino | Wine |
| 6 | Agostinella | White | Campania | Castelvenere , Benevento | Wine |
| 7 | Arilla | White | Sicily | Eboli, Salerno | Wine |
| 8 | Arilla | White | Sicily | Ischia, Naples | Wine |
| 9 | Barbera | Black | Piemonte | Ischia, Naples | Wine |
| 10 | Barbera del Sannio | Black | Campania | Castelvenere , Benevento | Wine |
| 11 | Barbera del Sannio | Black | Campania | Pietradefusi , Avellino | Wine |
| 12 | Cacamosca | White | Campania | Eboli, Salerno | Wine |
| 13 | Cacamosca | White | Campania | Ischia, Naples | Wine |
| 14 | Cannamelo | Black | Campania | Eboli, Salerno | Wine |
| 15 | Cardinal | Red | North America | Pietradefusi , Avellino | Table grape |
| 16 | Catalanesca | White | Campania | Eboli, Salerno | Wine |
| 17 | Chardonnay | White | France | Pietradefusi , Avellino | Wine |
| 18 | Coda di Volpe bianca | White | Campania | Pietradefusi , Avellino | Wine |

| | | | | | |
|---|---|---|---|---|---|
| 19 | Coda di Volpe nera | Black | Campania | Paternopoli , Avellino | Wine |
| 20 | Coglionara | White | Campania | Ischia, Naples | Wine |
| 21 | Don Lunardo | White | Campania | Ischia, Naples | Wine |
| 22 | Falanghina beneventana | White | Campania | Pietradefusi , Avellino | Wine |
| 23 | Fiano di Avellino | White | Campania | Pietradefusi , Avellino | Wine |
| 24 | Ginestrello | White | Campania | Amalfi, Salerno | Wine |
| 25 | Greco di Tufo | White | Greece | Pietradefusi , Avellino | Wine |
| 26 | Guarnaccia | Black | Campania | Ischia, Naples | Wine |
| 27 | Guarnaccia | Black | Campania | Eboli, Salerno | Wine |
| 28 | Lambrusco Salamino | Black | Emlia-Romagna | Pietradefusi , Avellino | Wine |
| 29 | Lambrusco Maestri | Black | Emlia-Romagna | Pietradefusi , Avellino | Wine |
| 30 | Livella | Black | Campania | Ischia, Naples | Wine |
| 31 | Livella | Black | Campania | Eboli, Salerno | Wine |
| 32 | Malaga | White | Spain | Amalfi, Salerno | Wine |
| 33 | Mennavacca | White | Campania | Pietradefusi , Avellino | Wine |
| 34 | Merlot | Black | France | Pietradefusi , Avellino | Wine |
| 35 | Montepulciano | Black | Abruzzo | Pietradefusi , Avellino | Wine |
| 36 | Montuonico | White | Calabria | Pietradefusi , Avellino | Wine |
| 37 | Moscatello | White | Apulia | Ischia, Naples | Wine |
| 38 | Moscato bianco | White | Italy | Pietradefusi , Avellino | Table grape |
| 39 | Moscato d'Amburgo | Black | Italy | Pietradefusi , Avellino | Wine |
| 40 | Nocella | White | Campania | Ischia, Naples | Wine |
| 41 | Olivella | Black | Lazio | Eboli, Salerno | Wine |
| 42 | Passolara | White | Campania | Eboli, Salerno | Wine |
| 43 | Pellecchiona | Black | Campania | Amalfi, Salerno | Wine |
| 44 | Piedirosso | Black | Campania | Pietradefusi , Avellino | Wine |
| 45 | Pizzutello bianco | White | Lazio | Pietradefusi , Avellino | Table grape |
| 46 | Pizzutello nero | Black | Lazio | Pietradefusi , Avellino | Table grape |
| 47 | Procidana | White | Campania | Ischia, Naples | Wine |
| 48 | Regina | White | Lazio | Pietradefusi , Avellino | Table grape |
| 49 | Rosè | Red | Campania | Paternopoli , Avellino | Wine |
| 50 | Rovello | Black | Campania | Eboli, Salerno | Wine |
| 51 | Royal | White | Campania | Pietradefusi , Avellino | Table grape |
| 52 | Sanginella | Black | Campania | Ischia, Naples | Wine |
| 53 | Sangiovese | Black | Emlia-Romagna | Pietradefusi , Avellino | Wine |
| 54 | Sciascinoso | Black | Campania | Pietradefusi , Avellino | Wine |
| 55 | Serpentaro | Black | Campania | Amalfi, Salerno | Wine |
| 56 | Sommarello | Black | Apulia | Pietradefusi , Avellino | Wine |
| 57 | Suppezza | Black | Campania | Eboli, Salerno | Wine |
| 58 | Tintore | Black | Campania | Eboli, Salerno | Wine |
| 59 | Trebbiano | White | Tuscany | Pietradefusi , Avellino | Wine |
| 60 | Uva chiena | White | Campania | Ischia, Naples | Wine |
| 61 | Uva fragola white[a] | White | America | Pietradefusi , Avellino | Table grape |
| 62 | Uva fragola black[a] | Black | America | Pietradefusi , Avellino | Table grape |

a It belongs to cultivar "Isabella", an hybrid between V. labrusca and an unidentified V. *vinifera* subsp. *vinifera* variety (Munson, 1909)

The expression analysis was performed on *V. vinifera* cv Aglianico del Taburno, clone Ampelos TEA 22 grafted on rootstock 1103 Paulsen – *V. berlandieri* x *V. rupestris* – clone ISV 1. Samples were collected from a 7-year-old vineyard (41°13'43.00'' N, 14°33'37.56'' E, 145 slm, Castelvenere, Benevento Province, Italy) during the 2012 growing season.

### 2.2.2 Sample collection

For molecular marker analysis, young leaves of all genotypes were collected in the field and immediately frozen in liquid nitrogen. For expression and chemical analysis of poly-phenol compounds, Aglianico del Taburno berries were collected at five developmental time points and immediately frozen in liquid nitrogen. The first stage (15 days after flowering [DAF]; Eichhorn-Lorenz System [E-L] 29) corresponded to the fruit set, when young berries are enlarging (>3 mm diameter); the second stage (35 DAF; E-L 32) was the post-fruit set, when berries start touching (>7 mm diameter); the third stage (70 DAF; E-L 35) was the *veraison*, when berries begin to change colour and enlarge; the fourth stage (84 DAF; E-L 36) corresponded to the mid-ripening stage; and the final stage (115 DAF; E-L 38) represented complete ripening. Starting from the *veraison* stage, berries were manually dissected into skin, pulp and seeds starting from frozen berries. Three biological replicates were sampled. Aglianico del Taburno cuttings were collected and maintained in greenhouse for further collection of material.

### 2.2.3 Microsatellite analysis

Total genomic DNA was extracted from young leaves using the Qiagen Plant DNeasy Maxi Kit (Qiagen, Valencia, CA, USA), following the manufacturer's procedure. Microsatellite analysis was carried out with 20 nuclear and 10 chloroplast markers. They were chosen from six sources: eight (VVMD7, VVlb01, Vvlh54, Vvln16, VVIp60, VVIq52, VVMD25 and VVMD5) from Laucou *et al.* (2011), eight (VrZAG series) from Sefc *et al.* (1999), three (VVS series) from Thomas *et al.* (1993), nine (CCMP series) from Weising *et al.* (1998), VVIC05 from Merdinoglu *et al.* (2005) and ccSSR5 from Chung *et al.* (2003). All SSRs characteristics are reported in Supplementary Table 2. PCR reactions were performed in a 20 µL volume containing 1× reaction buffer with 1.6 mM $MgCl_2$, 0.2 mM of each dNTP, 30 pM FAM-labelled forward SSR primer and reverse SSR primer, 1 unit of go*Taq* polymerase (Promega, Madison, WI, USA) and 30 ng of genomic DNA. Amplification consisted of 5

cycles at 94°C for 45 sec, Ta°C + 5 for 60 sec, 72°C for 30 sec; 30 cycles at 94°C for 45 sec, Ta°C for 60 sec, 72°C for 30 sec with the annealing temperature reduced by 1°C per cycle (touchdown PCR), then one elongation cycle at 72°C for 20 min. Amplicons were separated with the ABI PRISM® 3130 DNA Analyzer system (Life Technologies, Carlsbad, California, USA). Size calibration was performed with the molecular weight ladder GenScan® 500 ROX™ Size Standard (Life Technologies, Carlsbad, California, USA). SSR alleles were detected and scored by using Peak Scanner® software (Applied Biosystems, Foster City, California, USA). The results validation was carried out with two biological replications and PCR reactions were performed in triplicate. The allele's sizes were normalized using SSR data reported in the *Vitis* International Variety Catalogue (http://www.vivc.de/).

### 2.2.4 Retrotransposon-based genotyping

For REMAP and IRAP marker amplification, four primers were chosen: two were designed on the *Gret1* LTR regions, (5'-LTR: 5'-CGAGTTTGTGTAGATTACAC-3', and 3'-LTR: 5'-GCATTTAGAAGGATTTAGCTT-3'), and two on microsatellite repeats (Microsat-GA [ $(GA)_9$ C] and Microsat-CT [ $(CT)_9G$]). We set up four REMAP markers combining LTR primers with microsatellite primers and one IRAP marker amplifying with both LTR primers as described in Pereira *et al.* 2011. PCRs were performed in a 20 µl reaction mixture containing 20 ng of genomic DNA as previously reported (Kalendar *et al.,* 1999). PCR products were separated in a 2% agarose gel electrophoresis at 50V for 30 min, 70V for 30 min and 100V for 3 h. Bands were detected by GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, California, USA). For image acquisition and identification of band size, the Quantity One® 1-D Analysis Software (Bio-Rad Laboratories, Hercules, California, USA) was used. Results were confirmed with three technical replicates.

### 2.2.5 Molecular Markers Data Analysis

The statistical software GenAlex 6.5 (Peakall *et al.,* 2006) was used for data analysis. To estimate the degree of polymorphism, the number of alleles per locus for single-locus markers (SSR) and the number of bands per primer combination for multi-locus markers (REMAP and IRAP) were calculated. The statistical parameters useful for genetic diversity analysis were the expected heterozygosity ($He= 1 - \sum pi^2$, where pi is the frequency of the $i^{th}$ allele) (Nei *et al.,* 1972), the fixation index ($F = 1/1 - Ho/He$) (Wright *et al.,* 1951), the Shannon's Informative Index ($I = -1 * Sum\ (pi * Ln\ (pi))$ (Shannon *et al.,* 1949), the Power of Discrimination ($PD= 1 - \sum C$, where $C = pi * \{[ (N*pi) - 1]/N - 1\}$), the probability of

identity ($PI = [1 - \sum pi^4 + \sum (2pipj)^2$, where *pi* and *pj* are the frequency of the $i^{th}$ and $j^{th}$ alleles, respectively) (Peatkau *et al.,* 1995). For the chloroplast microsatellite data the Gene Diversity equation was adapted to haploid data ($He = 1 - \sum pi^2$, where pi is the frequency of the $i^{th}$haplotype) (Castro *et al.,* 2012). Because the chloroplast genome is uniparentally inherited and thus not recombining, it was treated as one locus and the different haplotypes were treated as alleles. We also identified marker-specific and genotype-specific alleles, called private alleles. These are alleles found in a single genotype among the complete collection of genotypes studied.

A genetic distance matrix was calculated by using the Dice coefficient (Dice *et al.,* 1945). Phylogenetic clustering trees were constructed by unweighted pair group method with arithmetic mean (UPGMA) (Sneath *et al.,* 1973) using MEGA 5 software (Tamura *et al.,* 2011). Clusters robustness was tested by bootstrap resampling ($n$=1000) with the software package WINBOOT (Yap *et al.,* 1996). To confirm the accuracy of clustering, we conducted a Bayesian analysis using the software STRUCTURE 2.3.1 (Pritchard *et al.,* 2000). For this analysis we used the admixture model (because of the grape mating system and biology), correlating allele frequencies twenty times for each K (number of populations assumed), with a burn-in of 500.000 interactions followed by 750.000 interactions MCMC (Markov Chain Monte Carlo). The most likely number of clusters was chosen using the ΔK method, as described in (Evanno *et al.,* 2005). Pearson correlation between matrices of genetic and geographic distances, as well as between genetic distance matrices for SSR and retrotransposon-based markers, was calculated using the statistical tool XLSTAT 2013.2.05 (Addinsoft, Paris, France). Significance was evaluated by Mantel test (Mantel *et al.,* 1967).

### 2.2.6 Gene expression analysis

Total RNA was isolated from 40 mg of ground leaves using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's protocol with some modifications. Quantity and quality of the isolated RNA was measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). For cDNA synthesis, 100 ng of each RNA sample was reverse transcripted using the SuperScript® III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Before use in Real-Time PCR experiments, cDNA reactions were diluted 5-fold to 200 µL with UltraPure DNase/RNase-Free Distilled Water (Lonza Group, Basel, Switzerland). Expression analysis was conducted by real-time PCR analysis using a SYBR Green method on a 7900HT Fast Real-Time PCR System

(Applied Biosystems, Foster City, CA. USA). Each 15 µL PCR reaction contained 330 nM of each primer, 2 µL of diluted cDNA and 7.5 µl of Sybr Green Mix (Applied Biosystems, Foster City, CA, USA). The SDS 2.3 and RQ Manager 1.2 softwares (both Applied Biosystems, Foster City, CA, USA) were used for data elaboration. The primer pairs used in the quantitative analysis are reported in Table 3.

Table 3. List of primers used in expression analysis of the phenyl-propanoid pathway. For each primer, genes, sequences, amplicons length, reference and GenBank accession number are reported.

| **Genes** | **Primers sequence (forward/reverse)** | **Amplicon lenght (bp)** | **Reference** | **GenBank accession number** |
|---|---|---|---|---|
| *PAL* | TCTGGTGGAAGGAATCCAAG/ CAAAGTGCCACCAGGTAGGT | 230 | Ageorges *et al.,* 2006 | GU585850 |
| *F3'H* | ATTCGCCACCCTGAAATGAT/ AGCCGTTGATCTCACAGCTC | 196 | Castellarin *et al.,* 2006 | GU585856 |
| *F3'5'H* | GAAGTTCGACTGGTTATTAACAAAGAT/ AGGAGGAGTGCTTTAATGTTGGTA | 156 | Castellarin *et al.,* 2007 | GU585857 |
| *LDOX* | AGGGAAGGGAAAACAAGTAG/ ACTCTTTGGGGATTGACTGG | 109 | Jeong *et al.,* 2004 | GU585861 |
| *UFGT* | GGGATGGTAATGGCTGTGG/ ACATGGGTGGAGAGTGAGTT | 152 | Jeong *et al.,* 2004 | GU585862 |
| *LAR1* | AAATGAACTCGCATCTGTGT/ CTGTGGGATGATGTTTTCTC | 109 | Fujita *et al.,* 2007 | GU585865 |
| *ANR* | GCTGCTGTTACCATCAATCA/ GCAGGATAGCCCCAAGTAGG | 113 | Fujita *et al.,* 2007 | GU585867 |
| *C4H* | ATCCACCGCCACAACCAT/ GGCCAGAATTATAGCGCAGAA | | | |
| *4CL* | CTGCCGCTGTTCCACATATACTC/ CCAATCTCGAACTTCTGCATCA | 198 | Lijavetzky *et al.* 2008 | AF002257 |
| *FLS4* | AGGCACACTTTTCCCACGTT/ ACCCCCTACGAACCCAGAAG | 99 | Kobayashi *et al.,* 2011 | AB092591 |
| *FLS5* | GCATGTCATGGGCTGTGTTT/ ACTTGGCAGGGTTTGGTTCA | 99 | Kobayashi *et al.,* 2011 | AB213566, |
| *DFR* | ACCTGGATGACCTCTGCAATG/ CAATCGTGGGAGGAGCAAAT | 84 | Kobayashi *et al.,* 2011 | X75964 |
| *LAR2* | TCAAGCAGCAGCCGAGAAG/ CGAGAGAAGTGGCGGTGATC | 99 | Kobayashi *et al.,* 2011 | XM_002273879 |
| *MybPA1* | TTGACGGGGTTGACTTCTTC/ GAGTAGTGATTCGGCGAAGG | 188 | Terrier *et al.,* 2008 | CAJ90831 |
| *MybPA2* | CCAAGATGAAGAGATGGGAGA/ AAAGATGCCAGTGTCTTGAGC | | | |
| *β-Tubulin* | TGAACCACTTGATCTCTGCGGACTA/ CAGCTTGCGGAGGTCTGAGT | 86 | Reid *et al.,* 2006 | EC922104 |

The expression of each target gene was normalized with the expression level of the housekeeping gene (*β-Tubulin*) and the ΔCt values were calculated using the Livak method calibrating each gene expression against the ΔCt values obtained in the fruit set. For each

gene and all the different tissues, fold change values were calculated using this formula $2^{\Delta\Delta Ct}$. The down-expression values were indicated by results ranging from 0 to 1, while over-expression values were over 1. Heatmap graphs were carried out using the phenyl-propanoid pathway as background and reporting the fold change values in a colorimetric scale ranging from blue (-3) to red (7).

### 2.2.7 Polyphenols chemical analysis

The analyses were carried out with Prof. Luigi Moio and Dr. Alessandra Rinaldi at the Department of Agriculture of the University of Naples "Federico II" (division of Vine and Wine, Avellino). For three developmental stages of Aglianico del Taburno (*veraison*, mid-ripening and ripening), twenty berries were collected and immediately frozen in liquid nitrogen. Skin and seeds were separated and grounded using mortar and pestle. The polyphenols compounds were extracted using the protocol reported in Lorrain *et al.* (2011). Afterwards, monomers, oligomers and polymers of proanthocyanidin were separated and lyophilized (Sun *et al.,* 1998). A sonication step was performed on 1 g/L of extracted solutions after a protein precipitation assay developed by Harbertson and collaborators (2002), consisting in a precipitation step with BSA and a following detection of phenol compounds by a UV-VIS spectrophotometer.

### 2.2.8 Wounding treatment

Aglianico del Taburno young leaves were detached from greenhouse vines and sterilized under laminar hood with a solution 1:1 Amuchina and autoclaved MilliQ Water for 5 minutes. Three washing steps were performed using autoclaved MilliQ water. The sterilized leaves were dried for one hour and separated in three biological replicates. Using a sterilized cookie cutter, discs of 2 cm diameter were punched from leaves avoiding central ribs. The punching of discs was considered as a wounding treatment. Control discs corresponded to an unwounded leaf straight detached from a healthy vine (Vine control) and to sterilized unpunched leaves (0 h). The punched discs, at least five for sample, were collected 1, 2, 4, 6 and 24 h after treatment. The expression level of nine genes involved in the phenyl-propanoid pathway was investigated following the same procedure previously described. Four of them are candidate genes of the stilbene synthase family, *VvSTS6*, *VvSTS16*, *VvSTS36* and *VvSTS22*, and two of them are transcription factors (*VvMyB14* and *VvMyB15*). Their amplification was set up as reported in Vannozzi (2012).

## 2.3 Results

### 2.3.1 SSR analysis

SSR used in this study allowed the differentiation of 62 grapevine accessions. We detected 183 alleles with size ranging from 62 bp (CCMP8) to 331 bp (VVIp60) and an average of 12.35 alleles per *locus* for nuclear microsatellites (Table 4) and 3.6 for chloroplast microsatellites (Table 2). For nSSR, *He*, *F*, *I* and *PD* mean values were 0.794, 0.258, 1.927 and 0.806, respectively. *He* was significantly higher than 0.5 for 80% of markers and *F* value was negative for one marker, VrZAG21, and higher then 0.85 for six markers. F negative values indicate an excess of heterozygotes and values above zero an excess of homozygotes. This suggested that many apparent homozygotes are likely to be heterozygotes with one amplified and one null allele, as suggested by Pelsy *et al.* (2007). The Shannon's index was greatly higher than two in nine loci (VrZAG62, VrZAG79, VrZAG112, VVIC05, VVS2, VVS5, Vvlp60, VVMD25 and VVMD5), with five of them sharing a high *PD* value (>0.86) (Table 4). For cpSSR, the Gene Diversity values ranged from 0.031 (Ccmp7) to 0.716 (Ccmp10) and the Shannon's Index was between 0.082 (Ccmp7) and 1.391 (Ccmp10) (Table 5).

Table 4. Genetic parameters of 30 nuclear and cytoplasmatic microsatellites used to differentiate 62 grape varieties. Numbers in bold indicate statistic significant values.

| Locus | Allele size range, bp | Alleles per locus, no | Expected heterozygosity | Fixation index | Shannon's Index | Power of discrimination |
|---|---|---|---|---|---|---|
| VrZAG79 | 104-180 | 12 | 0.700 | 0.332 | 1.534 | 0,715 |
| VrZAG12 | 138-172 | 10 | 0.739 | 0.586 | 1.608 | 0,754 |
| VrZAG21 | 178-214 | 14 | 0.776 | -0.060 | 1.816 | 0,787 |
| VrZAG29 | 102-116 | 7 | 0.491 | 0.573 | 1.048 | 0,493 |
| VrZAG47 | 148-182 | 11 | 0.841 | 0.463 | 1.976 | 0,852 |
| VrZAG62 | 185-213 | 10 | 0.838 | 0.173 | **2.007** | 0,852 |
| VrZAG79 | 226-262 | 15 | 0.874 | 0.114 | **2.306** | 0,852 |
| VrZAG112 | 220-262 | 17 | 0.882 | 0.177 | **2.373** | **0,888** |
| VVIC05 | 142-169 | 14 | 0.823 | 0.334 | **2.047** | **0,895** |
| VVMD27 | 173-203 | 10 | 0.846 | 0.161 | 1.980 | 0,836 |
| VVS2 | 120-156 | 13 | 0.853 | 0.017 | **2.187** | **0,864** |
| VVS4 | 154-176 | 9 | 0.671 | 0.158 | 1.345 | 0,686 |
| VVS5 | 85-157 | 17 | 0.829 | 0.455 | **2.191** | 0,847 |
| VVIb01 | 288-304 | 8 | 0.794 | 0.248 | 1.723 | 0,808 |
| VVIh54 | 144-186 | 14 | 0.798 | 0.313 | 1.944 | 0,813 |
| VVIn16 | 139-175 | 10 | 0.676 | 0.356 | 1.555 | 0,680 |
| VVIp60 | 303-331 | 20 | 0.911 | 0.097 | **2.633** | 0,925 |
| VVIq52 | 75-89 | 8 | 0.771 | 0.415 | 1.657 | 0,786 |
| VVMD25 | 232-264 | 15 | 0.881 | 0.140 | **2.335** | **0,895** |
| VVMD5 | 223-249 | 13 | 0.883 | 0.105 | **2.279** | **0,897** |
| | *average* | 12.35 | 0.794 | 0.258 | 1.927 | 0.806 |

Table 5. Genetic parameters of 10 cytoplasmatic microsatellites used to differentiate 62 grape varieties.

| Locus | Allele size range, bp | Alleles per locus, no | Gene diversity | Shannon's Index |
|---|---|---|---|---|
| CCMP1 | 123-127 | 4 | 0.432 | 0.811 |
| CCMP2 | 207-208 | 4 | 0.091 | 0.191 |
| CCMP3 | 100-106 | 2 | 0.471 | 0.917 |
| CCMP4 | 117-127 | 4 | 0.470 | 0.887 |
| CCMP5 | 99-102 | 4 | 0.675 | 1.186 |
| CCMP6 | 106-109 | 4 | 0.482 | 0.876 |
| CCMP7 | 142-144 | 2 | 0.031 | 0.082 |
| CCMP8 | 62-86 | 3 | 0.282 | 0.610 |
| CCMP10 | 104-112 | 5 | 0.716 | 1.391 |
| ccSSR5 | 250-258 | 4 | 0.582 | 1.099 |
| | *average* | 3.6 | 0.423 | 0.805 |

Allele frequencies (*Af*) were useful to distinguish common alleles (*Af*>0.5) and specific alleles (*Af*<0.1). Out of 47 marker-specific alleles identified, 19 were present in only one genotype, and were, therefore, named private alleles. VrZAG21 and VVIp60 revealed the highest number private alleles (4). Private alleles were detected in 14 grapes: 4 in ''Catalanesca'', 2 in ''Mennavacca'', and ''Sommarello'' and ''Pellecchione'' and 1 in ''Coda di Volpe Bianca'', ''Ginestrello'', ''Rose'', ''Malaga'', ''Coda di Volpe Nera'', ''Montepulciano'', ''Sanginella'', ''Abbuoto'', ''Piedirosso'', ''Aglianico del Taburno'', ''Serpentaro'', ''Arilla Ischia'' and ''Pellecchione''(Table 6).

Table 6. Locus specific alleles identified in 24 SSR loci. Private alleles and the corresponding genotypes are reported in bold and brackets, respectively.

| SSR locus | | Allele (genotype) |
|---|---|---|
| Nuclear | VrZAG7 | 112, 152, 158, 170 |
| | VrZAG12 | **140** (**Mennavacca)**, 158 |
| | VrZAG21 | 178, **188** (**Mennavacca), 196 - 204** (**Catalanesca), 212** (**Coda di Volpe nera)** |
| | VrZAG47 | 148, 152, 164 |
| | VrZAG79 | **226** (**Pellecchione)** |
| | VrZAG112 | **220** (**Ginestrello), 222** (**Rose')**, 238 |
| | VVIC05 | 144, 150, 164 |
| | VVMD27 | 185 |
| | VVS2 | 138, **146** (**Catalanesca)** |
| | VVS4 | 154, 160, **163** (**Malaga),** 172 |
| | VVS5 | 129, **133** (**Coda di Volpe bianca),** 139, 153 |
| | VVIh54 | 144, 152, 156, **180** (**Catalanesca)** |
| | VVIn16 | 139, 147 |
| | VVIp60 | **313** (**Montepulciano), 314- 328** (**Sommarello), 329** (**Sanginella)** |
| | VVIq52 | **87** (**Abbuoto)** |
| | VVMD25 | 232, 254 |
| | VVMD5 | **243** (**Piedirosso)** |
| Plastidial | ccmp8 | **62** (**Aglianico del Taburno)** |

Variety distribution in the UPGMA dendrogram was organized in five major clusters (Figure 3). Two of them contained few grapevines: Cluster IV included two genotypes “Uva Fragola Bianca” and “Uva Fragola Nera” and Cluster V included only one genotype, “Catalanesca”. Thirty grapevines were included in Cluster I, seven in Cluster II and twenty two in Cluster III. Clustering robustness of some nodes was supported by bootstrap values higher than 70%. Based on our molecular data (phylogenetic classification and microsatellite allele mismatches), it was possible to distinguish cases of synonymy (cultivars having more than one name) and homonymy (different cultivars mentioned under the same name) in the entire group of cultivars. For example, “Procidana” showed a genetic constitution similar to “Coglionara”. They are both grapevines mainly cultivated in the island of Ischia and based on our data they resulted synonymous. In must be noticed that synonymous cases are likely originated by mutation rather than sexual reproduction. Therefore they share a common genetic origin but a different ampelographic classification. We also fund cultivars identically coded but molecularly different to each other. For instance, the two “Barbera del Sannio” biotypes sampled in Castelvenere and Pietradefusi clustered apart to the one sampled in Ischia, highlighting their genetic diversity. Similarly, the two “Livella” genotypes sampled in Ischia and Eboli did not group together, as well as the two “Cacamosca” genotypes, both sampled in the same location.

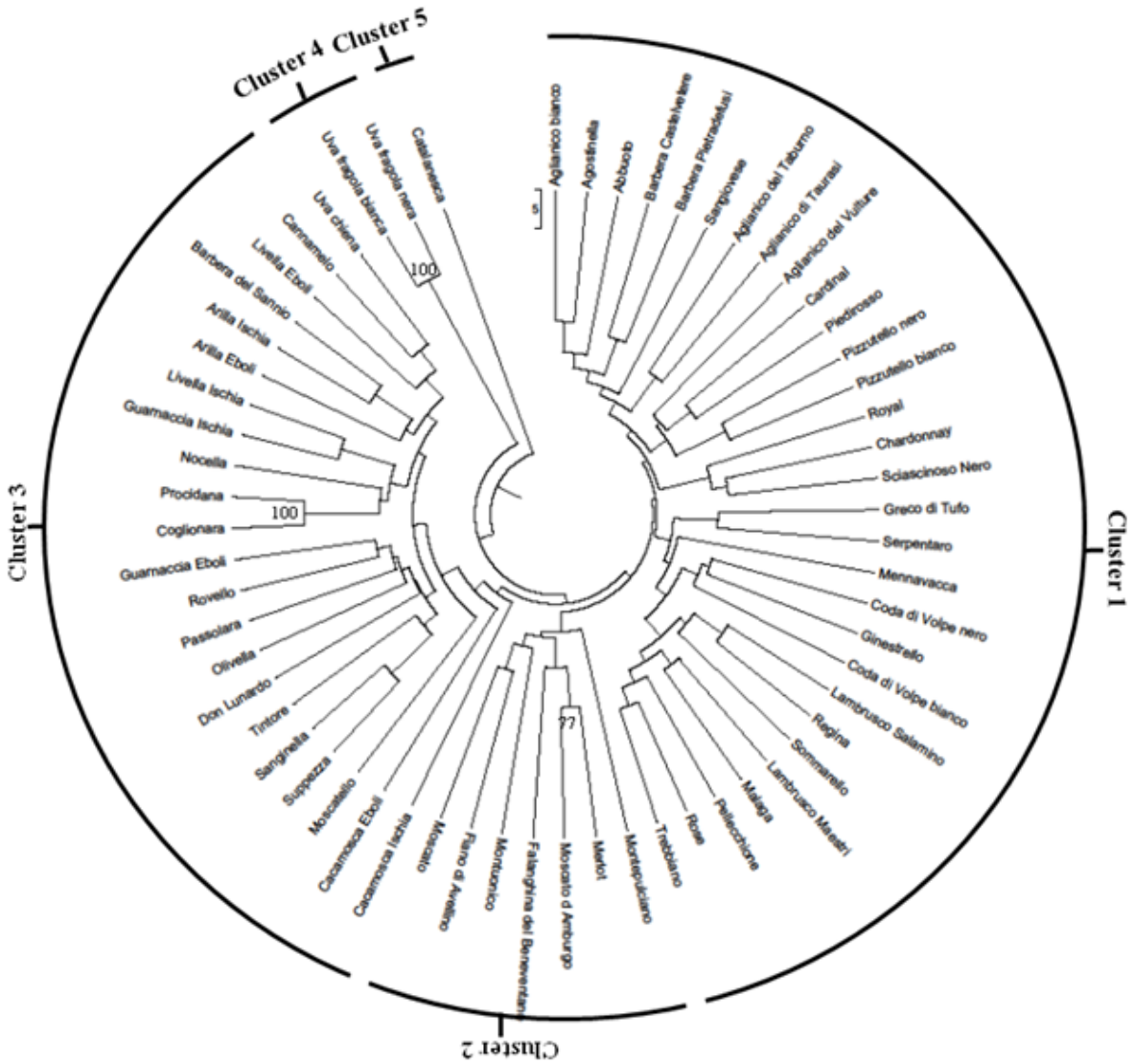


Figure 3. Dendrogram of 62 grape genotypes obtained using UPGMA cluster analysis of SSR marker data. Bootstrap values higher than 70% are indicated at nodes.

The number of subpopulations was inferred by the model-based Bayesian clustering procedure. The Δk method is based on the rate of change in the log probability of data between successive *K* values and is a good estimator of the number of clusters (*K*) (Pritchard *et al.,* 2000). Δk values ranged from 0.1 (9 populations assumed) to 42.98 (3 populations assumed) (Figure 3) and suggested three (K = 3) as optimal number of population sampled. The three populations were divided in bar plots and labelled as A, B and C (Figure 4). Comparing Bayesian and phylogenetic analyses, we associated population A to Cluster II and a Cluster I's sub-cluster, population B to Cluster III and population C to Cluster IV, V and a second Cluster I's sub-cluster. The bar plot graph (Figure 4), based on STRUCTURE analysis, showed similar clusterization of UPGMA dendrogram.

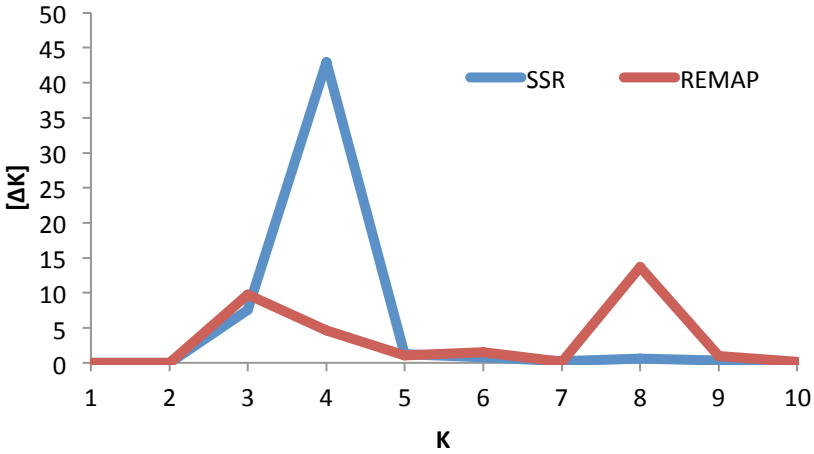


Figure 1. Optimal Δk deduced with a Bayesian analysis of microsatellite (SSR) and retrotransposon-based markers (REMAP) data. The graph shows a clear peak at the true value of K (number of populations assumed).

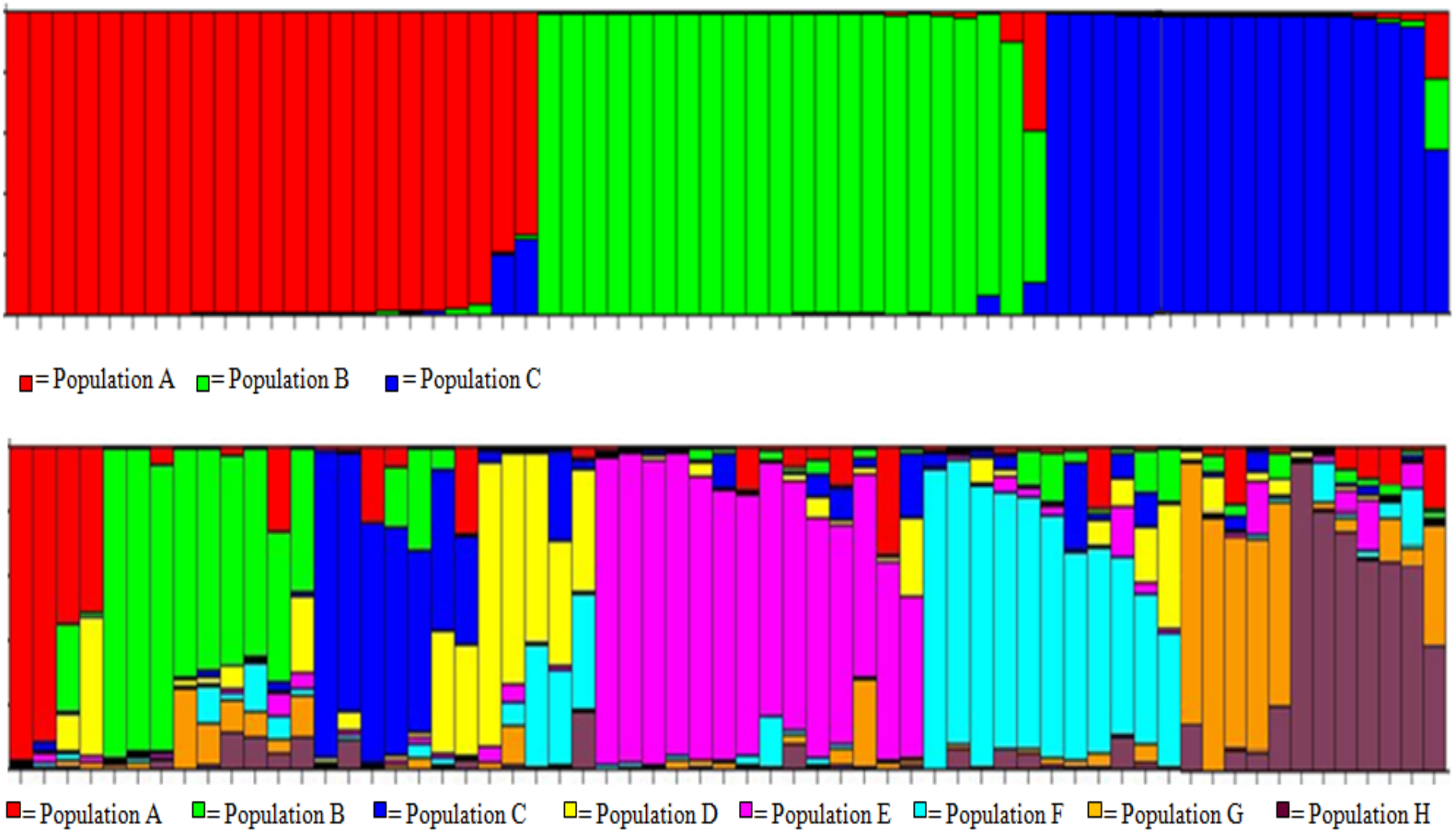


Figure 2. Inferred population structure of the 62 grapevines genotypes using SSR (top), REMAP and IRAP (bottom) markers through the model-based program STRUCTURE. Each individual is represented by a horizontal bar, which is partitioned into coloured segments that represent the individual estimated levels of the populations.

### 2.3.2 Retrotransposon-based genotyping

The *Gret1* LTR element amplification revealed a unique multilocus pattern for each accession. This is due to the retrotransposons integration ability in either orientation, as head-to-head, head-to-tail and tail-to-tail. Our analysis with retrotransposon-based markers was performed with 5 primer combinations, generating a total of 44 bands, of which 90% were polymorphic (data not shown). Primer combinations amplifying the highest and lowest number of bands were 5'LTR/ $(CT)_9G$ (13 bands) and 3'LTR/ $(CT)_9G$ (4 bands), respectively. The *He* value was higher than 0.3 in 5'LTR/ $(CT)_9G$ and in 5'LTR/3'LTR. These primer combinations shared also the highest *I* value (Table 4). Among REMAP patterns, three private bands were identified: two with the 5'LTR/ $(GA)_9C$ combination in "Moscatello" and "Sangiovese" and one with 3'LTR/ $(GA)_9C$ combination in "Pizzutello bianco". The IRAP patterns revealed one private allele in "Cacamosca Ischia". All genotypes were clusterized in eleven groups thought UPGMA analysis (Figure 5). Cluster IV was the largest one with 15 genotypes, while Clusters VI, VII, VIII, IX, X and XI were the smallest, with an average of three genotypes each and a minimum of one genotype in Cluster XI. The estimated membership of each individual in each cluster was compared with the Bayesian analysis. Using the REMAP and IRAP dataset, the highest $\Delta K$ value was 13.69 in the eighth K (Figure 2). Eight different bar plot colours explained the groups (Figure 3). The populations were explained by eight distinctive bar plots differentiated by different grey gradations (Figure 3). Six Bayesian populations assorted the same genotypes grouped in the UPGMA clusters: Population B was comparable with the Cluster II, C with Cluster II, D with Cluster V, A with Cluster VIII, E with Cluster IX and G with Cluster X.

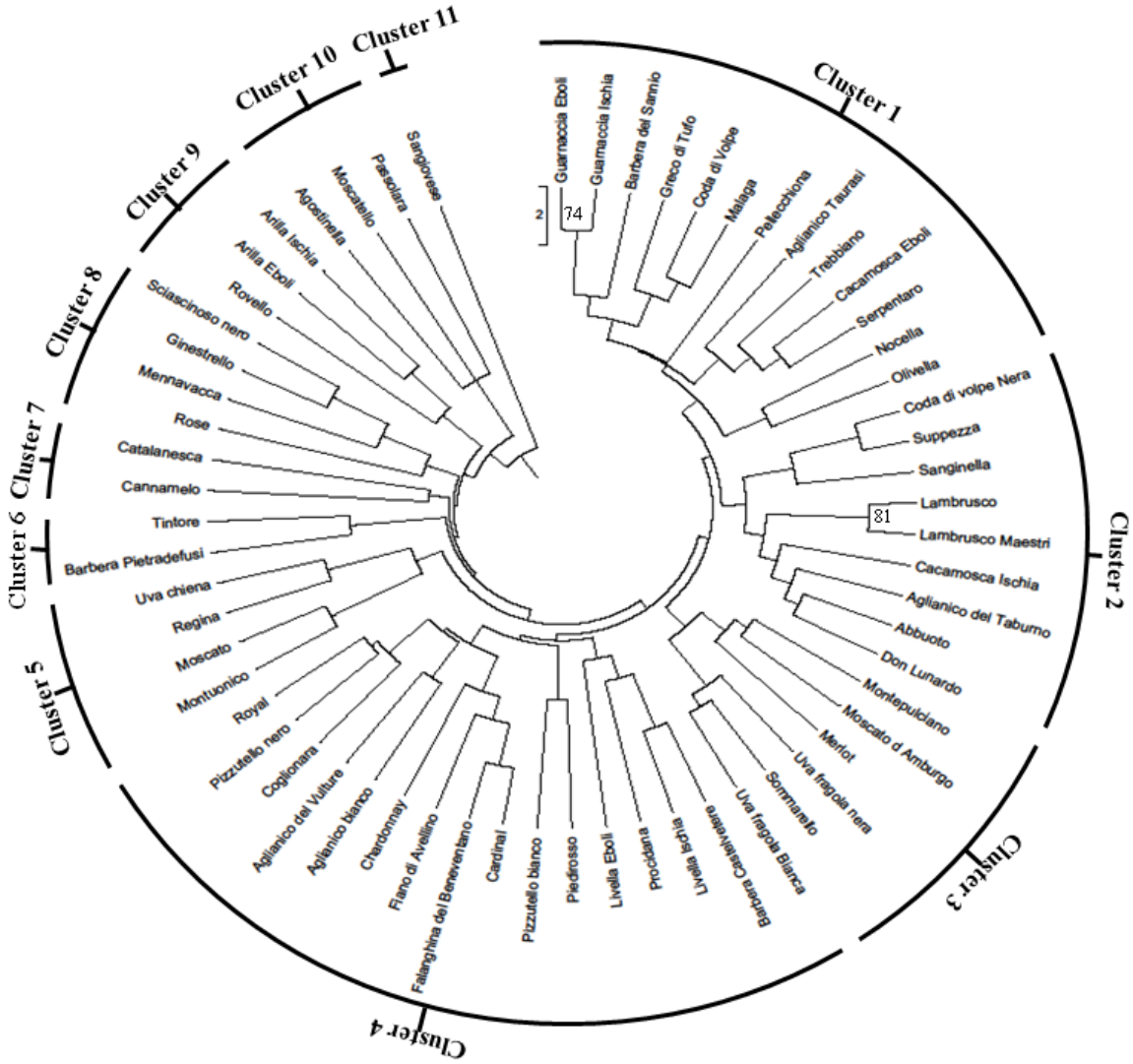


Figure 3. Dendrogram of 62 grape genotypes obtained using UPGMA cluster analysis of IRAP and REMAP marker data. Bootstrap values higher than 70% are indicated at nodes

### 2.3.3 Phenyl-propanoid pathway genes expression analysis

To investigate the genetic regulation of tannins, anthocyanins and flavonoids in Aglianico del Taburno, the transcript level of genes involved in the phenyl-propanoid pathway was monitored using a quantitative RT-PCR in whole berry, berry skin and seeds. For dissertation clarity, the pathway was divided in three parts, namely early, middle and late pathways; each of them is composed by a group, in particular the genes *PAL, C4H, 4CL, F3'H* and *F3'5'H* were grouped in the early-pathway; the middle-pathway was formed by *FLS4, FLS5, DFR, LDOX* and *UFGT*, *ANR, LAR1* and *LAR2* were included in the late-pathway. The transcriptional factors *MybPA1* and *MybPA2* were taken separately.

The selection of reference genes to normalize the cDNA represents a critical step in any quantitative RT-PCR analysis. In this analysis, β-Tubulin (*TUB),* β-actin and elongation factor

were tested as housekeeping genes. The expression of β-Tubulin was the most stable among the three candidates in different tissues and therefore was selected as reference gene.

The expression values obtained from the whole berry in the three parts of the phenyl-propanoid pathway are reported in Figure 6 as binary logarithm of the fold change value. Among the set of genes analyzed no genes were up-regulated in all steps of berry development. By contrast *PAL*, *ANR*, *LAR2* and *MybPA2* showed a down-expression during all developmental stages. The lowest expression have been detected during ripening for the gene *PAL* (-9.4), post-fruit-set for the gene *LAR2* (-7.8) and mid-ripening for the genes *ANR* (-7.8) and *MybPA2* (-7.9). Among them, *PAL* was the only gene showing a gradual decrease of expression from post-fruit set to ripening. The other genes showed an uneven expression across the ripening times. Indeed, *4CL*, *F3'H*, *DFR* and *MybPA1* were down-expressed in the first three developmental stages; increasing their expression in ripening time with an over expression of 1.3, 3.5, 3 and 3.4, respectively. The highest expression value was detected for *UFGT*, which showed an increasing of expression from *veraison* (-2.2) to ripening(14.7). *F3'5'H* and *LDOX* were over-expressed only in mid-ripening (2.7 and 1.3, respectively) and in ripening (4.9 and 3.9, respectively). Regarding *C4H, FLS5* and *LAR1* we observed down-expression in only one developmental stage (*veraison* for *C4H* and *FLS5* and mid-ripening for *LAR1*).

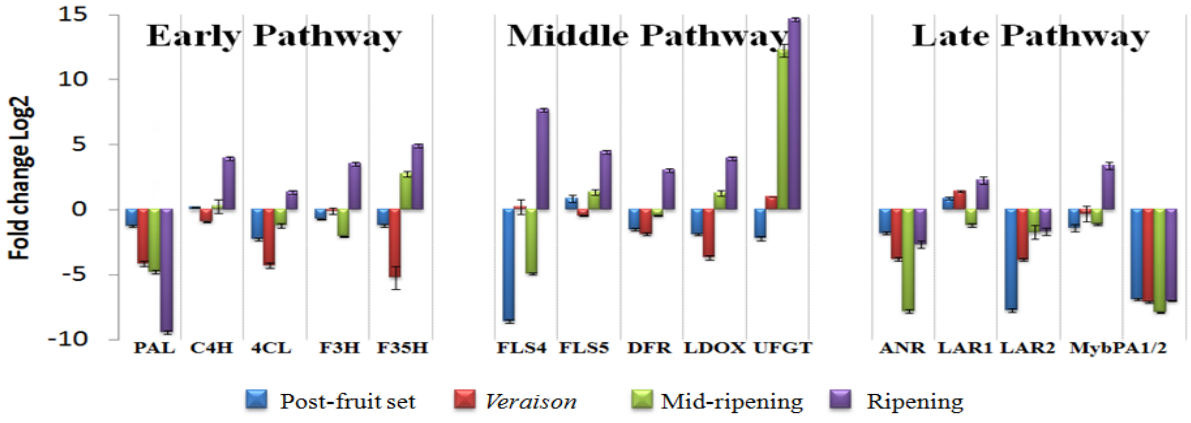


Figure 4. Expression values of genes involved in the early, middle and late pathway of phenyl-propanoid analyzed in four developmental stages: Post-fruit set, *veraison*, mid-ripening and ripening in the whole berry.

Gene expression data were graphically represented through heatmap for helping to immediately make sense of the expression level differences across developmental stages for each phenyl-propanoid gene analyzed, both in skin and seeds (Figure 7 and 8). Different colors were assigned to different expression value (from -3 (blue) to red +7 (red)) so that genes with similar or vastly different expression levels are easily visible. The analysis carried out on skin berries showed that the highest expression was identified in the middle pathway

specifically in mid-ripening stage for *F3'5'H* (5), *FLS5* (0.6) and *UFGT* (5.1) and ripening time for *DFR* (0.9), *LDOX* (2.9) and *LAR1* (1.5). Other five genes showed a down-expression in both stages with minimum values at mid-ripening for *C4H* (-1.9), *FLS4* (-0.4) and *ANR* (0.8) and ripening for *LAR2* (-1.2) and *MybPA2* (-3). The remaining genes reveal a down-expression in the first stage, *PAL* (-1.1), *4CL* (-0.4), *F3'H* (-1.9) and *MybPA1* (-0.1), and an over-expression in the ripening time, *PAL* (0.4), *4CL* (0.6), *F3'H* (0.7) and *MybPA1* (0.4).

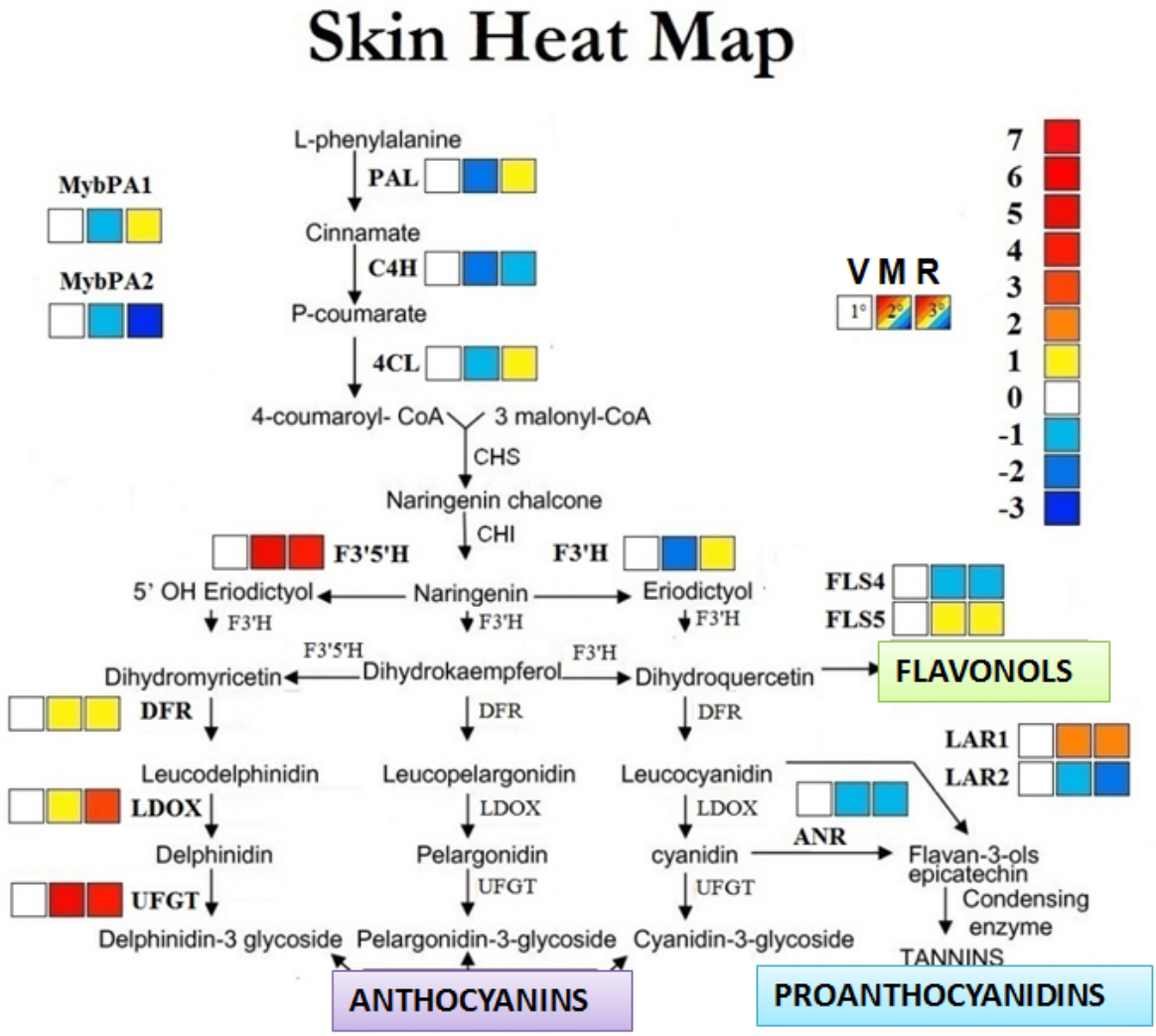


Figure 7. Heat Map representation of expression level (colorimetric scale) of phenyl-propanoid genes in seeds. Each gene expression is shown in three boxes corresponded to the three developmental time studied, veraison (V), mid-ripening (M) and ripening (R).

The phenyl-propanoid pathway expression course were investigated also in seeds. The data were reported in a Heat Map (Figure 8). Compared to the data obtained in the whole berries and in the skin, in seeds nearly all genes showed an over-expression in the ripening stage, except for *LAR1* which is down-expressed with a value of -1.4. The expression values at ripening time ranged from 0.1 (*PAL*, *UFGT*) to 6.6 (*FLS4*) and were mostly grouped in the middle and late pathways.

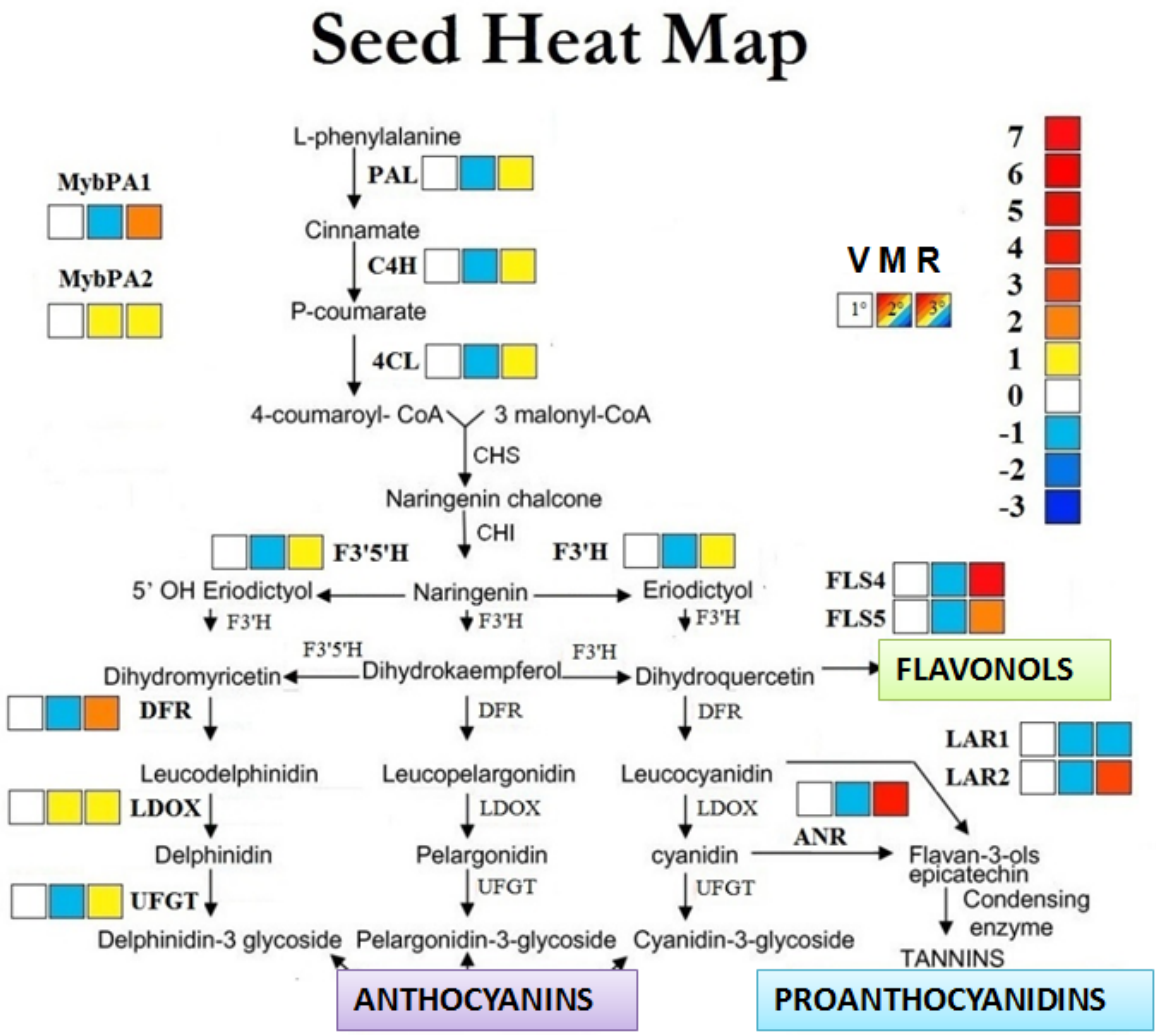


Figure 8. Heat Map representation of expression level (colorimetric scale) of phenyl-propanoid genes in seeds. Each gene expression is shown in three boxes corresponded to the three developmental time studied, veraison (V), mid-ripening (M) and ripening (R).

### 2.3.4 Phenyl-propanoid chemical analysis

A quantitative analysis of phenol compounds in skin and seeds during three developmental stages (veraison, mid-ripening and ripening) was carried out. The results of total phenols, flavans, Folin–*Ciocalteu* Index (FCI), tannins and anthocyanins are reported in Table 7. In skin analysis, the highest total phenols content were obtained at *veraison* (7 mg/g) and the lowest at ripening (573 µg/g). The spectrophotometric analysis allowed the identification of three fractions per each compound, monomers, oligomers and polymers. The amount of phenol compounds seemed to decrease exponentially from *veraison* to ripening, especially in polymers which represent the most abundant extracted fraction. In seeds, the total phenol were much higher than skin (65 mg/g in *veraison* and 40 mg/g in ripening); the higher total phenol fraction in skin was represented by monomers. Tannins were highly identified in skin as polymers, with values ranging from 2.4 mg/g (ripening) to 4.3 mg/g (mid-ripening). By contrast, monomers varied from 10 µg/g (mid-ripening) to 32 µg/g (ripening).

Table 7. Phenol compounds analysis in skin and seeds of Aglianico del Taburno berries collected during three developmental stages, *veraison*, mid-ripening and ripening. The fractions analyzed were total phenolics, flavans, FCI, tannins and anthocyanins, and for each of them monomers, oligomers and polymers were distinguished.

| **SKINS (µg/g)** | **VERAISON** | | | **MID-RIPENING** | | | **RIPENING** | | |
|---|---|---|---|---|---|---|---|---|---|
| Fractions | Monomers | Oligomers | Polymers | Monomers | Oligomers | Polymers | Monomers | Oligomers | Polymers |
| **Total phenolics** | 67,8 ± 1,77 | 2304,00 ± 8,49 | 4683,24 ± 8,97 | 246,61 ± 20,86 | 3359,63 ± 51,72 | 4538,79 ± 108,94 | nd | 252,37 ± 23,53 | 320,85 ± 43,42 |
| **Flavans** | 43,02 ± 5,51 | 719,13 ± 19,40 | 1602,43 ± 17,72 | nd | 645,61 ± 12,11 | 540,98 ± 83,23 | 8,84 ± 0,99 | 107,79 ± 5,83 | 419,96 ± 18,81 |
| **FCI** | 91,52 ± 0,63 | 954,54 ± 5,76 | 1828,55 ± 6,51 | 119,28 ± 1,77 | 813,21 ± 16,00 | 1671,35 ± 56,40 | 136,82 ± 2,07 | 489,06 ± 6,41 | 1291,72 ± 18,69 |
| **Tannins** | 17,67 ± 5,05 | 1870,37 ± 20,52 | 3495,0 ± 56,51 | 10,59 ± 12,23 | 1764,67 ± 26,75 | 4336,17 ± 162,38 | 31,63 ± 5,71 | 507,04 ± 12,58 | 2392,65 ± 50,23 |
| **Anthocyanins** | nd | nd | nd | 4,56 ± 0,95 | 91,23 ± 2,12 | 967,83 ± 20,56 | 8,8 ± 0,45 | 331,46 ± 10,78 | 771,6 ± 15,43 |

| **SEEDS (mg/g)** | **VERAISON** | | | **MID-RIPENING** | | | **RIPENING** | | |
|---|---|---|---|---|---|---|---|---|---|
| Fractions | Monomers | Oligomers | Polymers | Monomers | Oligomers | Polymers | Monomers | Oligomers | Polymers |
| **Total phenolics** | 39,31 ± 0,32 | 19,34 ± 0,07 | 7,46 ± 0,10 | 23,25 ± 0,28 | 21,22 ± 0,27 | 3,5 ± 0,03 | 18,51 ± 0,19 | 15,97 ± 0,23 | 6,3 ± 0,09 |
| **Flavans** | 11,80 ± 1,16 | 7,82 ± 0,15 | 2,87 ± 0,09 | 9,31 ± 0,21 | 8,46 ± 0,23 | 1,27 ± 0,02 | 6,12 ± 0,07 | 6,12 ± 0,19 | 1,95 ± 0,07 |
| **FCI** | 15,41 ± 0,35 | 7,12 ± 0,08 | 2,69 ± 0,03 | 10,04 ± 0,25 | 8,05 ± 0,47 | 1,43 ± 0,02 | 7,94 ± 0,08 | 6,11 ± 0,11 | 2,49 ± 0,03 |
| **Tannins** | 3,97 ± 0,19 | 16,47 ± 0,10 | 7,95 ± 0,07 | 1,44 ± 0,07 | 17,15 ± 0,18 | 3,64 ± 0,04 | 1,7 ± 0,15 | 10,74 ± 0,25 | 5,24 ± 0,13 |

In seeds, the amount of oligomers were higher than monomers and polymers; indeed, the mg/g of monomers in the three developmental stages were 16, 17 and 11 respectively compared to the 4, 1 and 2 of monomers and the 8, 4 and 5 of polymers. Anthocyanins were estimated only in skin, since they lack in seeds. As expected, they were mostly identified as polymers only in mid-ripening and ripening; the amount of anthocyanins polymers were 968 µg/g in mid-ripening and 772 µg/g in ripening.

### 2.3.5 Wounding treatment

The Aglianico del Taburno was used also to set up an experiment of wounding stress carried out on young leaves in sterile conditions. The time course was analyzed with a relative Real-Time method using five genes involved in phenyl-propanoid pathway (*PAL*, *4CL*, *C4H*, Myb14 and Myb15) and four candidate genes of stilbene synthase (*VvSTS6*, *VvSTS16*, *VvSTS22* and *VvSTS36*) as reported in Vannozzi A. (2012). The results are reported in binary logarithm of fold change in Figure 9. The genes *PAL*, *4CL* and *C4H* showed a variable expression along the time course. In particular *PAL* and *C4H* were highly over expressed 24 hours after wounding with a 2.9 and 2.2 fold change, respectively. Four genes (*4CL*, *VvMyB15*, *VvSTS22* and *VvSTS36*) were down expressed during the whole treatment. Among them *VvSTS36* showed the minimum expression value with -3.5 fold change at 24 hours post treatment. The two transcription factors, *VvMyB14* and *VvMyB15*, showed an opposite behavior. *VvMyB14* was over-expressed, while *VvMyB15* was not. This confirmed an expected result useful to confirm the validity of the treatment and the induction of the wounding stress. Among the stilbene synthase candidate genes, only *VvSTS16* and *VvSTS6* was over-expressed, with the highest value of 4.1 at 1 hour post treatment in *VvSTS16*.

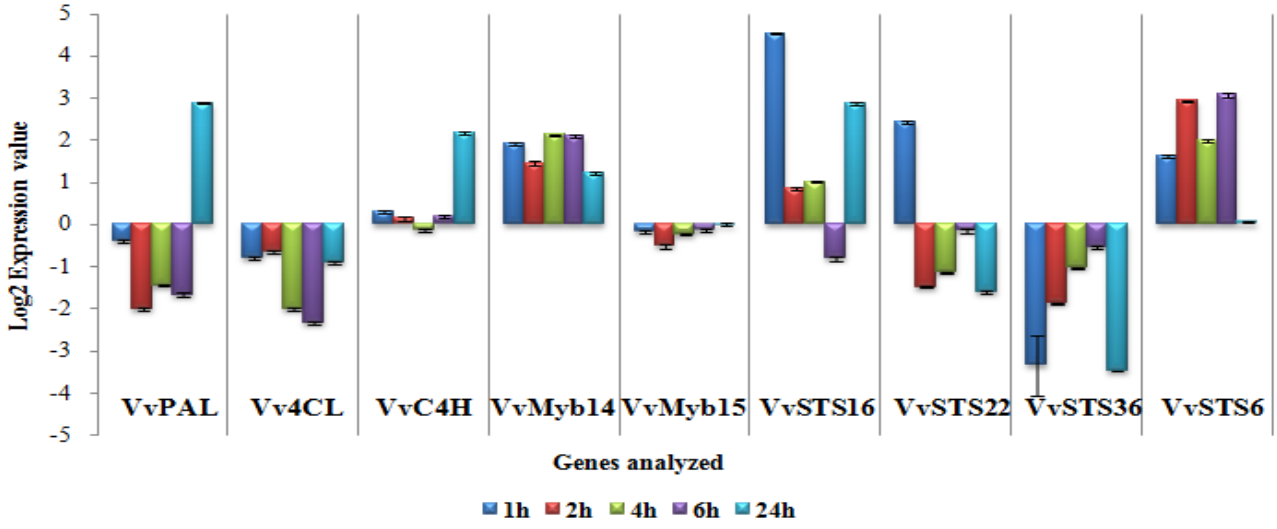


Figure 9. Expression values of nine genes involved in phenyl-propanoid pathway and plant immunity during a wounding stress time course of 1, 2, 4, 6 and 24 hours.

## 2.4 Discussion

The genetic diversity of 62 grape accessions including 38 autochthonous grapevines from Campania was investigated using SSR and retrotransposon-based molecular markers. The gene pool of cultivated grapes has significant amounts of genetic variation, which needs to be characterized to increase knowledge on the available genetic resources. To provide further insights into the genetic structure and differentiation within and among grapevine samples, the analysis of the same genotypes with different techniques is likely the best approach (Bacilieri *et al.,* 2013). Therefore, in this study we combined SSR with REMAP and IRAP markers. Based on their different nature, they can detect similar diversity patterns, but with differences in sensitivity. Marker sensitivity is intended as the capability of distinguishing inter and intra specific variability. Through SSR analysis it is possible to investigate inter-specific variability and identify genetically different varieties (Laucou *et al.,* 2011). On the other hand, transposon-based markers can investigate intra-specific variability and identify different accessions within the same species (Pelsy *et al.,* 2007). Our results showed that five markers (VrZAG79, VrZAG112, VVS2, VVMD25 and VVMD5) should be recommended for the rapid and unambiguous identification of grapes from Campania region as they proved to be the most discriminating loci, with PD values ranging between 0.852 and 0.897 and Shannon's Index values from 2.187 and 2.373. This confirms findings by Costantini *et al.* (2005), Zoghlami *et al.* (2009) and Laucou *et al.* (2011), who reported and used the same set of microsatellite in grape genotyping studies. With an average value of 9.9 alleles per locus, our microsatellite results are consistent with those of Santana *et al.* (2008), who found an average of 8.7 alleles per locus studying 65 grape accessions. It is interesting to note that out of 30 markers used, only 6 showed a FI value higher than 0.6 (Table 1) suggesting that 80% of the microsatellite markers used are highly heterozygous. Our data were consistent with reports by Sant'Ana *et al.* (2012), Lopes *et al.* (1999) and Riahi *et al.* (2012), who confirmed the high individual heterozygosity due to breeding programs applied to improve quality and productivity. Through statistical and phylogenetic analysis, synonymies, homonymies and unique genotyping cases were identified. The three "Aglianico" biotypes ("Taburno", "Taurasi" and "Vulture") analyzed in this study confirmed the findings of Muccillo *et al.* (2013); suggesting that "Aglianico Taurasi" and "Aglianico Taburno" biotypes are closer than the "Aglianico Vulture". One genotype, "Catalanesca", resulted unique and highly heterozygous and also produced the highest number of private alleles. "Uva Fragola Nera" and "Uva Fragola Bianca", called also "Isabella" (Robinson *et al.,* 2012), are the only two

*Vitis labrusca* genotypes in the grapevine collection analyzed; it clarified the clustering on their own in Cluster IV. In SSR dendrogram grouping all the native grapes of Ischia along with grapes sampled in the Ischia area were grouped in Cluster III (Fig.1); this is explained by the presence of ancient genes correlated to the low rate of crossing occurring in the island of Ischia. Several studies reported the successful use of combined dominant and co-dominant markers for grapevine clone identification (Moisy *et al.,* 2008, Ocanã *et al.,* 2013). Microsatellites are usually the most frequently used genetic markers in intra-specific *Vitis* studies (Laucou *et al.,* 2011), while retrotransposon-based markers, classified as epigenetic markers, result the best for intra-species studies or rather to distinguish clones generated by vegetative propagation. For this reason, the genetic diversity of our 62 grapevines was investigated also using four REMAP and one IRAP markers designed on the gypsy-type retroelement *Gret1*. The chosen methods have the characteristics of using fewer primers but providing sufficiently high polymorphisms to allow detection of inter-varietal diversity and heterotic groups, as already demonstrated in apricot (Yuying *et al.,* 2011), bread wheat (Nasri *et al.,* 2013) and alfalfa (Mandoulakani *et al.,* 2010). We identified 44 different bands with five markers in a profile complexity ranging from 4 to 13 bands per marker. This is comparable with findings by Carcamo *et al.* (2010), who detected a significant lower number of bands in 28 clones of grapevine "Tempranillo". This result could be related to a point mutation occurring in the primer sequence. Relatively few bands were obtained also in other grape studies (Pereira *et al.,* 2005; D'Onofrio *et al.,* 2010). As far as we know, this is the first time this technique has been used to produce complex multi-locus profiles. Because of the nature of these markers, they could be successfully employed to build a cultivar identification diagram (CID). This is an open diagram successfully used in different plant species, as grapevine (Zhao *et al.,* 2013) and *Ginkgo* (Li *et al.,* 2013). It can be very helpful for genetic resource conservation and utilization and plant variety protection. The complete set of REMAP and IRAP markers was able to distinguish different biotypes of the same genotype as "Aglianico" or "Barbera del Sannio" series, but not the same genotype sampled in different areas like "Arilla Ischia" and "Arilla Eboli" or "Guarnaccia Ischia" and "Guarnaccia Eboli". This confirms the capability of these markers to discriminate the grape accessions but not structured sub-groups. Even if specific sequences have been identified, these molecular data cover only part of the grapevine genome, meaning that even when all the DNA sequences we analyzed look totally identical, it might turn out that genetic differences are present in other part of the genome and they might be more widespread than we believe. Indeed, the accessions sharing the same SSR/REMAP profile must be evaluated further before being

eliminated from standard grapevine collections, because they might not be redundant at all. Our molecular findings represent a peculiar starting point for whole-genome profiling of varieties native of Campania region.

The chosen genotype for gene expression analysis is Aglianico del Taburno, one of the three main biotypes of Aglianico grape cultivar (Taburno, Vulture and Taurasi) and the main variety used in the production of Taburno DOCG (Denominazione di Origine Controllata e Garantita) wines. It is well known not only for the excellent wine it produces, but also for its phenotypic plasticity (the capacity of a given genotype to render different phenotypes under different environment conditions) and its ability to adapt to different environments and managements of *vine vigor* (Rinaldi *et al.,* 2014). The characteristic high content of total flavonols and anthocyanins in Aglianico made it the perfect candidate for the present study. A complete characterization of Aglianico del Taburno could be an interesting starting point to introduce this grapevine in the world market and preserve it as an elite product. As true for the molecular characterization, the expression profile analysis can create a pattern of results able to identify the uniqueness of this genotype especially if the chosen genes are involved in phenol production and if the analysis is made on fruit tissues. Molecule production in plant tissues can be regulated at different levels, transcriptional post transcriptional and translational. Focusing on transcriptional regulation, key genes expression of the poly-phenolic pathway has been investigated during five developmental stages. The first stage (15 days after flowering [DAF]) corresponds to the fruit set, when young berries are enlarging (>3 mm diameter); the second stage (35 DAF) is the post-fruit set, when berries (>7 mm diameter) start touching; the third stage (70 DAF) is the *veraison*, when berries begin to change color and enlarge; the fourth stage (84 DAF) corresponds to the mid-ripening stage; and the final stage (115 DAF) represents complete ripening. The pathway considered can be divided in three parts, called Early, Middle and Late pathways; each of them is characterized by a group of genes used in this analysis. The genes *PAL*, *C4H*, *4CL*, *F3'H* and *F3'5'H* are grouped in the early-pathway, the middle-pathway is formed by *FLS4*, *FLS5*, DFR, *LDOX* and *UFGT* and the rest of the genes (*ANR*, *LAR1*, LAR2, *MybPA1* and MybPA2) represent the late-pathway. All the analyzed genes showed expression values strictly correlated with the developmental stage. Indeed some phenol compounds, as anthocyanins were not produced in berry until *veraison* and their production can be tissue specific, especially in red berry grapes. Among the all gene studied, *PAL* (Phenylalanine Ammonia Lyase) exhibits a gradational decrease of expression from fruit set to ripening confirming his role as entry point into the pathway. *PAL* catalyses the non-oxidative deamination of phenylalanine to trans-cinnamic

acid and ammonia. It has been demonstrated that increased activity of *PAL* is correlated with increased production of phenyl-propanoid and levels of *PAL* activity vary with developmental stage, cell and tissue differentiation, and exposure to different stress stimuli (Bagal *et al.,* 2012). Together with *4CL* and *C4H*, *PAL* activity is highly correlated with the total contents of phenolic acids. For this reason, the exponential decrease of expression of *PAL* during grape berry development may trigger the decrease of total phenolic compounds in berry skin and seeds. Another gene closely related to the developmental stages is the *UFGT* (UDP-glucose: Flavonoid 3-O-glucosyl transferase), which is committed to anthocyanin synthesis. *UFGT* catalyses the glucosylation of anthocyanidins into stable colored anthocyanins. Its expression levels in middle ripening and ripening were higher in the whole berry (12.3, 14.7) and in the skin (5.1, 4.3) then in the seeds (-0.2, 0.1). Furthermore, the chemical analysis show an incredibly increase of anthocyanins content in skin (412 µg/g) compared to seed content (0) during ripening time. Our expression and chemical data confirmed the results obtained from Castellarin and Di Gaspero (2007), who demonstrate that the anthocyanin content in skin is dependent on the expression level of *UFGT*. Another gene that is involved in anthocyanin production is *F3'5'H* (Flavonoid 3', 5'-hydroxylase). This gene is highly expressed in maturation stages from *veraison* to ripening in the whole berry and in the skin, but down-expressed in seeds. Jeong and collaborators (2004) demonstrated the over-expression of this gene in the ripening time of Cabernet Sauvignon berries confirming our results. In the phenyl-propanoid pathway, besides *UFGT* there are two additional enzymes that have been demonstrated to be involved in anthocyanin production: *DFR* (Dihydroflavonol 4-Reductase) and *LDOX* (Leucoanthocyanidin Dioxygenase). *DFR* diverts the substrate from flavonols formation to the anthocyanin and proanthocyanidin pathway, while *LDOX* is involved in anthocyanin biosynthesis and catalyses the conversion of colorless leucoanthocyanidin to colored anthocyanidin. *DFR* expression has been shown to be spatially and developmentally regulated, organ-specific, and induced the accumulation of anthocyanin in different plant tissues (Zhang *et al.* 2008). Similarly, the expression of the *LDOX* gene has been detected in different organs of Shiraz grapevine, such as leaves, roots, seeds, flowers, berry skin, and flesh (Boss *et al.* 1996a, b). In fact, those genes are over-expressed in all tissues during middle ripening and ripening but with variable levels. Proanthocyanidins (PAs), or condensed tannins, are flavonoid polymers synthesized from leucoanthocyanidin reductase (*LAR1* and *LAR2*) and anthocyanidin reductase (*ANR*), two specific enzymes for the formation of flavan-3-ols, respectively (+)- (gallo)catechin and (-)-epi (gallo)catechin. We found that the expression values of these genes were higher in seeds than in skin and this result was

confirmed by chemical analysis. Interesting data were obtained with the two homologous genes *LAR1* and *LAR2*, that showed an opposite expression level in all tissues and stages. Our findings concerning the predominant amount of polymeric tannins in skin rather than seeds are in agreement reported by Vivas *et al.* (2004). In seed chemical analysis, tannin monomers decreased drastically along with berries ripening (90%). This seemed to be correlated with tannin oxidation and structural modifications of phenol molecules as consequence of protein, carbohydrates and other poly-phenolics interactions (Kennedy *et al.,* 2000). *MybPA1* and *MybPA2* are two transcription factors that regulate the expression of phenyl-propanoid pathway genes, in particular *ANR* and *LAR*. In grapevine it has been reported that the expression levels of *MybPA1*, *ANR* and *LAR1* are correlated and that they are highly increased following *veraison* (Bogs *et al.* 2007). Another transcriptomic study of those genes in grapevine has been described from Terrier and collaborators (2009); they demonstrated a tissue-specific expression of *MybPA1* and *MybPA2* in young leaves and berries skin. Compared with these studies, similar results were observed in our tests. Indeed, the expression of *MybPA2*, *ANR* and *LAR2* was correlated in all tissues and developmental stages; also *MybPA1* and *LAR1* were co-expressed in the whole experiment. The findings of our expression data on *MybPA1* and *MybPA2* suggest that these transcription factors regulate different genes of the same family. However further research is required to understand the positive or negative nature of this transcriptomic regulation. Additional plant secondary metabolites investigated in this study were flavonols, classified as proanthocyanidins. They are commonly associated to the antioxidant and nutriceutical properties of wine and grape juice products. Their expression is regulated from flavonols synthase 4 (*FLS4*) and 5 (*FLS5*) and can be tissue specific. Fujita *et al.* (2005) demonstrated that the biosynthesis of flavonols possesses a control system different from that of anthocyanins. Indeed, the expression levels of *UFGT* and *F3'5'H* were different from that of *FLS4* and *FLS5*. These genes seem to be highly expressed in seeds and the data were confirmed by chemical analysis, that showed higher levels of flavonols in seed (55 mg/g) rather than skin (4 mg/g). In detail, the great part of seed flavonols were represented by monomers and oligomers. This is an interesting finding since it has been demonstrated that monomeric and oligomeric flavanols (MOF) are involved in cardiovascular disease prevention and have ontioxidant properties. Their mechanism of action is still under investigation (Weseler *et al.,* 2011). The first attempts to analyze the phenyl-propanoid pathway in grapevine were able to identify only few genes that tended to modulate dramatically during berry development and ripening. An example is given in Terrier et al (2001) and da Silva et al. (2005), that demonstrated that genes ancoding cinnamyl-

alcohol dehydrogenase (*CAD*), caffeic acido O-methyltransferase (*COMT*), chalcone-flavanone isomerase (*CHI*), chalcoene synhtase (*CHS*) and leucoanthocyanidin dioxygenase (*LDOX*) were the only genes expressed during early berry development and at veraison. By contrast, our data showed an over-expression of LDOX only in the late berry development stages. These gene was strictly specific to skin tissues together with *F3'5'H* and *UFGT*. On the counterpart, some genes (*FLS4*, *FLS5*, *LAR2* and *ANR*) were found to be specific to seed tissue. Recent studies demonstrate that the phenyl-propanoid metabolism is under strict transcriptional control, indeed two transcription factors (*VvMybPA1* and *VvMybPA2)* were put in the set of genes chosen for the transcriptional analysis. Water et al. (2005) found 11 secondary metabolism cDNAs, like *PAL, 4CL, CHS, F3'H* and *ANR*, that were up regulated during the herbaceous stage of Shiraz berry development but non from berry skin that were up-regulated during ripening. Terrier et al. (2005) identified approximately 20 developmentally modulated phenylpropanoid pathway genes in Shiraz berries, but only five of these were involved in ripening and suddenly induced at the very beginning of berry colouration (as *FLA4, FLS5*) and two genes involved in anthocyanin synthesis (as UFGT). The remaining transcripts (*PAL, CHS, CHI, F3'H, DFR, F3'5'H* and *LDOX*) were expressed predominantly during early development and later at ripening, confirming a bimodal pattern of transcript accumulation as initially described by Boss et al. (1996a) for the flavonoid pathway genes. This profile previdesan explanation for the asynchronous accumulation of proanthocyanidins and anthocyanins in the berries of red cultivars. Nowadays, an interesting phenomenon not intensely studied, is the increasing of phenolic compounds in response to wounding stress. A stress or an injury to a plant cell will trigger two types of responses in phenolic metabolism (Rhodes and Wooltorton, 1978). The first response is the oxidation of the existing phenolic compounds as a result of rupture of the cell membrane; this causes the phenolics to combine with the oxidative enzyme systems. The other response involves the synthesis of monomeric or polymeric phenolics to repair the wounding damage. These second responses are caused by changes in phenylalanine ammonia lyase activity. In this study the expression levels of *PAL*, *4CL* and *C4H* were investigated in a wounding stress time course of Aglianico del Taburno leaves. At one day post treatment, an over-expression of *PAL* and *C4H* was registered with an increase of 5 and 2 fold change unites, respectively. These data confirm the findings of Surjadinata *et al.* (2012), albeit with slight differences. They demonstrated that wounded carrots could be promoted as an inexpensive rich source of phenolic antioxidants for the regular human uptake simply increasing wounding stress intensity. The phenolic amount and the *PAL* enzyme activity were identified and an increase

of *PAL* activity (200%) was registered at four hours after wounding in three different carrot cultivars. These findings of increased antioxidant activity with wounding stress have been demonstrated only in few plants: lettuce leaves (Kang and Saltveit, 2002), purple-flesh potato (Reyes *et al.,* 2003) and in a variety of fresh vegetables (Reyes *et al.,* 2007).

Another molecule highly accumulated in plant cells in response to abiotic stress is the stilbenes, which represent a relatively small group of phenyl-propanoid compounds. In peanut and grapevine the accumulation of stilbenes have been correlated with an increased transcription of both STS genes and upstream enzymes in the phenyl-propanoid pathway such as *PAL* and *C4H* (Lanz *et al.,* 1990; Bais *et al.,* 2000). A peculiar role in phenyl-propanoid regulation is covered from the transcription factors, in particular the R2R3-MYB transcription factors, responsible for the regulation of flavonols, lignin and anthocyanin metabolism (Boudet 2007). The R2R3-MYB TF group is the largest MYB sub-family in plants (Du *et al.,* 2009) with the grapevine genome estimated to contain 108 R2R3-MYB members (Matus *et al.,* 2008). Höll *et al.* (2013) demonstrated that two R2R3-MYB accessions displayed similar expression patterns to the inducible VvSTS genes. These two accessions were previously designated *VvMyB14* and *VvMyB15* by Matus *et al.* (2008) based on their homology to the *A. thaliana* MYB14 gene. Along with the transcription factors, four stilbene synthase candidates were chosen from the 36 identified in Höll *et al.* (2013) and used in our expression analysis. The strong difference between *VvMyB14* and *VvMyB15* expression is due to the treatment used. Indeed, the induction of *VvMyB15* seemed to be restricted to UV-C treatment, while *VvMyB14* showed a dramatic increase in the expression upon all treatments in grapevine (Höll *et al.* 2013). Our results confirmed data indicating a consistent over expression of *VvMyB14* during the all treatment, while *VvMyB15* did not show any significant expression. These data can be correlated with the over-expression of *VvSTS6* and *VvSTS16* and the down-expression of *VvSTS22* and *VvSTS36*.

## 2.5 Conclusions

In conclusion, we have used and integrated two marker systems to detect genetic diversity and population structure in *V. vinifera* cultivars from a relatively small area. Using two appropriate techniques we were able to classify this heterogeneous group on origin and spread with microsatellite markers or on vegetative propagation with retrotransposon-based markers. Data suggested that a vast genetic variability is still present in grape germplasm. Homonymies

and synonymies were found, reinforcing the knowledge that molecular evaluations can provide further insights into genetic structure and differentiation of *Vitis* germplasm accumulated during centuries of cultivation and selection. These molecular data are a powerful tool to preserve the Campania grape biodiversity and protect producers and consumers from food frauds. The grape collection established during this PhD research project represent part of the material carried out during the regional project SALVE (Safeguarding of the plant biodiversity of Campania), which considers the use of molecular markers for the identification, selection and conservation of autochthonous plant genetic resources in the agricultural system of the Campania region. Among the collection, Aglianico del Taburno is a model for astringency and the strong resveratrol content confer high positive nutriceutical properties to their grapes and wines. For this reason, a transcriptomic analysis of key genes of the phenyl-propanoid pathway were carried out in whole berries, skin and seeds during the maturation and in leaves after wounding treatment. The results obtained in different tissues, seeds and skin, make us able to classify a tissue specificity of the analyzed genes. The expression study was correlated with the amount of total phenols, flavans, anthocyanins and tannins detected in skin and berry using a spectrophotometric assay. The gene expression study suggested that tannins are produced to defend grape before maturity from predation, indeed astringent tannins declined with fruit parts ripening. Overlapping the transcriptomic and chemical data, anthocyanins are highly synthetized at ripening in skin tissues and are completed absent in seeds. Finally, wounding stress was found to induce an increase of antioxidant compounds and we speculate that their expression might be regulated by *VvMyB14*, a transcription factor.

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# Chapter 3

# Investigation of grapevine basal immunity

## 3.1 Introduction

### 3.1.1 The plant immune system

Protection of plants against infectious microorganisms depends on both passive and active defense mechanisms. Broadly speaking, passive defense mechanisms are those that are present before contact with the pathogen, while active defense mechanisms are activated only after pathogen recognition. In detail, to gain access to the nutrients or replication machinery available within the host cell, pathogens must first breach the natural barriers presented by healthy plants, the passive defense. These barriers may be physical (e.g. wax, cuticle, cell wall, stomatal aperture, lenticels, etc.) or chemical (e.g. inhospitable pH, inhibitory compounds, phytoanticipins, or the lack of stimulatory compounds needed for pathogen development). Sometimes, the aggressiveness of pathogens is directly related to the activity of them cutinolytic enzymes and to plant cell wall thickness and position (pathogens prefer young leaves). The current understanding of plant defense was described by Bent and Mackey (2007). This model is nicknamed the new "Central Dogma" of plant pathology (Figure 1). The more ancient, primary plant immune response is referred to PTI (PAMP-Triggered Immunity) and is based upon the recognition of conserved components of microbial surfaces termed PAMPs (Pathogen Associated Molecular Pattern), alternatively known as elicitors. The recognition is made up by specialized molecules, the PRRs (Pattern-Recognition Receptors), which can switch on the PTI response inducing cytoskeleton rearrangements, callose deposition and the induction of antimicrobial compounds. The PTI response usually terms with the resistance of plant to the pathogen attack, as in the case of *Vitis vinifera* with the non-adapted powdery mildews (Figure1, box 1). However, certain microbes become "adapted" pathogens of particular plant species by evolving virulence factors, called effectors, which actively suppress parts of the host general defense response (Figure 1, box 2). For instance, *E. necator* appears to be the only powdery mildew species which has become adapted to *V. vinifera* . The initial stages of the plant-pathogen 'arms race' starts with the plant immunity defense evolution (Figure 1, box 3). It consists in the restoring of the host species resistance status through specific resistance (R) genes. Commonly, the result of defense activation involving R genes is the programmed cell death (PCD), termed a hypersensitive response (HR), which prevents the pathogen from obtaining nutrients and

completing its life cycle (Mur *et al.,* 2008). The pathogen can be able to evolve further and escapes detection by the *R* protein by modifying or eliminating the effectors that triggers those defenses (Figure 1, box 4). PTI is effective against most pathogens and generally operates in all individuals of a plant species since both PRRs and their corresponding PAMPs are highly conserved in plants and (potential) pathogens, respectively. In contrast, ETI (Effector-Triggered Immunity) in most cases is only effective against one or a few strains of a particular pathogen that possesses an Avr (Avirulence) protein recognized by an R protein (Dry *et al.,* 2009). ETI is also less stable and may be overcome by pathogens in a short period of time (e.g. many resistant cultivars last only 5-10 years in field). As a consequence of the co-evolutionary arms race between plants and pathogens, both the R gene and Avr gene loci are highly polymorphic. In the next paragraph the state-of-art of the current knowledge of the elicitors, PRRs and R genes involved in the interaction plant-microbe in grapevine is given.

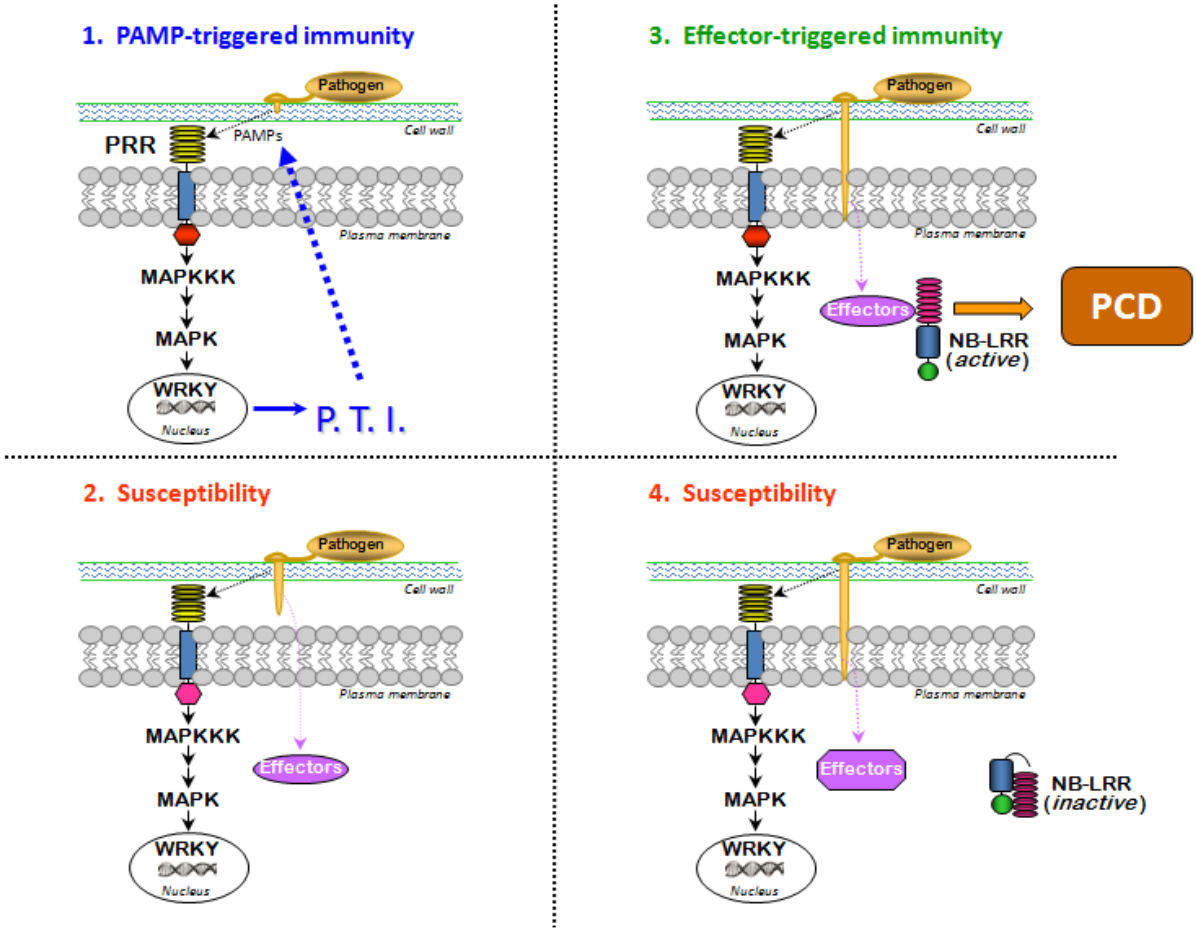


Figure 5. The new "Central Dogma" of plant pathology (Bent and Mackey, 2007).

### 3.1.2 Elicitors, effectors and R genes in grapevine

Over the course of the last few decades, there has been a great increase in the economic competitiveness of grape production. The competitive challenge is associated with the need to maintain quality and productivity, as well as adapt production to new geographical areas. Climate changes, the advent of new diseases and the increasing market demand have led to a remarkable boost in studies related to the grape physiology and pathology of this species (Wang *et al.,* 2014). In particular much attention has been payed on the better understanding of defense mechanisms to either increase resistance or decrease susceptibility in *V. vinifera*. Nowadays, a lot is known about the ETI, but not much about PTI. In the PTI scenario, PAMPs constitute abundant, conserved structures typical of whole classes of pathogens. These structures, known as elicitors of cultivar-non-specific defenses, have been identified both for bacteria, such as lipopolysaccharides (LPS), flagellin (Flg), elongation factor Tu (EF-Tu), cold shock protein (CSP), peptidoglycan (PGN) and Harpin (HrpZ) (Meyer *et al.,* 2001; Newman *et al.,* 2002; Zeidler *et al.,* 2004; Furukawa *et al.,* 2014; Felix and Boller, 2003; Erbs *et al.,* 2008) and for fungi, such as chitin and ergosterol (Wan *et al.,* 2008a; Granado *et al.,* 1995). Focussing on grapevine, grey mould (*Botrytis cinerea* Pers.), powdery mildew (*E. necator*), downy mildew (*P. viticola* Berk.), fruit rot (*Rhizopus stolonifer* Ehrenb. Fr. Lind) and some *Aspergilli* spp. are some of the main fungi that attack the grapevine and lead to the production of defense molecules, like phitoalexins (from the Greek *phyton*=plant and *alexein*=to defend). The elicitors able to induce tolerance in grape epidermal cells after *B. cinerea* or *P. viticola* attacks have been identified and they are oligosaccharides and glucans, such as α -1,4-cellodextrins (Aziz *et al.,* 2007), cyclodextrins (Bru *et al.,* 2006), laminarin extracted from algae (Aziz *et al.,* 2003), β-1,3-glucan sulfate (Trouvelot *et al.,* 2008), botrycin and cinerein (Repka *et al.,* 2001, 2002, 2006). Besides saccaride molecules, also lipids are known to act as elicitors in plants. Among all, ergosterol is the most described one in grapevine. This sterol was described as an inducer of a specific set of defense-related genes and associated signal transduction pathways (Kasparovsky *et al.,* 2004; Lochman and Mikes 2006, Rossard *et al.,* 2006). In addition have been reported that some enzymes of the stilbene biosynthesis pathway are highly induced in grapevine by ergosterol treatment, most probably through the activation of *WRKY* transcription factors (Gomès *et al.,* 2003, Laquitaine *et al.,* 2006; Marchive *et al.,* 2007). Other typical fungal elicitors are chitin and its fragments, chitin oligosaccharides or *N*-acetylchitooligosaccharides. It has been shown that they elicit a variety of plant defense reactions such as the stimulation of phenylalanine ammonia lyase (*PAL*),

peroxidase and lipoxigenase activities, as well as the accumulation of phytoalexins (Aziz *et al.,* 2007). The putative specific receptors of chitin remain to be identified.

Some pathogens, however, have evolved and acquired mechanisms to escape PTI via effectors, causing effector-triggered susceptibility (ETS), that is assumed to allow pathogens to grow and multiply in a potentially hostile plant environment (Alfano and Collmer, 2004; Chisholm *et al.,* 2006; Jones and Dangl, 2006). However, the host contains a back up detection system governed by *R* genes for races and biotypes of a pathogen species. Avirulence genes (Avr) encode for specific effectors that interacts with and recognized by specific cognate receptors (R genes) situated either in the intracellular space or at the plasma membrane of host plants in accordance with Flor's gene-for-gene hypothesis (Flor, 1956). These effectors induce an ETI response. A number of avirulence genes have been identified in plant pathogenic bacteria, although their gene products are yet to be characterised. The majority of characterized plant *R* genes can be grouped into 5 classes (Ellis *et al.,* 2000, Dangl and Jones 2001) encoding for: (i) cytoplasmic serine/threonine kinases (Figure 2A); (ii) extracellular leucine-rich repeats (LRRs) proteins anchored to a trans-membrane domain (Figure 2B); (iii) receptor-like kinases (RLKs) with an extracellular LRR and an intracellular serine/threonine kinase (Figure 2C), (iv) proteins with an N-terminal transmembrane anchor and a cytoplasmic coiled-coil (CC) domain (Figure 2D) genes; and (v) proteins with a nucleotide binding site (NBS), and a LRR domain in their C-terminus (NBS-LRR proteins, Figure 2E). That latter class of *R* genes can be sub-divided in two sub-classes based on their N-terminal domain (Bai *et al.,* 2002), which can either be a toll/interleukine-1 receptor (TIR-NBS-LRR, specific to dicotyledonous species) or a coiled-coil domain (CC-NBS-LRR, present in all angiosperms).

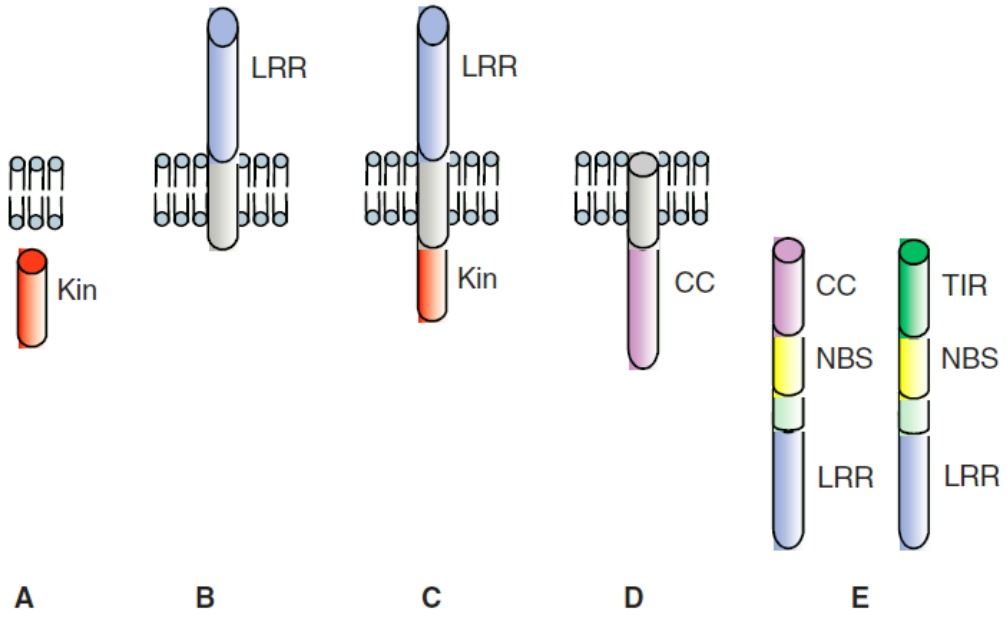


Figure 6. The majority of characterized plant R genes

*NBS-LRR* genes are the most largely represented *R*-genes in plant genomes. Grapevine is no exception and in a survey of the grape cv. Pinot Noir draft genome (Velasco *et al.,* 2007) 233 genes encoding for proteins containing both *NBS* and *LRR* domains were detected. Among them, 84 genes belong to the *CC-NBS-LRR* subfamily while the *TIR-NBS- LRR* subfamily includes 37 genes. A complete inventory of defense-related *RLKs* genes is not easy to make, because these proteins are also implicated in a wide range of developmentally related signaling pathways (Shiu and Bleecker 2001). Nevertheless, 53 genes encoding putative *RLKs* have been identified in grapevine and are arranged in clusters in plant genomes (Di Gaspero and Cipriani 2003). In grapevine, for example *TIR-NBS-LRR* gene clusters are preferentially located on linkage group (LG) 18, *CC-NBS-LRR* gene clusters on LG 9 and 14 and truncated *NBS-LRR* on LG 12 and 13 (Velasco *et al.,* 2007, Moroldo *et al.,* 2008). The LG 14 scored the highest number of *RLK* coding genes (Moroldo *et al.,* 2008).

In agreement with the role of *R* genes in plant innate immunity, several receptor-encoding genes were mapped in *V. vinifera*. The *Run1* locus (Resistant to *Uncinula necator* 1), originating from *M. rotundifolia* (Pauquet *et al.,* 2001) confers resistance to powdery mildew and has a counterpart in the *Vitis* genome physically located on LG 12 (Barker *et al.,* 2005). Additional loci for powdery mildew resistance have been also reported on LG 15 and 14 in *Vitis* hybrids (Dalbo *et al.,* 2001, Fischer *et al.,* 2004). Quantitative trait loci for downy mildew resistance have been mapped with SSR markers to the distal part of LG 18 (Fischer *et al.,* 2004), and in the middle of LG 7 (Grando *et al.,* 2003) in *Vitis* resistant accessions, nearby regions where *NBS-LRR* genes are clustered. Another major determinant responsible

for resistance to *P. viticola* has been identified on LG 12 (Merdinoglu *et al.,* 2003). In conjunction with the knowledge of the grape genome sequence, the availability of linkage maps based on transferable molecular markers (reviewed by Doligez *et al.,* 2006) will constitute valuable tools for pathogen resistance breeding in premium *Vitis* cultivars.

### 3.1.3 The most economically important grapevine disease: Powdery mildew

Powdery mildew afflicts vineyards worldwide. It is caused by the ascomycete fungus *E. necator*, which originates from North America. In the XIX century, American grapevines were introduced in Europe giving to this pathogen the possibility of spreading in the all European countries contributing to destroy the autochthonous grapevines (Gadoury and Pearson, 1991). Indeed, *V. vinifera* evolving in isolation from *E. necator* is highly susceptible to powdery mildew, while many non-*vinifera* grape species, endemic to North America, display varying levels of resistance (Pearson and Gadoury, 1992).

Powdery mildew, also called *oidium*, is a polycyclic disease caused by the gamic (*E. necator*) and the agamic (*Oidium tuckeri* Berk.) form of the fungus. The primary *inoculum* process begins with an *ascogonium* (female) and *antheridium* (male) joining to produce an offspring at the end of summer. This offspring, a young *cleistothecium*, is used to infect immediately or overwinter the host (Figure 3).

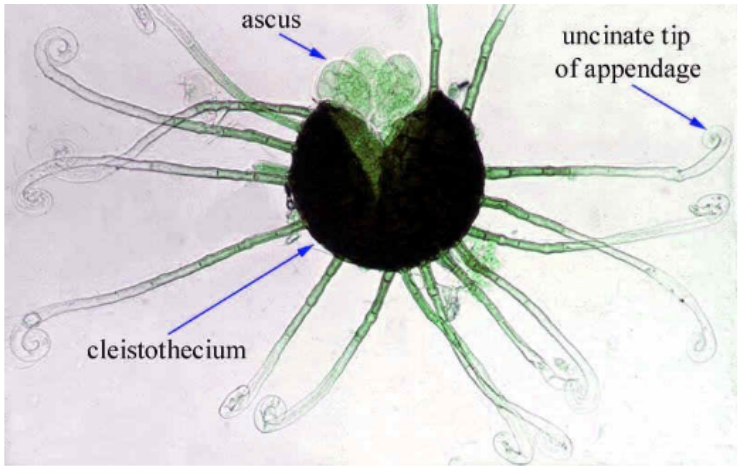


Figure 7. *Cleistothecium* of *E. necator.*

Cleistothecia are formed primarily on foliage, but also on berries, rachises or shoots (Pearson and Gadoury, 1987). They contain at least six asci each of them produce from four to eight spore mother cells of the fungus (ascospores). The long appendages radiating from the ascocarp are hooked at the end; the *cleistothecium* is the persistent (overwintering) stage of the fungus. Morphologically mature cleistothecia are dispersed during rain events to either the bark of the vine or to the soil (Gadoury and Pearson, 1988). They may also be dispersed by

extreme high winds (Grove, 2004). After overwintering, they start off a new cycle of disease in the new growing season. Indeed, when the environmental conditions are right (typically in spring) the cleistothecium ruptures the structure to release ascospores and initiate new infections representing the asexual reproduction or secondary infection. The ascospores land on a healthy tissue and produce the first mycelium hyphae. In the infected cells, the *hyphea* generate agamic spores, conidia, which are responsible of the secondary infections recurrent during summer time. The pathogenicity of *E. necator* is based on the capacity of its hyphae to penetrate the host epidermal cells. The hyphae are 4-5 mm in diameter, hyaline and superficial with multilobed appressoria at regular intervals. A penetration hypha pierces the cuticle and epidermal cell wall and is subtended by a globose haustorium, which envaginates the epidermal cell membrane. After haustorium formation, conidiophores are formed perpendicularly to the epidermis. Each conidiophores produce a single spore or conidia and chains of conidia may accumulate (Gadoury *et al.,* 2012) (Figure 4).

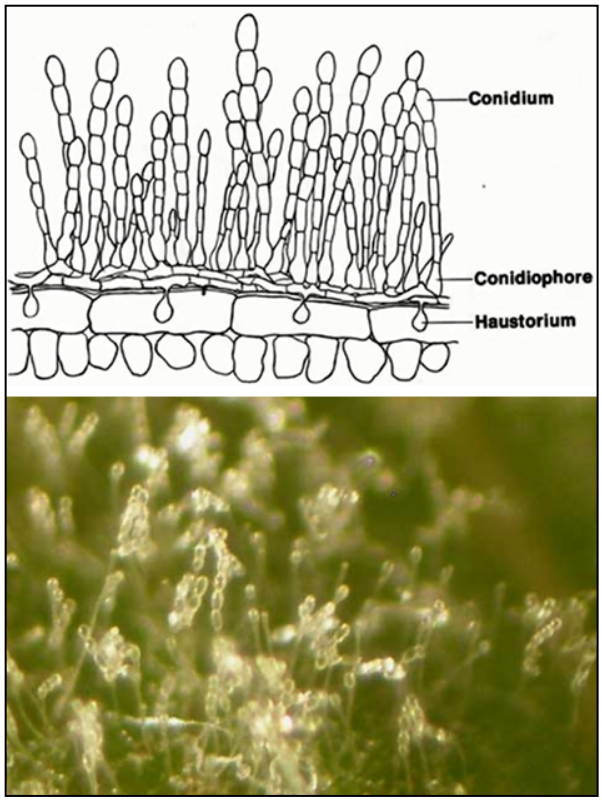


Figure 8. *E. necator* conidia, conidiophores and haustoria.

The pathogenic specialization in *E. necator* is the differential ability of isolates to infect a host species (Gadoury *et al.,* 2012). It was found on *V. rotundifolia*, a powdery mildew resistant grape. Out of 38 isolates, 10 were able to form colonies (Frenkel *et al.,* 2010). The race specificity is similar to the pathogenic specialization, but is conferred by single resistance

genes (R genes), it has been proven in several other host plants (Coffey *et al.,* 2006; Leus *et al.,* 2006; Sacristán *et al.,* 2009). Two breeding populations of *V. vinifera* were shown to segregate for powdery mildew R genes introgressed from wild *Vitis* species. Progeny were screened with diverse isolates collected by Frenkel *et al.* (2010), and individual isolates were differentiated in their ability to overcome the R genes. The isolates that overcame each resistance were collected from the same host species as the source of resistance. This raises the question of whether the pathogenic specialization observed previously was a result of the action of single *R* genes present in only some genotypes of a species, or to more complex non host barriers of all host genotypes of a given species. Another specialization of *Erysiphe* spp. is based on the plant host, indeed each *Erysiphe* species is specialized for one or few susceptible hosts. For example, *E. necator* is the grape powdery mildew, meaning that it can infect only grape species. If a *E. necator* conidia would land on potato leaves, the host immune system will fight and destroy the fungus. However, when *E.necator* land on grape leaves, the possibilities are various due to the fungus pathogenic specialization; on *V. vinifera* the infection occurs, on *V. rotundifolia* it does not. *E. cichoracearum* is the powdery mildew adapted for *Arabidopsis*, pumpkin, melon and squash, and successfully activate the plant immune defense in grapevine.

### 3.1.4 Functional genomics strategies to investigate grapevine defense to powdery mildew

Powdery mildew caused by the biotrophic *E. necator* is the most widespread and destructive disease of grapevines world-wide. However, grapevines are highly resistant to many powdery mildew of other species, called non-adapted (e.g *E. cichoracearum*). The first defense line is a PTI based on PAMPs recognition by surface or intracellular receptors (PRRs). In the model plant species, *A. thaliana*, the PRR *cerk1* (Chitin Elicitor Receptor Kinase 1) recognises the chitin component of fungal cell walls (Figure 5).

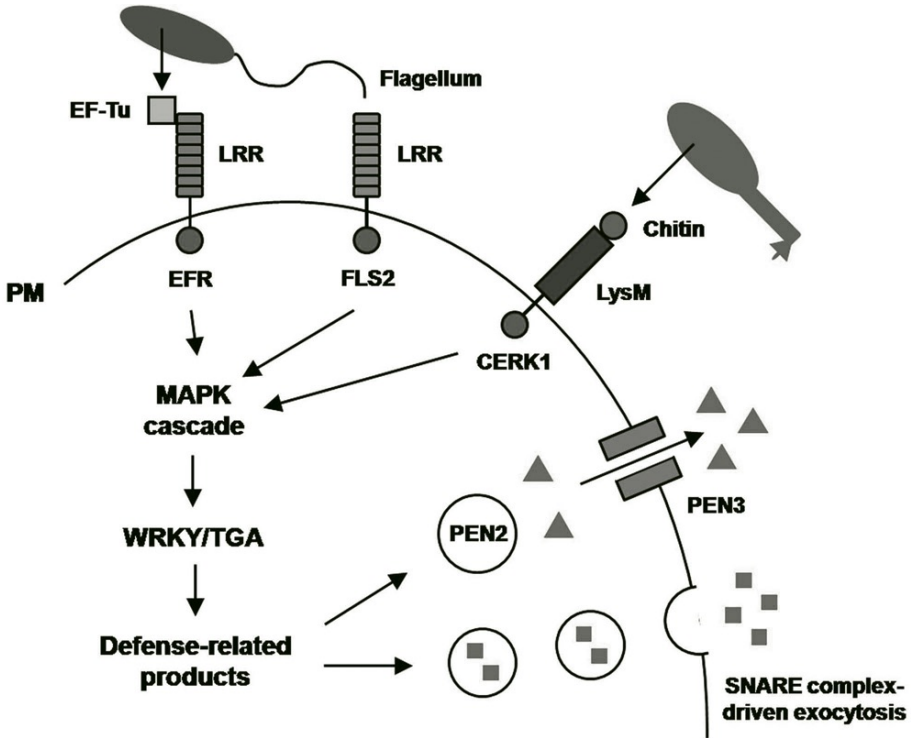


Figure 5. A schematic view of current understanding of PTI responses. Pathogen-derived molecules, bacterial flagellin and EF-Tu (Elongation factor Tu) and fungal chitin, are recognized by cognate PRRs, *fls2* (flagellin-sensitive 2), *efr* (EF-Tu receptor) and *cerk1*, respectively. This recognition drives infected host plant cells to reprogram by changing the gene expression patterns through the MAP kinase cascade. (Kwon, 2010)

*cerk1* is a plasma membrane receptor-like kinase protein with three LysM motifs (LysM RLK) in the extracellular domain which are responsible for chitin recognition. The immune response triggered by *cerk1* leads to the secretion of cell wall appositions called papillae at the site of attempted fungal penetration effectively blocking fungal ingress. The *Arabidopsis* defense pathway represented in Figure 6 starts with the perception of chitin by *cerk1*, followed by activation of the *MPK* pathway, leading to the activation of a variety of transcription factors, as *WRKYs*, and, ultimately, the induction of genes involved in pathogen defense (Wan *et al.,* 2014). Recently, Sánchez-Vallet *et al.* (2015) defined this mechanism as the chitin-triggered immunity, confirming that this carbohydrate plays a central role in plant-fungus interactions. The bioinformatics resources for the grapevine species has expanded in the past few years, with a variety of tools created for post-genomics era applications (Grimplet *et al.,* 2011). Most notably, the genomes of the heterozygous variety Pinot Noir and a near homozygous Pinot Noir derived inbred (PN40024) have been sequenced (Velasco *et al.,* 2007; Jaillon *et al.,* 2007). The sequencing and the assembly of the latter have been updated recently from 8X to 12X coverage. This new assembly is accompanied by the gene structure predictions, which included all the features associated to them, such as mRNAs, UTRs, introns, exons, and inter-genic spaces (Grimplet *et al.,* 2012). The predicted sequence of genes of interest can be downloaded and studied for functional analyses. This type of

studies can be carried out searching grapevine homologs of known and characterized genes in other plant species, like *Arabidopsis*.

### 3.1.5 Aim of the research

Our current knowledge of the interactions between grapevine and powdery mildew at the plant cell wall level and how this affects downstream signaling is still limited. The aim of the present research was gain new insights into mechanisms by which grapevines resists infection by the non-adapted powdery mildew, investigating both grape chitin perception and signal transduction pathways. Towards this goal, we in silico sought these genes putatively involved in the perception of chitin at the epidermal cell apoplast level through the characterization of the grape homologs genes, which are *AtCERK1* homologs. We cloned these homologs in *A. thaliana cerk1* mutant to get functional complementation and determine their role in the defense response of grapevine against non-adapted powdery mildew species. Using a similar in silico approach, signal transduction investigations were performed and grapevine MAPK and WRKY candidates were identified. To better understand the role of these genes in grapevine defense activation after chitin recognition, expression analyses in chitin spray and powdery mildew infection time courses were carried out. The different levels of expression at each time point are powerful indices of candidate genes behavior after fungus attack or fungal elicitor treatment.

## 3.2 Materials and methods

### 3.2.1 The plant material

The *A. thaliana cerk1-1* mutant in the Col-0 genetic background (Germplasm: 4515087882) was obtained from the germplasm collection of *Arabidopsis* Information Resource (TAIR). The *A. thaliana* wild type Columbia-0 was kindly provided by CSIRO (Adelaide, Australia). In time course studies, a Cabernet Sauvignon clone was used, while in the *Agrobacterium* - mediated transformations a Shiraz clone was considered based on recent studies about genetic transformations attitudes.

### 3.2.2 LYK and WRKY phylogenetic analysis

Bioinformatics analysis revealed the presence of 12 *LysM-RLK* genes within the grapevine genome (Zhang *et al.,* 2009). A phylogenetic analysis among these candidates and the 5

*AtLYKs* of *A. thaliana* was carried out using the free platform Phylogeny.fr (http://www.phylogeny.fr/).

A similar analysis was performed to identify the *WRKY* grape candidates. Using *AtWRKY33* as protein query, an *in silico* analysis of its homologs was carried out in the protein database using the algorithm Blastp in the free platform NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The results were screened using the p-values and some reference papers and the selected predicted protein sequences were analyzed using the free platform Phylogeny.fr (http://www.phylogeny.fr/) together with the all *Arabidopsis WRKY* proteins.

### 3.2.3 General CDS cloning protocol

The CDS sequences were amplified using the Phusion® High-Fidelity DNA Polymerase (New England Biolabs, England, UK) from grapevine cDNAs. The PCR products were purified from agarose gel using the PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA) and the concentration was quantified using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, US). A ligation step was performed in a pCR BLUNT Vector from the Zero BLUNT PCR Cloning kit (Invitrogen, Carlsbad, CA, USA). The ligation reaction was performed in a 5 µL volume containing 2.5 ng/µl of the vector, 0.5 µl of T4 DNA ligase, 1x Ligase Buffer and and 3.5 µl of purified product and incubated overnight at 16°C. Ligation reactions were precipitated with n-Butanol to eliminate salt from the ligation buffer, increase transformation efficiency and eliminate arcing of cuvettes. The ligation was transferred in a 1.5 ml tube and 10 volumes of n-butanol added. After a brief vortex (5s), the mixture was centrifuged at 11,300 g for 10 min at room temperature. The supernatant was decanted, 20 volumes of 70% (v/v) ethanol added and the sample re-centrifuged at 11,300 g for 10 min at room temperature. After decanting the supernatant, the pellet was dried under vacuum for 7 min and resuspended in 5 ul of nanopure water. Electro-competent *E. coli* DH5α cells were transformed by electroporation using a Gene-Pulsar apparatus (Bio-Rad, CA, USA). 3 µl of ligation reaction was mixed with a 50 µl aliquot of cells and transferred to an ice-cold cuvette (path length = 1mm; Invitrogen). The cuvette was given a single pulse in the Gene Pulser (1.8 KV, 125 µFD, 200 Ohms), and immediately resuspended in 700 µl of LB agar (Broth LB Agar Lennox 20 gr/L). After incubation at 37°C for 45 min with 200 rpm shaking, the transformed cells were spread on LB agar plates with Kanamycin 100 µg/mL, X-gal 80 µg/mL and IPTG 50 µg/mL and incubated at 37°C overnight. With the blue/white screening, 10 white single colonies were picked, grown in 5

mL of LB liquid media at 37°C with 180rpm shaking overnight and purified using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. A digestion reaction of purified sequences was performed using 1 µl of an appropriate restriction enzymes (generally *EcoRI*-HF (New England Biolabs, England, UK)), 4 µl of the purified colony, 2 µl of CutSmart Buffer (New England Biolabs, England, UK) and water up to a final volume of 20 µl. The reaction was incubated at 37°C for two hours. The purification of the specific fragments from agarose gels after visualization by trans-illuminator was achieved using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The purified fragments were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT, USA) according to the manufacturer's instructions. Extension products were precipitated by adding 80 µl of 75% (v/v) isopropanol, incubating at room temperature for 20 min in the dark and centrifuging for 15 min at maximum speed. After discarding the supernatant and adding 100 µl of 75% (v/v) isopropanol, the tubes were centrifuged for 5 min at maximum speed. The supernatant was aspirated carefully and samples dried in a vacuum centrifuge for 10-15 min. Sequencing reactions were analyzed at the Australian Genome Research Facility (Urrbrae, South Australia). Sequence chromatograms were analyzed by Vector NTI software (Invitrogen, Carlsbad, CA, USA).

### 3.2.4 *A. thaliana cerk1* mutant floral dipping

The pART27_VvLYK1 (Figure 6), pART27_VvLYK2 (Figure 7) and pART27_VvLYK3 (Figure 8) vectors in *Agrobacterium* EHA105 were kindly provided from Dr.Ian Dry (CSIRO, Adelaide, Australia).

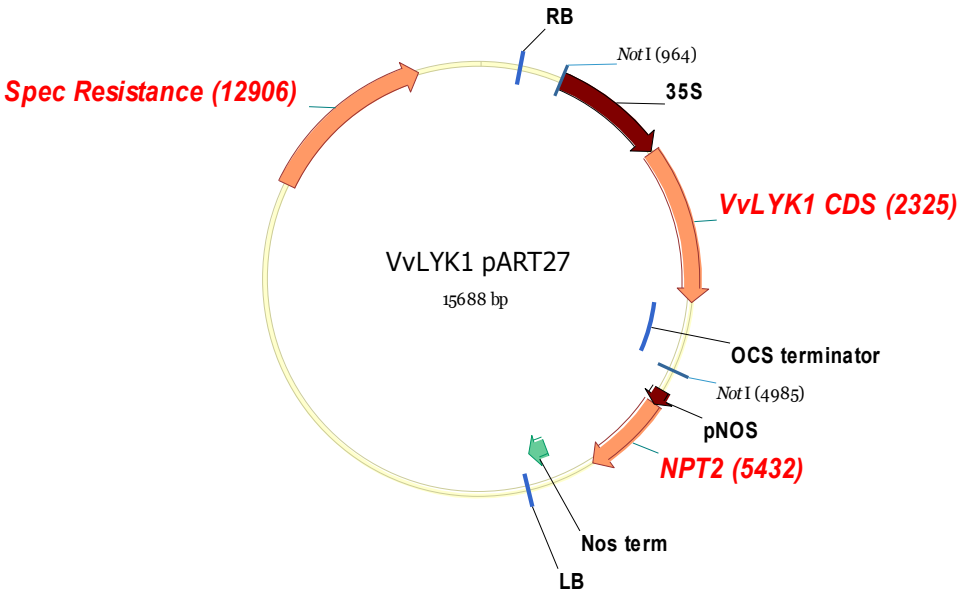


Figure 6. pART27 vector constructed with the expression cassette 35S-VvLYK1-OCS. NotI restriction sites, Right Border, Left Border, Spectinomycin resistance (Spec), and kanamycin resistance (NPTII) sites are reported.

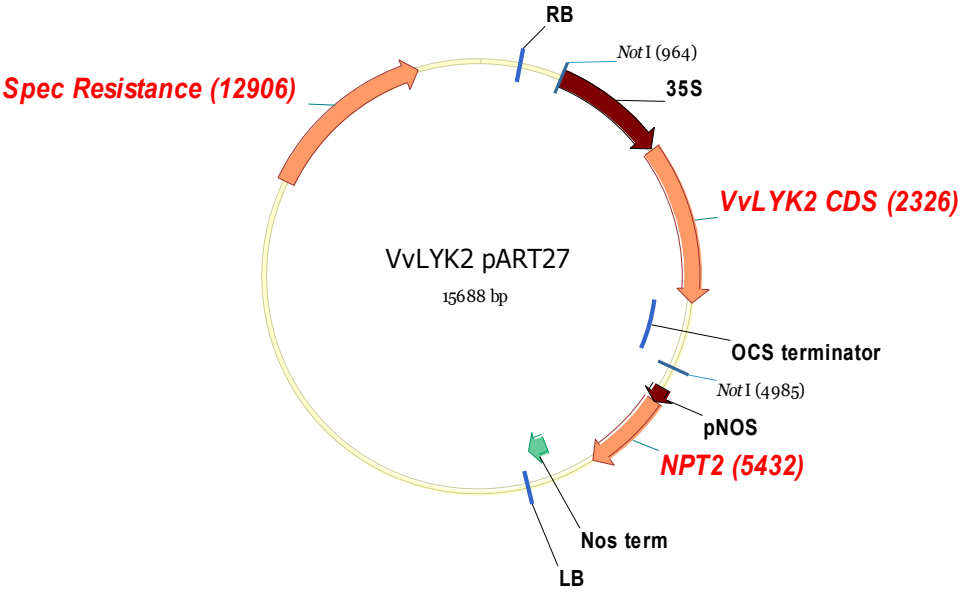


Figure 7. pART27 vector constructed with the expression cassette 35S-VvLYK2-OCS. NotI restriction sites, Right Border, Left Border, Spectinomycin resistance (Spec), and kanamycin resistance (NPTII) sites are reported.

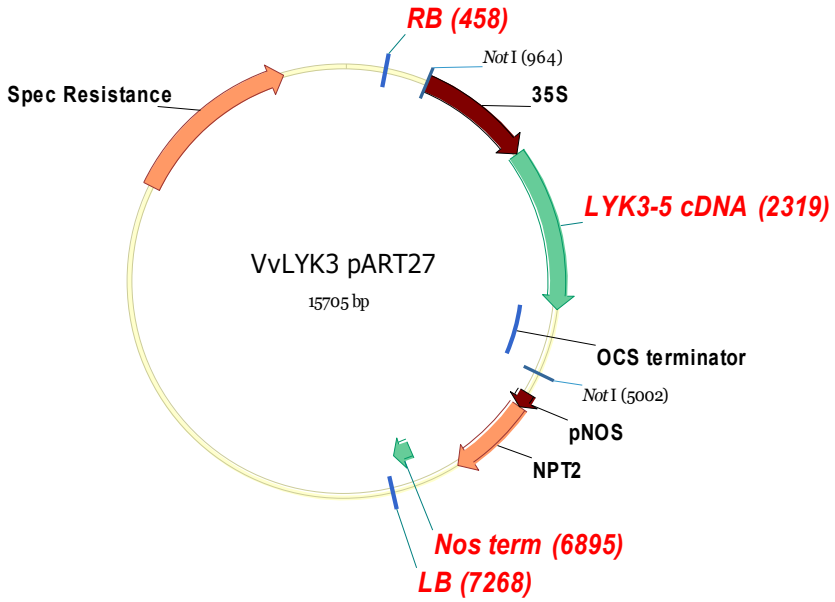


Figure 8. pART27 vector constructed with the expression cassette 35S-VvLYK3-OCS. NotI restriction sites, Right Border, Left Border, Spectinomycin resistance (Spec), and kanamycin resistance (NPTII) sites are reported.

The floral dipping was performed on at least six pots of *A. thaliana cerk1* mutants using a five-days protocol. At the beginning of the first day, an aliquot of the construct was taken from glycerol stock using a inoculation loop, put in 10 mL of LB (Broth LB Agar Lennox 20 gr/L) with Spectinomycin, Gentamicin and Rifampicin (0.1 mg/mL) and incubated at 28°C with 200 rpm shaking overnight. At the very end of the second day, the culture was equally distributed in two sterile flasks containing 160 ml of LB media and the appropriate antibiotics, and incubated overnight at 28°C with 180 rpm shaking. The third day, the culture were placed in centrifuge bottles and spin at 5000 rpm for 15 minutes. Poured off the surnatant, 80 mL of 5% sucrose were added to each bottle and the pellet resuspended. Once resuspended, 0.02% of Silwett was added to each bottle to increase the transformation efficiency. Afterwards, the developed siliques were removed from the *A. thaliana cerk1* mutant and the flowers were dipped twice for 15 seconds. Transformed plants were places in a humid container with a removable lid. This lid was opened in the fourth day and removed in the fifth one. The plants were dried to collect the seeds.

### 3.2.5 Seeds sterilization and plantlets screening

After floral dipping, the selfing (F1) generation seeds were collected, incubated at 37°C for one week and then sterilized. The sterilization consists of an incubation with Ethanol 70% (v/v) for 5 minutes, decant of the solution and another incubation with 2 parts of Ethanol

100%(v/v), 1 part bleach and 0.1% SDS for 5 minutes. The seeds were after washed three times with Ethanol 100%(v/v) and left to dry in laminar flow hood for at least two hours. Dried seeds were sprinkled on selection plates with MS media, Kanamycin (100 µg/mL) and Timentin (200 µg/mL) and incubated at 4°C for 48 hours for seeds synchronization. The plates were incubated in growing camera for two weeks and the positive plantlets (green) were distinguished from the negative ones (yellow) and transferred in new plates without antibiotics. After two weeks, the healthy plantlets fully formed were transferred in pots for seed collection. Four-week-old positive transformed plants were used for powdery mildew penetration test.

### 3.2.6 Semi-quantitative analysis of *VvLYK*s positive plantlets

The correct insertion of the *VvLYKs* sequences in the positive lines was confirmed trough semi-quantitative amplification of *VvLYK1*, *VvLYK2* or *VvLYK3* sequences. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and DNase-treated according to the manufacturer's instructions. cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technology). The semi-quantitative RT-PCRs were performed using the REDTaq® DNA Polymerase (Sigma-Aldrich) in a 25 cycles PCR reaction according to the manufacturer's instructions. The primer used for *VvLYK1*, *VvLYK2* and *VvLYK3* are reported in Table 1 and the internal standard used is the *Arabidopsis* housekeeping gene Elongation Factor (At*ELF*; At5g60390) (Table 1).

### 3.2.7 Functional characterization of VvLYKs

Two leaves per each positive plant were infected with one-week-old *E. necator* growing on Cabernet Sauvignon leaves or one-week-old *E. cichoracearum* growing on cucumber leaves. The infection was set up taking a little piece of the infected leaf (grape or cucumber) and putting it carefully in contact with the top page of the *Arabidopsis* leaf. Detached leaf material was sampled 48h post powdery mildew inoculation and stained in trypan blue with heat for 4 minutes according to Koch and Slusarenko (1990). Fungal structures were visualized using a Zeiss (Göttingen, Germany) Axioscop 2 light microscope and photographed using a Lecia (Wetzlar, Germany) DFC 500 digital camera. Successful penetration was determined by the presence of a fungal *haustorium*. As negative and positive controls, the *A. thaliana cerk1* mutant and the wild-type Columbia-0 (col-0) were used, respectively. The percentage of penetration (% PEN) was calculated counting a minimum of 100 germinated spores

presenting *appressoria* (Figure 9A), haustoria (Figure 9B) or secondary *hyphae* (Figure 9C) and applying the following formula:

$$\% \, PEN = \frac{(\#\, haustoria + \#\, secondary\ hyphae) * total\ number\ of\ germinated\ spores}{100}$$

Each experiment result was confirmed by triplicates. The Student's *t-test* was used for statistical significance of results using Excel add-in options.

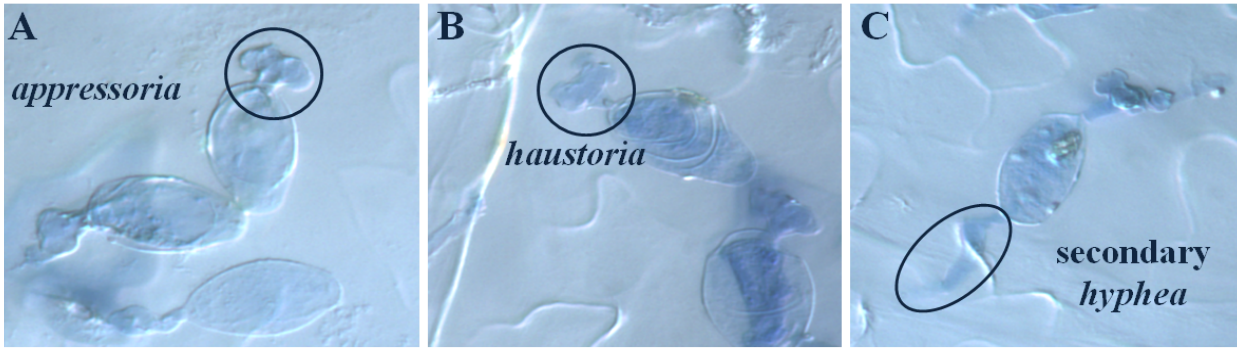


Figure 9. Germinated spores visualized on the light microscope. A. The conidia is germinated growing the *appressoria*, but didn't penetrate in the epidermal cell. B. The conidia is penetrated in the epidermal cell developing the haustoria. C. The conidia is penetrated in the epidermal cell developing the secondary *hyphae*.

### 3.2.8 Powdery mildew infection

*E. necator* (isolate APC, provided by E. Scott, University of Adelaide, SA, Australia) was maintained on detached leaves of *V. vinifera* Cabernet Sauvignon using an 8- to 10-day rotation as previously described (Donald *et al.,* 2002). *E. cichoracearum* was maintained on cucumber (*Cucumis sativus*) plants grown in chambers with a cycle of 16 h of light and 8 h of darkness and maintained at 22°C. Young no glossy Cabernet Sauvignon and Shiraz leaves were detached from glasshouse, sterilized and inoculated with adapted and non adapted powdery mildew. The infected leaves were maintained on agar medium in incubator and then sampled at 0, 4, 8, 12 and 24 hours post inoculation (h.p.i.).

### 3.2.9 Chitin treatment

The youngest no-glossy leaves of Cabernet Sauvignon were sprayed with water and Chitin 0.5 mg/ml diluted in water (YSK – Yaizu Suisankagaku Industry, Japan). The sprayed leaves were sampled at 0, 15, 30, 60, 120 and 240 minutes after spray (m.a.s.).

### 3.2.10 Gene expression analysis

Total RNA was isolated from 40 mg of ground leaves using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's protocol. RNA quality and quantity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). For gene-specific primers, 1 ug of each sample was reverse transcripted in cDNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocol. Semi-quantitative reverse transcriptase-polymerase chain reactions were performed in a 15 µL volume containing 1× $NH_4$ reaction buffer with 670mM Tris-HCl (pH 8.8 at 25°C) and 160mM $(NH_4)_2SO_4$, 2mM $MgCl_2$ Solution, 0.2mM dNTPs Mix, 0.7 µM primer forward, 0,7 µM primer reverse, 1 unit of BIOTAQ Red (Bioline, Alexandria, NSW, Australia), 0,5 µl of cDNA. PCR cycle included initial denaturation of 5 m at 94°C and 25 cycles of 20 sec at 94°C, of 30 sec at Ta°C, of 30 sec at 72°C. The PCR products were visualized on a 1,2% agarose gel. Before use in qRT-PCR experiments, cDNA reactions were diluted 10-fold to 200 mL with 10mM Tris–HCl, pH 7.6. Expression analysis was conducted by real-time PCR analysis using a SYBR Green method on a Light Cycler 480 thermal cycler (Roche). Each 7.5 µL PCR reaction contained 140 nM of each primer, 2 µL of diluted cDNA and 3.75 µL of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions used were 95°C for 15 min followed by 40 cycles of: 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by a melt cycle of 1°C increment per min from 65 to 96°C. The primer pairs used in quantitative analysis are reported in Table 1. All primer pairs amplified a single product of the expected size and sequence, which was confirmed by melt-curve analysis, agarose gel electrophoresis and DNA sequencing. The expression of each target gene was calculated relative to the expression of the housekeeping gene elongation factor, *VvELF*. The housekeeping expression values were used to calculate the multiplication factor of each cDNA (a good value was lower than 5). Afterwards the relative expression values were carried out multiplying the multiplication factors with the expression values. A Fold-change data was obtained dividing the relative expression of a treated sample on the no treated sample.

### 3.2.11 Overexpression of *VvWRKY*24 in Shiraz calli

For over-expression studies, the *VvWRKY24* CDS was amplified using the VvWRKY24_Xho_F and VvWRKY24_Xba_R primers and cloned in pCR BLUNT vector as reported in the Section 3.2.3. Afterwards, the purified constructs and the pART7 vector (Gleave *et al.,* 1992) were double digested with *Xba* and *XhoI* using 1.5 µl of each enzyme, 4 µl of the purified colony or 2 µg of plasmid, 3 µl of CutSmart Buffer (New England Biolabs,

England, UK) and water up to a final volume of 30 µl, following an incubation at 37°C for two hours. The products were purified from agarose gel and diluted to the concentration of 20 ng/µl. The ligation reaction was performed in a 5 µL volume containing 20 ng of vector, 20 ng of insert, 0.5 µl of T4 DNA ligase, 1x Ligase Buffer and incubated overnight at 16°C. The amount of vector and insert to use for ligation were calculated using the online tool Ligation Calculator (http://www.insilico.uni-duesseldorf.de/Lig_Input.html) considering a vector to insert ratio of 1 to 3. The electroporation and the cells growing were set up as reported in Section 3.2.3, but with different antibiotics. Without the blue/white screening, 10 random colonies were purified and the fragment insertion was checked with *XhoI/Xba* digestion. The next step consist in the purification of the expression cassette 35S-*VvWRKY24*-OCS and transformation in the pART27uGFP vector. Based on the pART7 vector restriction map, the *NotI* enzyme is able to cut the complete cassette, but another enzyme cutting outside the sequence is necessary to avoid restriction mistakes. A double digestion with *NotI* and *FspI* was performed as previously described. The *VvWRKY24* ORF (3700 bp fragment) was cutted from agarose gel, purified, treated with alkaline phospatase and diluted to 20 ng/µl. This fragment was inserted in the binary vector pART27uGFP trough ligation reaction, as previously described. The pART27uGFP binary vector is a modified form of the binary vector pART27 (Gleave, 1992) with the introduction of a GFP (Green Fluorescent Protein) reporter gene driven by the *Arabidopsis* ubiqutin-10 gene promoter (UBQ10). The ligation reactions were precipitated with n-Butanol (see Section 3.2.3). The vector is represented in Figure 10. Electro-competent *E. coli* DH5α cells were transformed by electroporation as previously described. The transformed cells were spread on LB agar plates with Spectinomycin 100 µg/mL, X-gal 80 µg/mL and IPTG 50 µg/mL and incubated overnight at 37 °C. Four white colonies were picked, purified and digested with *NotI* enzyme to check the insert size. One of them was used for transformation in *Agrobacterium* EHA105 cells using the Gene Pulser (2.0 KV, 125 µFD, 200 Ohms), electroporation. The cuvette was given a single pulse in and immediately resuspended in 700 µl of LB agar (Broth LB Agar Lennox 20 gr/L). After incubation at 28 °C for 90 min with 200 rpm shaking, the transformed cells were spread on LB agar plates with Spectinomycin 100 µg/mL and Rifampicin 10µg/mL and allowed to grow for 3-5 days at 28°C until single colonies were visible. To produce a culture for transformation, a single colony was inoculated into 3 ml of LB with the selective antibiotic and incubated with shaking at 28 °C for 24-48 h. Afterwards the colony was stocked with glycerol and stored at -80°C. The transformation in Shiraz callus was set up by Dr. Ian Dry group (Adelaide, Australia).

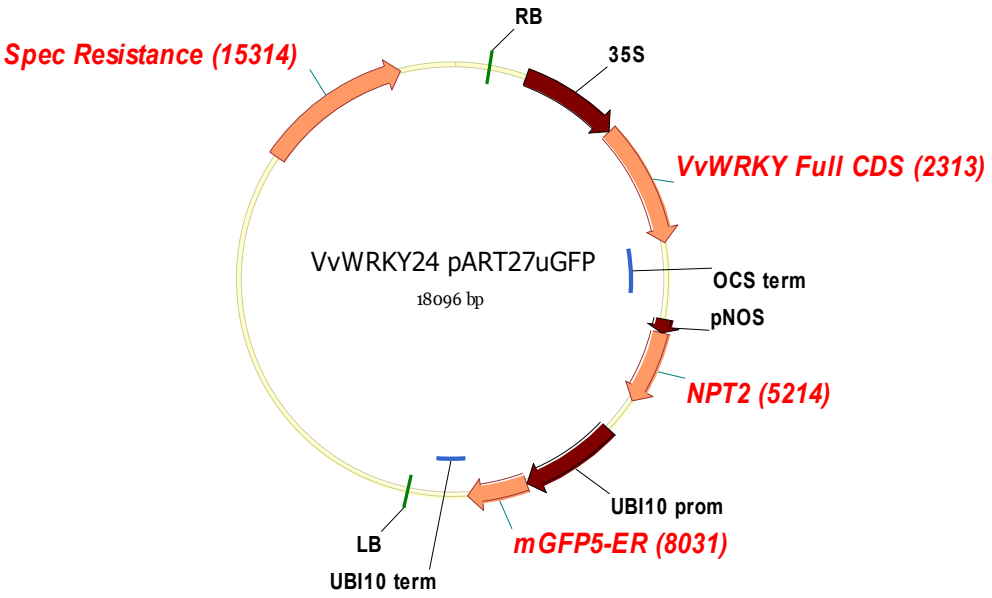


Figure 10. pART27uGFP vector constructed with the expression cassette 35S-VvWRKY24-OCS. NotI restriction sites, Right Border, Left Border, Spectinomycin resistance (Spec), kanamycin resistance (NPTII) sites, GFP reporter gene (mGFP5-ER) and its promoter (UBQ10) are reported.

### 3.2.12 Knock-out of *VvWRKY*24 in Shiraz calli

For production of a *VvWRKY24* silencing construct, 566 bp of the ORF of *VvWRKY*24 were inserted into the pGFP_HELLSGATE12 vector (Helliwell and Waterhouse, 2003). This vector use Gateway recombinational cloning (Invitrogen) for high-throughput construction of hairpins targeting and silencing the gene of interest. The *VvWRKY24* ORF fragment was amplified with flanking attB1 and attB2 sites using the VvWRKY24_RNAi_attB2_R and VvWRKY24_RNAi_attB1_F primers (Table 1) using Pfu DNA polymerase (Bioline). The fragment was cloned, purified and sequenced as described in Section 3.2.3 to check the sequence. The resulting fragment was recombined with the plasmid pDONR-221, which contains attP1 and attP2 sites. The clonase reaction was performed in 5 µl of final volume containing 70 ng of pDONOR221 vector, ~ 100 ng of purified fragment and 1 µl of BP clonase and incubated at 25°C for two hours. A treatment was set up. The reaction was treated with 1 µl of Proteinase K for 15 minutes at 37°C, precipitated with n-butanol and electroporated in E.*coli* DH5α cells as reported in Section 3.2.3. Ten colonies were purified and digested to check the fragment size. The clonase reaction gave rise to the pENTR vector with the inserted *VvWRKY24* ORF with flanking attL1 and attL2 sites. A second recombination reaction was then carried out to insert the target sequence into the attR1 and attR2 sites in the pGFP_HELLSGATE12 vector using 70 ng of pGFP_HELLSGATE12 vector, 70 ng of pENTR, 2 µl of TE buffer and 1 µl of LR Clonase. The reaction was

incubated at 25°C for two hours and cloned as previously described for the pENTR. The silencing construct (Figure 11) was electroporated into *Agrobacterium EHA105* strain and transformed in Shiraz callus as described in the section 3.2.11.

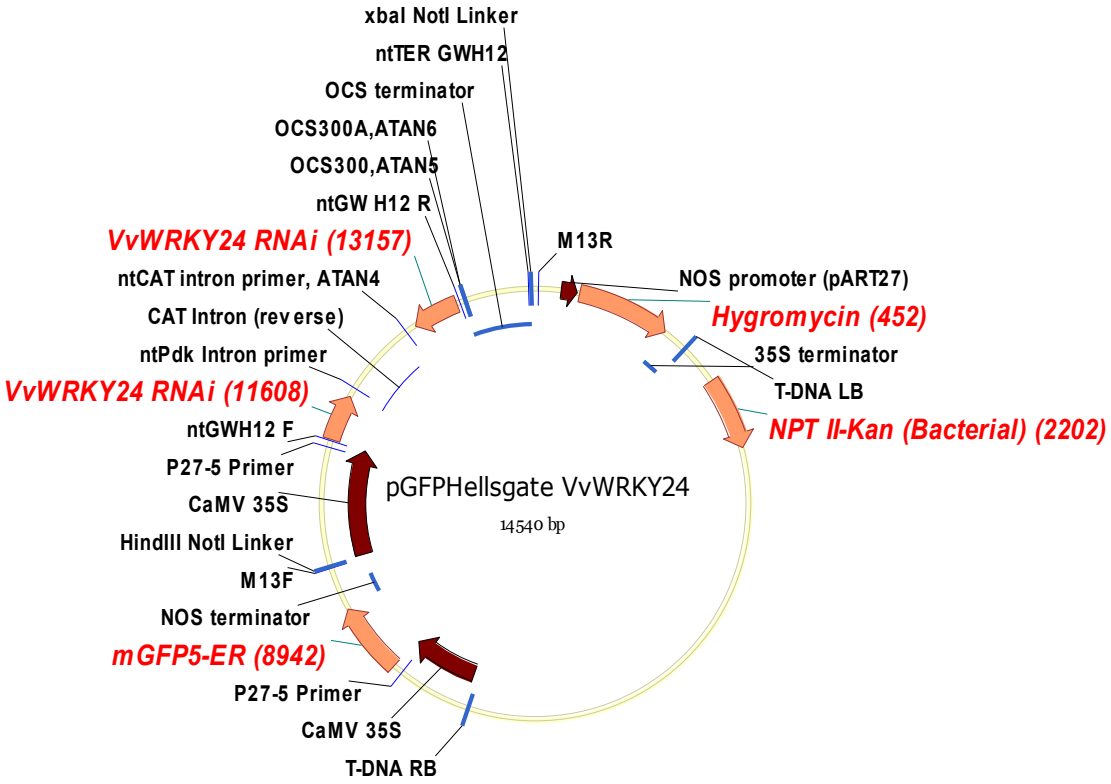


Figure 11. pGFPHellsgate vector constructed with the RNAi sequences of VvWRKY24 under the promoter CaMV 35S and the terminator NOS. NotI restriction sites, Right Border, Left Border, Hygromicin resistance, kanamycin resistance (NPTII) sites, GFP reporter gene (mGFP5-ER) and its promoter (CaMV35S) are reported.

### 3.2.13 Transient gene expression of *VvLYK*1 and *VvWRKY*24

*VvLYK1* (VIT_12s0059g01130) was amplified with VvLYK1_Xho_F and VvLYK1_Xba_R_NO_STOP primers and *VvWRKY24* (VIT_08s0058g00690) with VvWRKY24_Xho_F and VvWRKY24_Xba_R_NO_STOP primers to eliminate the stop codone (Table 1). The fragments amplified were cloned in pCR BLUNT as described in the Section 3.2.3. Afterwards, the positive colonies were separately cloned into pNgfp2 (a modified version of vector pART7 with an added in-frame GFP coding region) as reported in the Section 3.2.11. The sequences across junction of *VvLYK1/VvWRKY24* and GFP protein were sequenced to confirm the in-frame fusion. Young grape leaves and onion epidermal cells were bombarded with the pNgfp2_VvLYK1:GFP and the pNgfp2_VvWRKY24:GFP constructs coated on gold particles. For three shots, 10 mg of 1 µM gold particles (Bio-Rad, Gladesville, Australia) in 200 µl of ethanol were vortexed for 2 min, spun down for 10 s in a

microfuge, drained, washed twice with sterile water, and resuspended in 170 µl of 40% glycerol. While gently vortexing, 10 µl of the plasmid solutions (150 ng/µl), 10 µl of cold 0.1 M spermidine and 25 µl of 2.5 M CaCl2 were added drop wise and the resulting mixture was incubated on ice for 10 min. The particles were spin down, washed with 70% ethanol, and resuspended in 24 µl of cold 100% ethanol and 6-µl aliquots were placed onto sterile filter holders. After bombardment, tissue was stored in the dark for 24 h before fluorescence microscopy. The onion layers were floated on 0.5 M sucrose (to induce plasmolysis) and DAPI (4',6-diamidino-2-phenylindole) (to color the nucleus), incubated for 30 min and observed with a light microscope (Zeiss Axio Imager M1). The DIC (Differential interference contrast) microscopy, also known as Nomarski microscopy was used to enhance the cells contrast. An average of twenty cells were imaged for each experiment.

Table 8. Primers used in this research. For each primers, gene family, name used, sequence and usage and reported.

| Gene Family | Primers identity | Primer sequence (5'-3') | Usage |
|---|---|---|---|
| MAPK | VvMPK3_F1 | tatcatgcaactcccccaac | Real Time PCR |
| | VvMPK3_R2 | ctctgggcaaactggttcat | Real Time PCR |
| | VvMPK6_F1 | accaccacctcagaggacag | Real Time PCR |
| | VvMPK6_RT_Rv | gtccctgtgaagaacatttgc | Real Time PCR |
| WRKY | *VvWRKY*16_RT_Fw | ctgatggcttcggattctc | Real Time PCR |
| | *VvWRKY*16_Rv | gcagagcttcaacagagcag | Real Time PCR |
| | *VvWRKY*24_F2 | gcctataaggccctcagtca | Real Time PCR |
| | *VvWRKY*24_R | gttcttgagaaaaccccatctg | Real Time PCR |
| | *VvWRKY*24_Xho_F | gcg**ctcgag**ccatggcttcctctgctgctag | Over expression transformation |
| | *VvWRKY*24_Xba_R | gcg**tctaga**gcaattcagcaaaaaaatgactgg | Over expression transformation |
| | *VvWRKY*24_RNAi_attB1_F | ggggacaagtttgtacaaaaaagcaggctccttta ccaaataatagcaccac | Gateway knock-out |
| | *VvWRKY*24_RNAi_attB2_R | caacaatgcctcattcaaactatgacccagctttctt gtacaaagtggtcccc | Gateway knock-out |
| | *VvWRKY*24_Xba_R_NO STOP | gcg**tctaga**gcaaaaaaatgactggaacaagtc atc | Biolistic transformation |
| *VvLYK*1 | *VvLYK*1_RT_F | tacatgccaccagaatacgc | Real Time PCR |
| | *VvLYK*1_RT_R | aaggtcctctcgtggatcag | Real Time PCR |
| | *VvLYK*1-1_R2 | taaacagatccaaagccacc | RT-PCR |
| | *VvLYK*1_F2 | gtgggcttgtttacattcctac | RT-PCR |
| | *VvLYK*1_Xho_F | cgcctcgagcaaatgaaacagaaggtggg | Vector construction |
| | *VvLYK*1_Xba_R | cgctctagaaaggaaaaatggtgaagtattg | Vector construction |
| | *VvLYK*1_Xba_R_NO STOP | cgctctagaccttccagacattagattgac | Biolistic transformation |
| *VvLYK*2 | *VvLYK*2_RT_F | cgtcttgtgggtacatttgg | Real Time PCR |
| | *VvLYK*2_RT_R | tcctcaaacaaagcaacgag | Real Time PCR |
| | *VvLYK*2-1_F2 | cccgaatttgtaaaaggaag | RT-PCR |
| | *VvLYK*2_R2 | cacaggaactgtatgctcatg | RT-PCR |
| | *VvLYK*2_Xba_R | cgctctagatgctaccttcctgacattag | Vector construction |
| | *VvLYK*2_Xho_F | ggcctcgagaaacatggtcatttcatcaac | Vector construction |
| *VvLYK*3 | *VvLYK*3_RT_F | ttcttcattgcccactcgtc | Real Time PCR |
| | *VvLYK*3_RT_R | caagtcctcttgcttcagtgg | Real Time PCR |
| | *VvLYK*3-5_R2 | atatcctatcaggcgaaccag | RT-PCR |
| | *VvLYK*3_F2 | gttgatttcagccttggtagtg | RT-PCR |
| | *VvLYK*3_Xba_R | cgctctagacacactatcttcctgacatc | Vector construction |
| | *VvLYK*3_Xho_F | gcgctcgagccatctttgctagggtttc | Vector construction |
| Housekeeping genes | AtELF_F | tgagcacgctcttcttgctttca | RT-PCR |
| | AtELF_R | ggtggtggcatccatcttgttaca | RT-PCR |
| | VvELF_F | cgggcaagagatacctcaat | Real Time PCR |
| | VvELF_R | agagcctctccctcaaaagg | Real Time PCR |
| *Arabidopsis* genes | VvLYK3_FullcDNA_Fw | caaccacgtcttcaaagcaag | RT-PCR |
| | VvLYK3_FullcDNA_Rv | catgcgatcataggcgtctc | RT-PCR |
| | VvLYK3_InternalFw | gatgggaaaggcaccacttc | RT-PCR |
| | VvLYK3_InternalRv | cagagaaacagcagcaaccag | RT-PCR |
| | AtMPK3_F | ctcacggaggacagttcataag | RT-PCR |
| | AtMPK3_R | gagatcagattctgtcggtgtg | RT-PCR |
| | AtWRKY33_F | ctccgaccacaactacaactac | RT-PCR |
| | AtWRKY33_R | ggctctctcactgtcttgcttc | RT-PCR |
| | AtWRKY53_F | cctacgagagatctcttcttctg | RT-PCR |
| | AtWRKY53_R | agatcggagaactctccacgtg | RT-PCR |

## 3.3 Results

### 3.3.1 Functional characterization of grape chitin receptor

Certain members of the LysM-RLK receptor family have been shown to play a specific role in penetration resistance against powdery mildew, including the *AtCERK1* isolated from *Arabidopsis* (Wan *et al.,* 2008). In order to identify the candidate genes involved in chitin recognition and penetration resistance in grapevine, we carried out a cluster analysis using the 12 *VvLYK* orthologous proteins found in *V. vinifera* (Clone PN40024) genome sequence (Jallion *et al.,* 2007; Zhang *et al.,* 2009) and the 5 members of the *Arabidopsis* LysM-RLK family (Hyun *et al.,* 2010) (Figure 12). *AtCERK1* clustered with *VvLYK1* (VIT_12s0059g01130), *VvLYK2* (VIT_10s0116g00910) and *VvLYK3* (VIT_10s0116g00920).

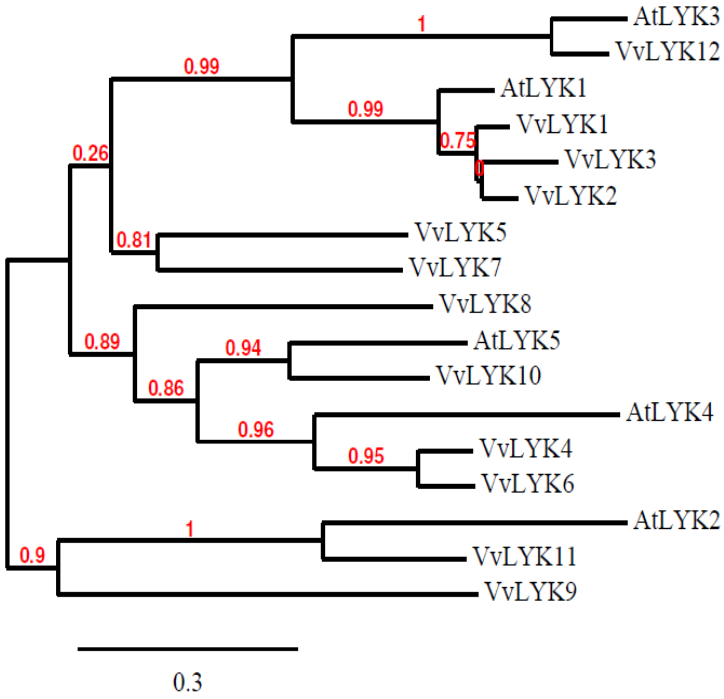


Figure 12. Philogenetic analysis of *V. vinifera* (*VvLYK*) and *A. thaliana* (*AtLYK*) LysM-RLK candidates. Bootstrap values are displayed.

To determine whether *VvLYK1*, *VvLYK2* and *VvLYK3* could contribute to penetration resistance, we generated transgenic lines in the *A. thaliana cerk1* mutant background. This mutant is compromised in penetration resistance and allow enhanced entry levels of non-adapted powdery mildew species (Miya *et al.,* 2007), for this reason was used as negative control. On the counterpart, the wild type Columbia-0 showing a high resistance to non-adapted powdery mildew species, was used as positive control. During one year, 15 positive and healthy selfing (F1) populations were collected from *VvLYK1* transformations, 5 for *VvLYK2* and 30 from *VvLYK3*. During the selection of positive transformed lines, *VvLYK2* showed growing difficulties both on plate media and on pots; indeed only five F1 populations

were collected and were used for functional studies. All the transformed populations selected on plate through antibiotic resistance were also tested using genomic PCR. The F1 and F2 lines checked to be positive are reported in Table 2. The F1 lines were usually indicated with two numbers, the first one is related to the construct used for the transformation (1 for pART27-VvLYK1, 2 for pART27-VvLYK2 and 3 for pART27-VvLYK3) and the second number represent a sequential numbering used to distinguish each line. For example, 1.2 is one of the F1 lines obtained from *VvLYK1* transformation.

Table 2. Identification number of the F1 lines of the three pART27-VvLYKs constructs and number of the F2 lines generated from them. Sometimes the F1 lines numbering is not sequential because the missed lines were tested to be negative.

| Construct | F1 line | # F2 lines |
|---|---|---|
| pART27-VvLYK1 | 1.1 | 2 |
| | 1.2 | 4 |
| | 1.4 | 3 |
| | 1.5 | 9 |
| | 1.6 | 4 |
| | 1.7 | 2 |
| | 1.8 | 2 |
| | 1.11 | 3 |
| | 1.12 | 5 |
| | 1.13 | 5 |
| | 1.15 | 3 |

| Construct | F1 line | # F2 lines |
|---|---|---|
| pART27-VvLYK2 | 2.2 | 5 |
| | 2.5 | 1 |
| | 2.10 | 4 |
| | 2.12 | 3 |
| | 2.14 | 2 |

| Construct | F1 line | # F2 lines |
|---|---|---|
| pART27-VvLYK3 | 3.1 | 3 |
| | 3.2 | 4 |
| | 3.3 | 3 |
| | 3.5 | 3 |
| | 3.6 | 5 |
| | 3.7 | 2 |
| | 3.8 | 5 |
| | 3.9 | 5 |
| | 3.10 | 5 |
| | 3.11 | 3 |
| | 3.15 | 3 |
| | 3.16 | 6 |
| | 3.19 | 2 |
| | 3.22 | 8 |
| | 3.23 | 5 |
| | 3.24 | 8 |
| | 3.25 | 1 |
| | 3.26 | 2 |
| | 3.27 | 1 |
| | 3.28 | 7 |
| | 3.30 | 5 |

Another test used to check the correct insertion of the *VvLYK* sequences in the *A. thaliana cerk1* mutant genome, was the semi-quantitative amplification of the inserted CDS in the positive lines after RNA retro-transcription. In Figure 13 is reported an example of the data obtained in six *VvLYK1* F1 lines (1.1, 1.2, 1.5, 1.6, 1.7, 1.8), five *VvLYK2* F1 lines (2.2, 2.5, 2.10, 2.12, 2.14) and five *VvLYK3* F1 lines (3.1, 3.2, 3.5, 3.7, 3.15). The expression was evaluated on agarose gel trough the bands presence/absence and intensity. The bands amplified in the *VvLYK1* lines showed a miscellaneous expression with the higher intensity in the 1.2, 1.5 and 1.7 lines. On the counterpart, in the *VvLYK3* lines the bands are uniform and

highly intense in all line analyzed. Instead, in the *VvLYK2* lines the amplification was scanty or completely missed.

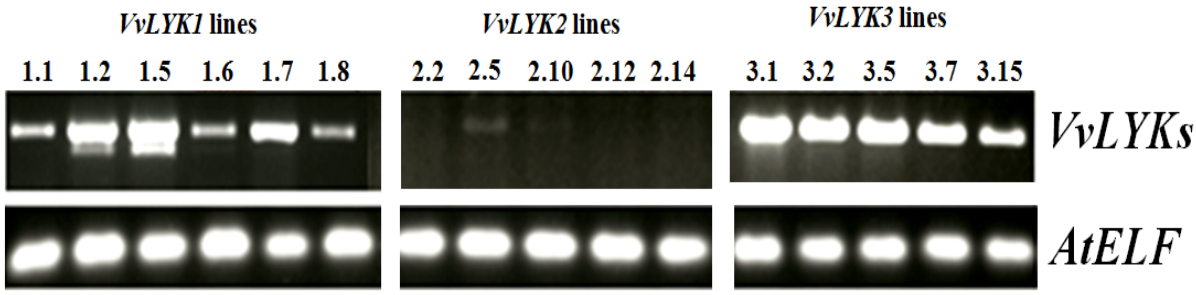


Figure 13. Semiquantitative reverse-transcription-PCR analysis of *VvLYK*s expression in 6 transgenic *VvLYK*1 lines, 5 *VvLYK*2 lines and 5 *VvLYK*3 lines. *AtELF* was used as internal control. Results shown are representative of three technical replicates.

To study the behavior of the transgenic lines in a plant-pathogen interaction, a penetration test was set up with non adapted powdery mildew, *E. necator*. The experiment plan used for inoculations is represented in Figure 14. In the experiment, a minimum of three positive F2 plantlets were considered for each selfing line and three or more leaves of this plantlets were functionally studied with powdery mildew inoculations. The inoculations were carried out more than one time on each transformed lines.

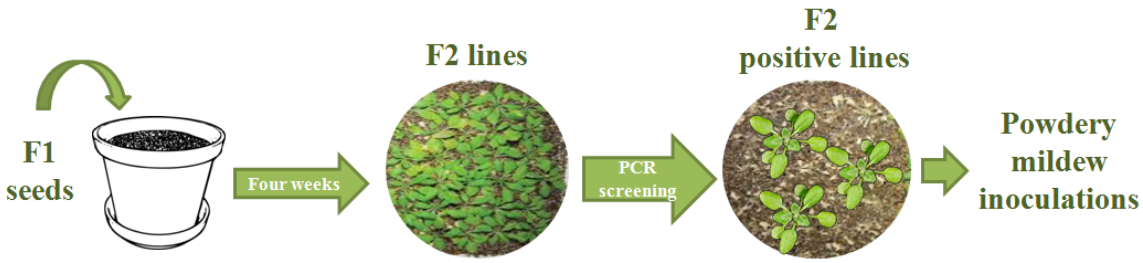


Figure 14. Experimental scheme of F2 lines screening for powdery mildew inoculations.

The inoculations were always set up also on the positive and the negative control, the wild-type Columbia-0 and the *A. thaliana cerk1* mutant, respectively. The results obtained on six *VvLYK1* F1 lines (1.1, 1.2, 1.5, 1.6, 1.7 and 1.8), five *VvLYK2* F1 lines (2.2, 2.5, 2.10, 2.12 and 2.14) and five *VvLYK3* F1 lines (3.1, 3.2, 3.5, 3.7 and 3.15) are representative of the all sampled lines and are reported in Figure 15. The number of leaves infected in the experiments is reported in brackets and ranged from 2 for 1.1 and 2.14 lines to 11 for 3.2 and 3.15 lines. In each graph, are reported the penetration percentages of controls, positive lines and two negative lines. The latter are F2 lines resulted negative at the PCR screening, they were considered as negative controls. In the *VvLYK1* lines, the penetration percentages ranged from 30.7 for 1.1 to 39.9 for 1.5 and resulted significantly similar to the positive control (38.8) ($p = 0,3811$). The *VvLYK1* negative lines results were highly different than the positive control and

similar to the negative control, as expected. For the other two transgenic lines, the percentages of penetration were highly similar to the negative control. Indeed, in *VvLYK2* and *VvLYK3* lines, the lowest values were 55.87 (2.14 line) and 58.4 (3.1 line) and the highest values were 69.1 (2.5) and 72.2 (3.15). There were no significant differences among the positive and the negative lines of *VvLYK2* and *VvLYK3*.

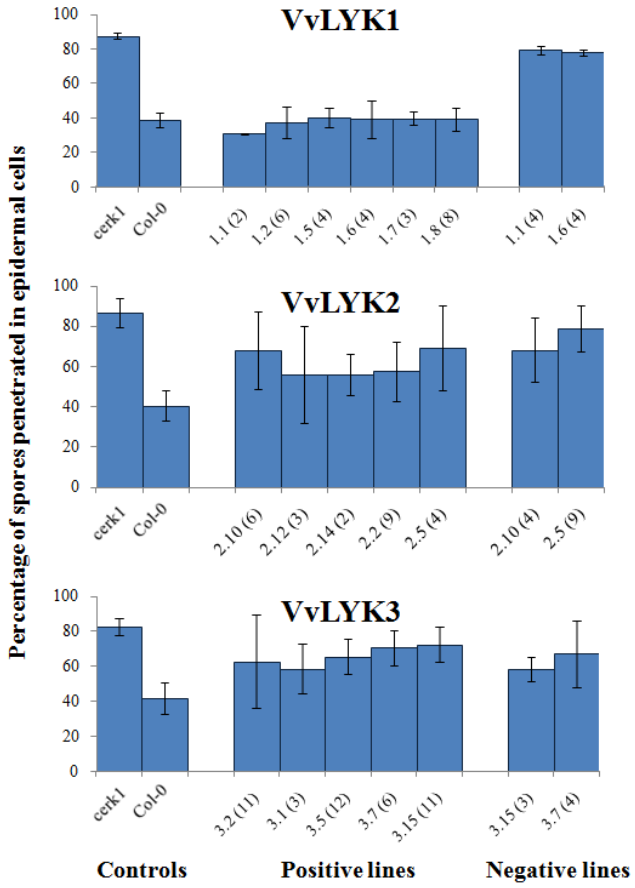


Figure 15. Penetration efficiency of *E. necator* in the negative and positive controls, the *A. thaliana cerk1* mutant and the wild type Columbia-0, and on positive and negative lines of *VvLYK1*, *VvLYK2* and *VvLYK3*. The number of leaves infected are bracketed.

In order to confirm the data obtained on the *VvLYK1* transgenic lines, a penetration test was arranged using the adapted powdery mildew, *E. cichoracearum*. The results obtained in the controls (the *A. thaliana cerk1* mutant and the wild type Columbia-0) and in four *VvLYK1* positive lines (1.1, 1.2, 1.6 and 1.8) are represented in Figure 16. The average of the percentage of penetration in the *VvLYK1* lines was 75.7 and the data recorded in Col-0 was

73.4. This finding suggested that the penetration levels in the positive lines were significantly similar to the positive control, Columbia-0 (p= 0,817), as expected.

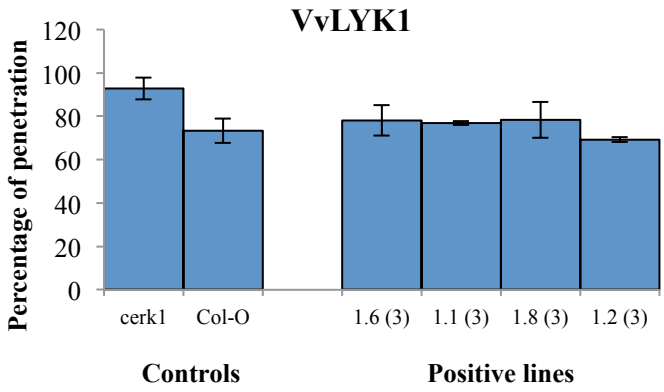


Figure 16. Penetration efficiency of the adapted powdery mildew *E. cichoracearum* on two controls, the *cerk1* and the wild type Col-0, and on the positive *VvLYK*1 lines. In brackets, the number of leaves infected.

For a further investigation of the chitin perception in the transgenic lines, a semi-quantitative analysis of *Arabidopsis* signal transduction genes was carried in *VvLYK1* and *VvLYK3* positive lines The *AtMPK3*, *AtWRKY*33 and *AtWRKY*53 genes were selected because have been shown to be involved in *Arabidopsis* signal transduction after chitin recognition (Wan *et al.,* 2004). A pilot experiment was set up on the *A. thaliana cerk1* mutant and the wild-type Columbia-0 to compare the response to soluble and insoluble chitin after 30 minutes of treatment (Figure 17). The results showed a higher expression of *AtWRKY*33 and *AtWRKY*53 genes after treatment with insoluble chitin (bands more intense). Contrariwise, the *AtMPK3* gene didn't showed any differences along the time course.

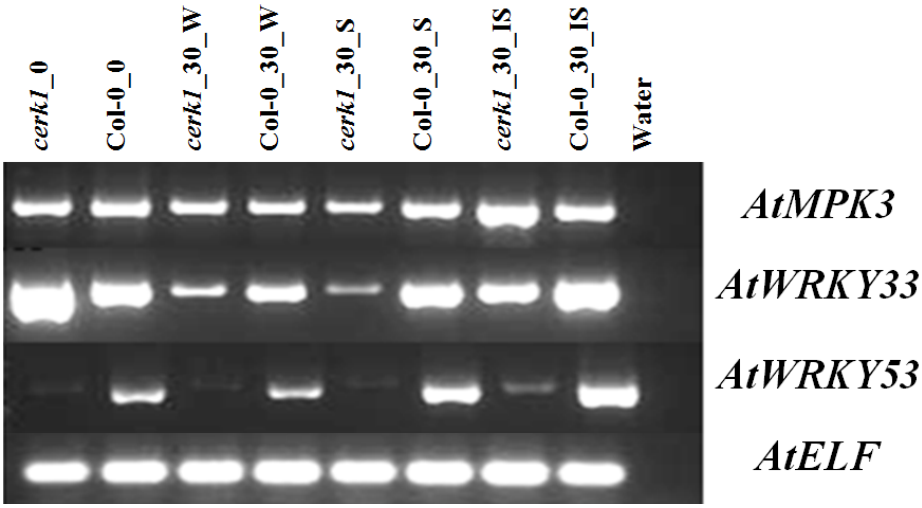


Figure 17. Semi-quantitative analysis of *AtMPK3*, *AtWRKY*33 and *AtWRKY*53 in *A. thaliana cerk1* mutant and the wild-type Columbia-0. The plantlets were sampled at 0

time (*cerk1*_0 and Col-0_0), after 30 minutes treatment with water (*cerk1*_30_W and Col-0_30_W), soluble chitin (*cerk1*_30_S and Col-0_30_S) and insoluble chitin (*cerk1*_30_IS and Col-0_30_IS). A PCR water control was included (Water). *AtELF* was used as internal control.

Three *VvLYK1* (1.1, 1.5 and 1.6) and *VvLYK3* (3.1, 3.2 and 3.15) transgenic lines were used in a 0, 15 and 30 minutes time course treatment with insoluble chitin. The time courses were semi-quantitative analyzed using the *AtWRKY*33 and *AtWRKY*53 genes (Figure 18) The results showed a heterogeneous expression in the *VvLYK*3 lines and a more reliable data in the *VvLYK*1 lines. Indeed, in the three *VvLYK*1 lines the expression was activated only following chitin treatment (no bands at point 0) and it was higher than the water controls.

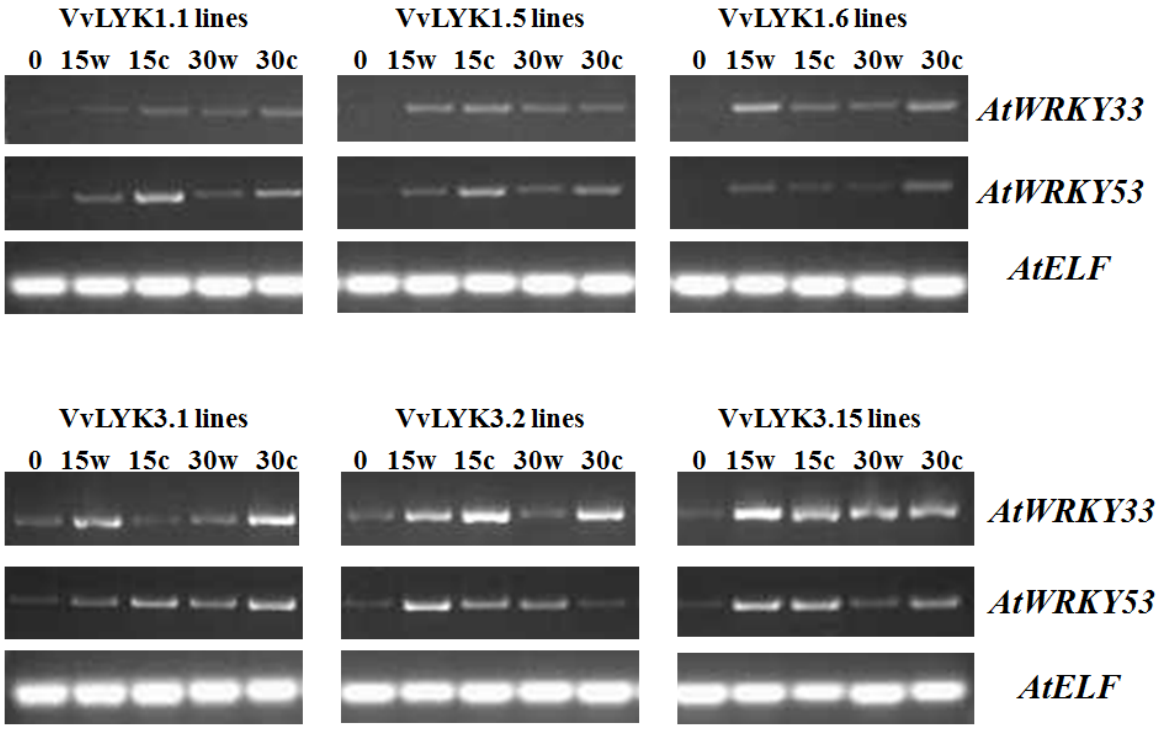


Figure 18. Semi-quantitative expression analysis of *AtWRKY*33 and *AtWRKY*53 genes in three *VvLYK1* (1.1, 1.5 and 1.6) and *VvLYK3* (3.1, 3.2 and 3.15) transgenic lines in a 0, 15 and 30 minutes time course treatment with insoluble chitin. 0 = 0 Time (no treated); 15w = 15 minutes treated with water; 15c = 15 minutes treated with chitin; 30w = 30 minutes treated with water; 30c = 30 minutes treated with chitin. *AtELF* was used as internal control.

Based on our data only *VvLYK*1 proved to be involved in chitin recognition and signal transduction. To confirm the data and avoid the possibility of a functional mutation in *VvLYK*3 lines due to the transformation process, we amplified and sequenced the *VvLYK3.1*, *VvLYK3.2* and *VvLYK3.15* sequences in cDNAs and gDNAs. The amplified full-length and partial sequences of *VvLYK3.1* and *VvLYK3.2* are reported in Figure 19. The amplicons obtained in cDNA (Figure 19, lane 1, 3 and 5) and in gDNA (Figure 19, lane 2, 4 and 6) didn't show any difference, suggesting the lack of modifications both in the genomic and transcript sequence. The data were confirmed by sequencing (data not shown).

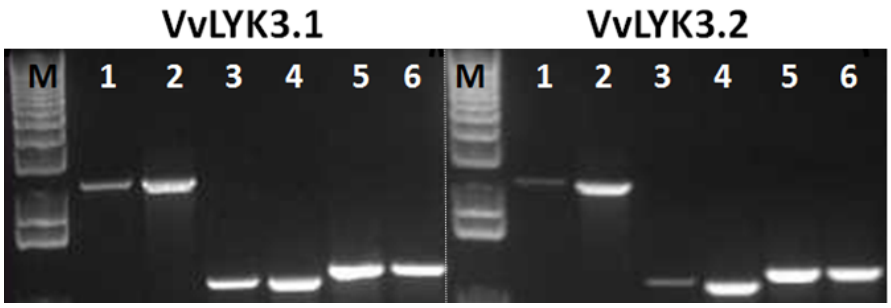


Figure 19. Amplification of two *VvLYK*3 lines on cDNAs and gDNAs. M = Molecular marker 1kbPlus; 1 = cDNA Full Length; 2 = gDNAs Full Length; 3 = cDNA partial sequence; 4 = gDNA partial sequence; 5 = cDNA partial sequence; 6 = gDNA partial sequence.

To localize the *VvLYk1* protein, a biolistic transformation in onion layers was set up co-expressing the *VvLYK*1 and the GFP proteins using the pNgfp2_VvLYK1:GFP vector. Those products were found to be localized in the cell periphery, certainly in the plasma membrane (Figure 20). The cell wall localization was excluded due to the plasmolysis induced by sucrose treatment, which caused the distinction of the cell wall from the cell membrane. Evenly, the *vacuola* localization was excluded due to internal *nucleus* position (DAPI staining).

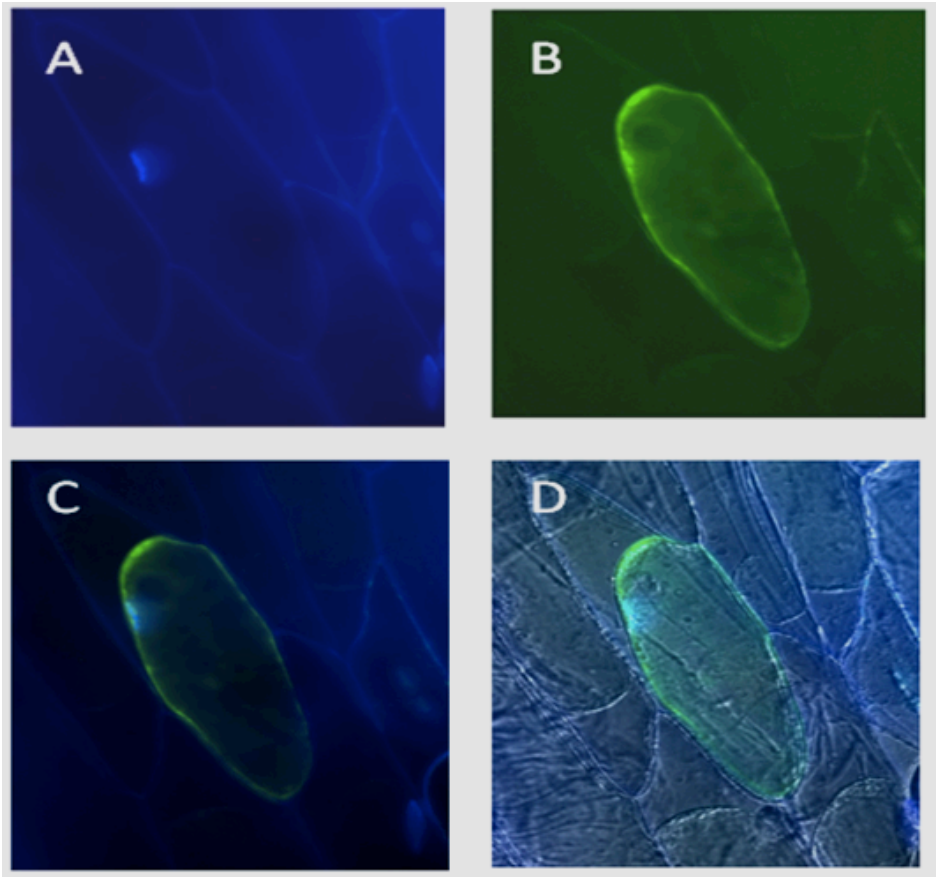


Figure 20. Biolistic transformation of pNgfp2_VvLYK1:GFP vector in onion layers. Each image is captured with different filters: (A) DAPI , (B) GFP, (C) GFP e DAPI e (D) DIC, GFP e DAPI.

### 3.3.2 Functional characterization of grape intracellular signal transduction

Using *Arabidopsis* as model plant, we looked at the genes induced in response to chitin treatment and powdery mildew infection, focusing on *MAPK* and *WRKY* gene families. Based on the *VvMPK* sequenced characterized by Hyun *et al. (*2010), specific primers were designed on the *VvMPK3* and *VvMPK6* sequences for expression analysis (Figure 21).

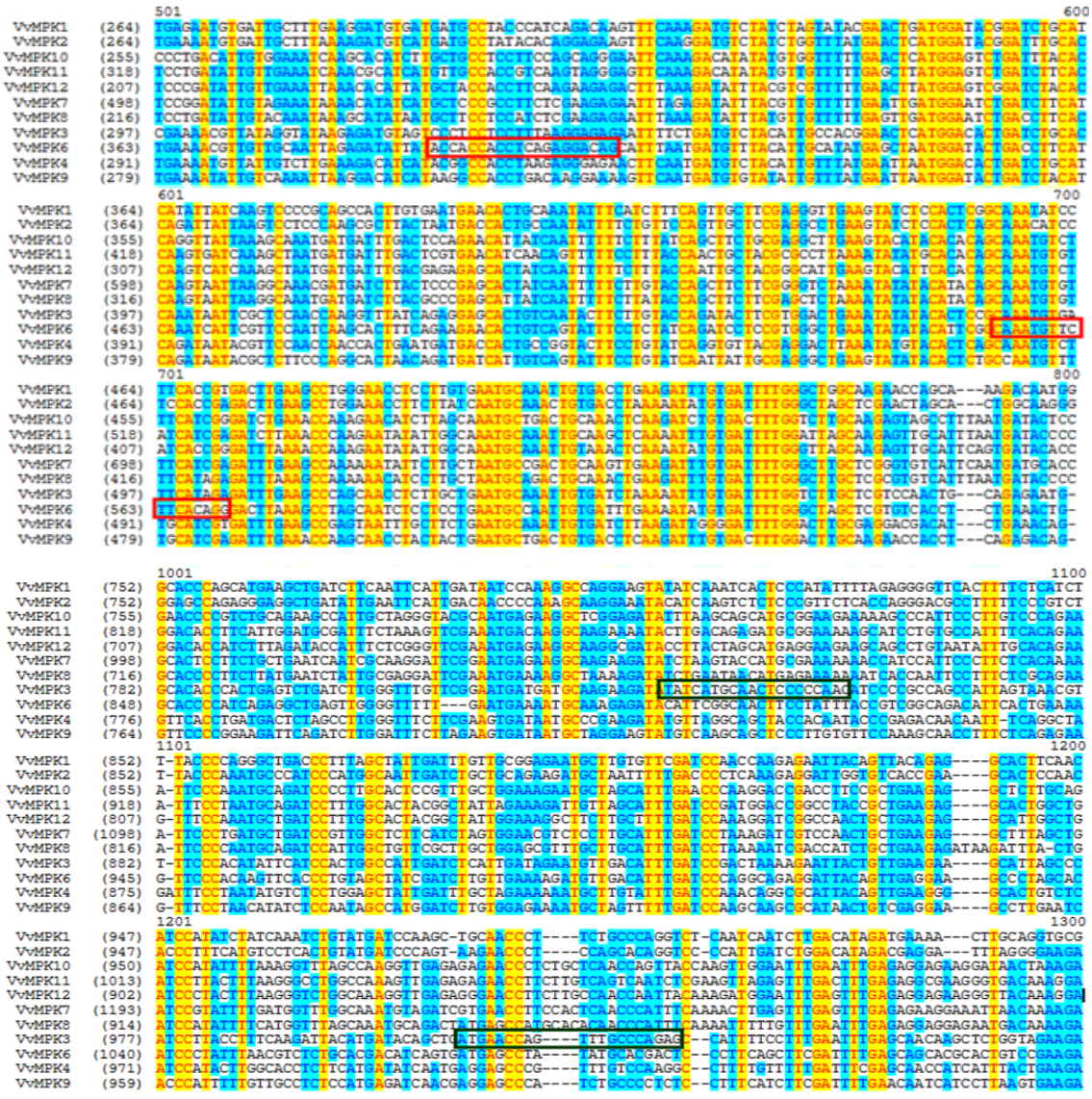

Figure 21. Part of the alignment of the 12 VvMPKs sequences reported by Hyun *et al. (*2010). specific primers were designed on the VvMPK3 (green boxes) and VvMPK6 (red boxes) sequences for expression analysis

Considering *Arabidopsis* as model, the grapevine *WRKY* transcription factors homologs were identified. The *Arabidopsis WRKY33* positively regulates the plant defence after biotic and abiotic stress, in particular fungal infections. *In silico* analysis allow us to identify the 78 *AtWRKY* sequences and among them the *AtWRKY*33 predicted protein sequence (Accession Number AEC09541). A Blastp search (See Section 3.2.2) of the *AtWRKY33*-like sequences was carried out allow us to identify the grape, tobacco, rice, parsley and *Brassica* homologs.

A phylogenetic analysis of all identified sequences was performed and a cladogram was built (Figure 22). The phylogenetic tree showed a similarity among the two grape homologs, *VvWRKY26* and *VvWRKY24*, and *AtWRKY33* and the other *AtWRKY*-like sequences (Figure 22, red box).

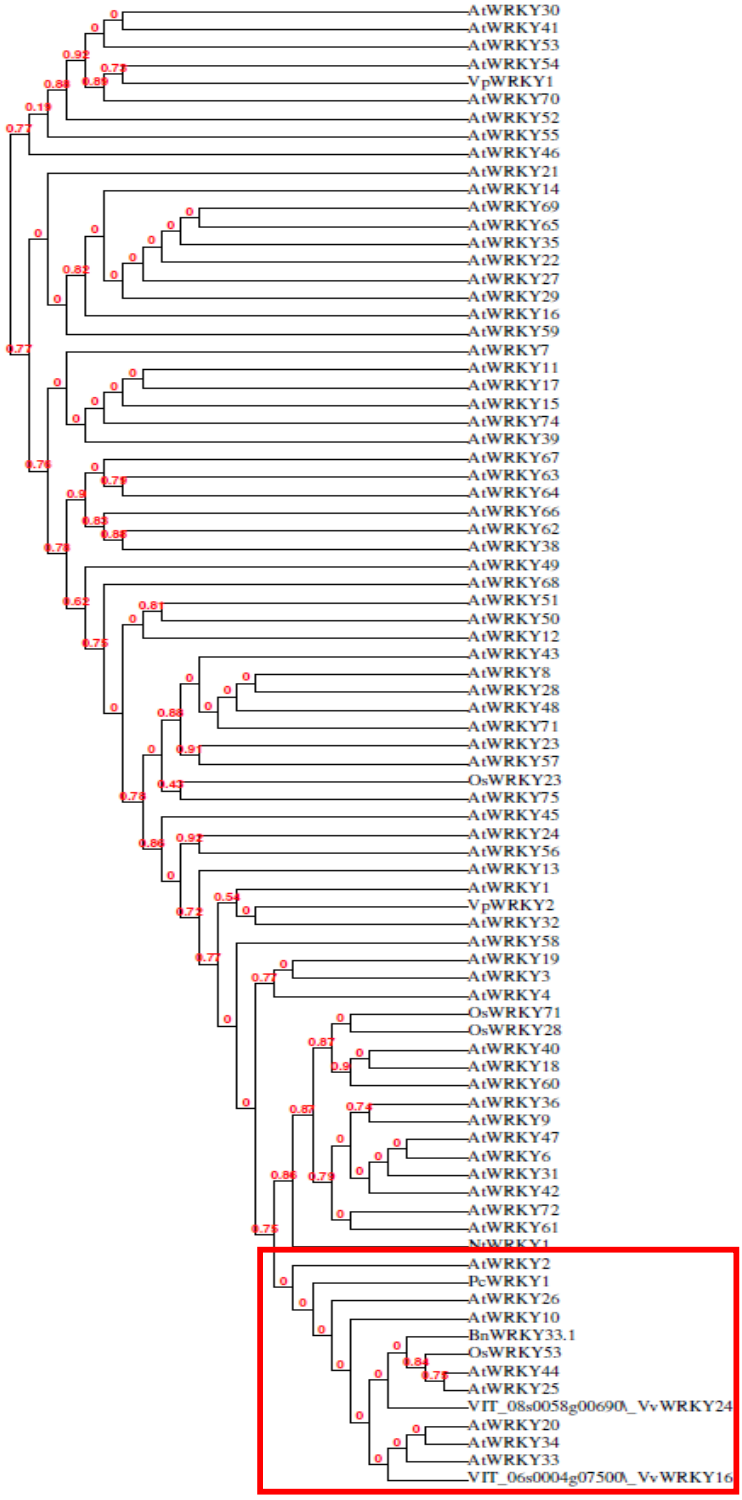


Figure 22. Phylogenetic tree of 78 *AtWRKY*s and all the *AtWRKY33* homologs identified trough *in silico* analysis.

The grape VIT_06s0004g07500 and VIT_08s0058g00690 genes were considered as candidates and renamed *VvWRKY16* and *VvWRKY*24, respectively, according to the nomenclature proposed by Wang *et al. (*2014). To investigate the possible biological roles of *VvMPKs* (*VvMPK3* and *VvMPK6*) and *VvWRKYs* (*VvWRKY16* and *VvWRKY*24) candidates, an expression analysis was conducted on Cabernet Sauvignon leaves treated with insoluble chitin as well as infected with grapevine powdery mildew (Figure 23).

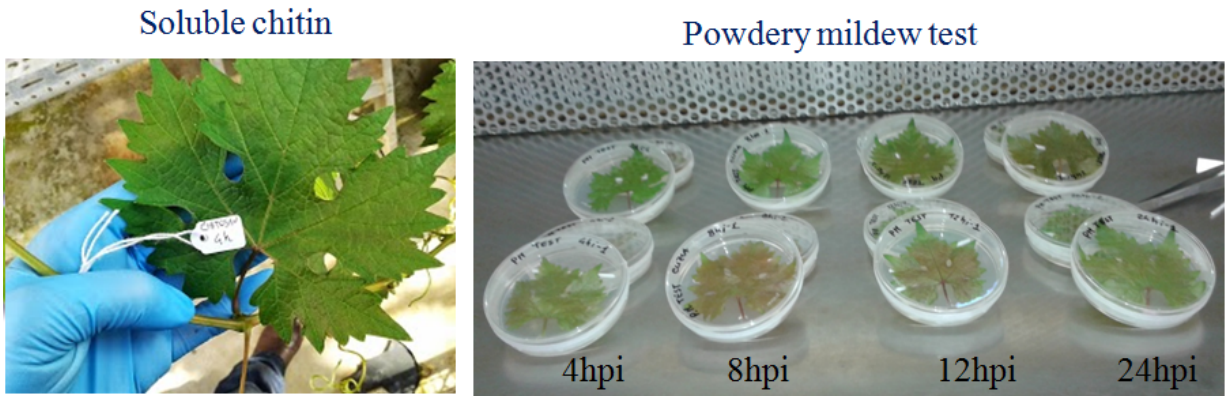


Figure 23. Cabernet Sauvignon leaves were sprayed with insoluble chitin (left) and infected with powdery mildew (right) following specific time courses.

*VvMPK3, VvMPK6*, *VvWRKY*16 and *VvWRKY*24 gene expression was evaluated both on powdery mildew and chitin time course (Figure 24). The relative expression values were reported in fold change. The time courses were referred to in minutes after spray (m.a.s.) for chitin and hours post infection (h.p.i.) for powdery mildew. The results showed a changeless expression of *VvMPK3, VvMPK6*, and *VvWRKY*16 in both experiments, with values ranging between 0.67 (*VvMPK3* at 60 m.a.s.) and 5.04 (*VvWRKY33* at 15 m.a.s.) in chitin treatment and between 0.74 (*VvMPK6* at 24 h.p.i.) and 1.62 (*VvMPK3* at 24 h.p.i.) in powdery mildew infection. The highest expression was obtained with *VvWRKY24*, which showed 28.20 (30 m.a.s.) and 6.87 (15 h.p.i.) expression values in chitin spray and powdery mildew infection, respectively.

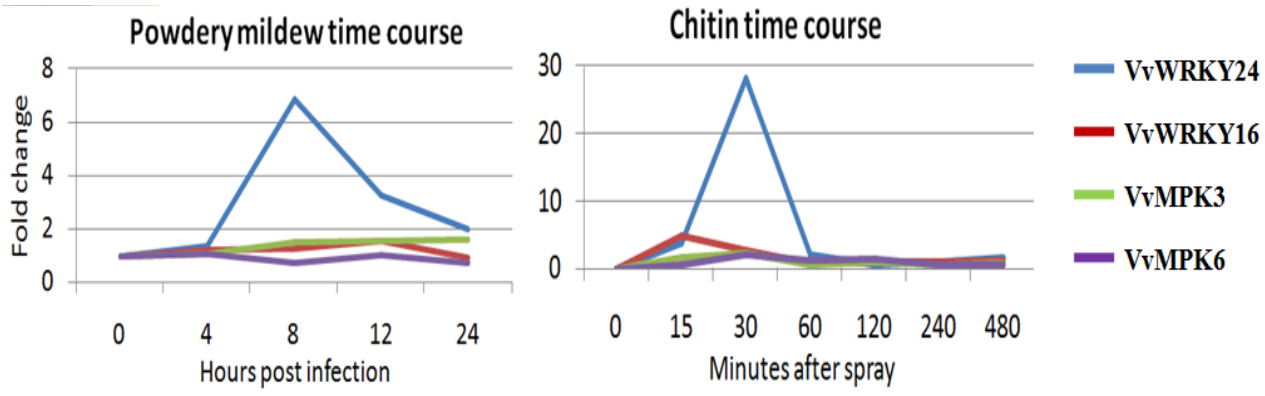


Figure 24. Expression analysis of four candidate genes in powdery mildew and chitin time courses.

As shown in Figure 25, the *VvWRKY24* protein localization in onion and grape cells, after co-expression with the green fluorescent protein, was restricted to the nucleus . This is consistent with its role as transcription factor.

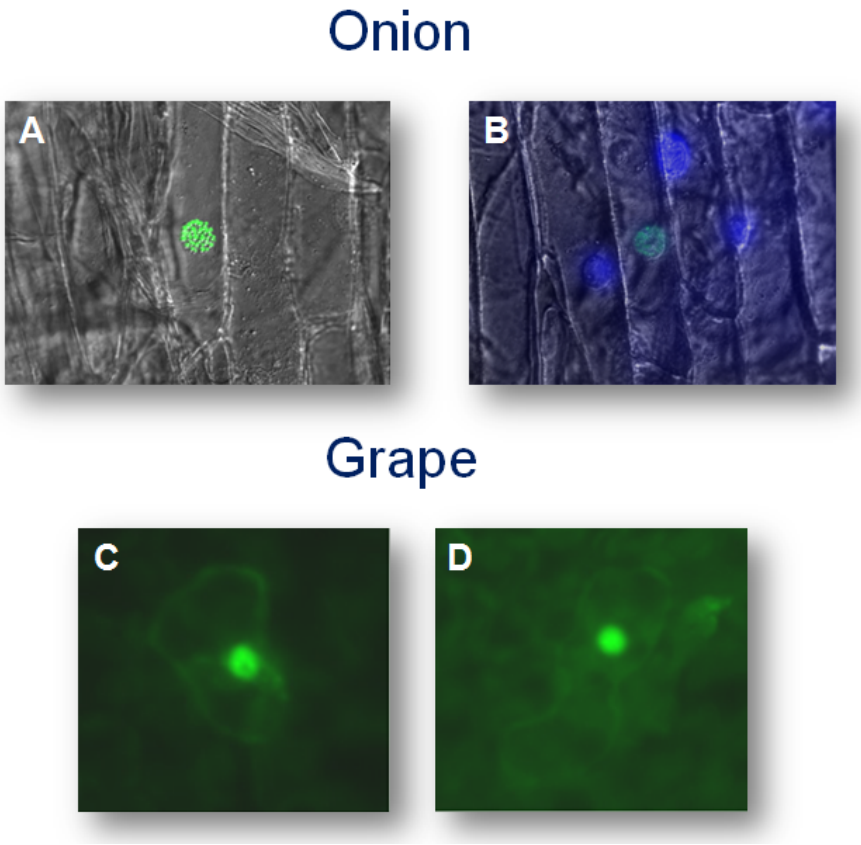


Figure 25. Biolistic transformation of *VvWRKY*24 in frame with GFP. Each image is captured with different filters and in different tissues: (A) onion with GFP, (B) onion with GFP and DAPI, (C) and (D) grape with GFP.

### 3.3.3 Grapevine genetic transformation

A grapevine *Agrobacterium* -mediated transformation was begun to a complete functional characterization of the candidate genes identified. Towards this goal, both an over-expression and knock-out vectors were built with the *VvWRKY*24 candidate. Embryogenic *calli* were initiated from anther filaments. The transformation was started using the construct pART27uGFP-*VvWRKY*24 for over-expression in the *Agrobacterium* strain EHA105. The embryogenic callus derived from Shiraz. The first positive embryos obtained are reported in Figure 26, in which the GFP fluorescence indicates that their embryos are positive.

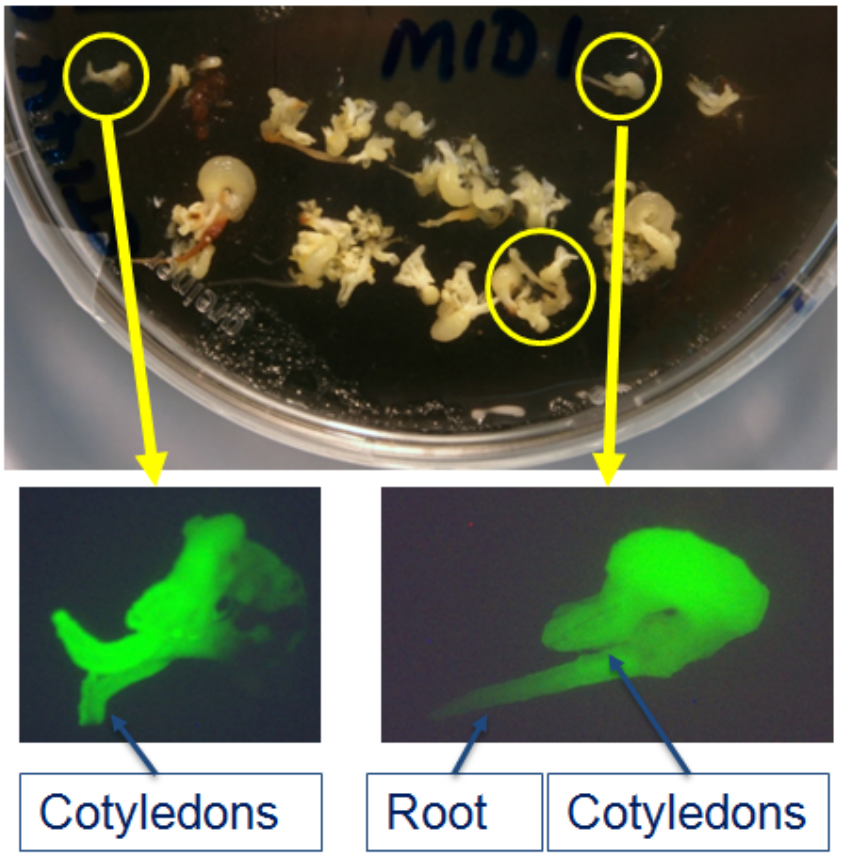


Figure 26. GFP positive embryos together obtained from over-expression of pART27uGFP-VvWRKY24 vector in Shiraz calli. Cotyledons an roots of the embryos are underlined.

## 3.4 Discussion

According to the central dogma of plant pathology proposed by Bent and Macket (2007), the first inducible responses during a plant-microbe interaction are a consequence of the perception of chemical elicitors, microbe-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs). PAMPS are a subgroup of MAMPs (Maffei *et al.,* 2012). All of these molecules, which could universally be described as "patterns that elicit immunity" (PEIs), are often recognized by trans-membrane pattern recognition receptors (PRRs) in plant cells (Maffei *et al.,* 2012; Newman *et al.,* 2013). A PAMP defines an evolutionarily conserved structural unit present only in a pathogen species. In particular, chitin is a major component of fungal cell walls and serves for the detection of various potential pathogens in innate immune systems of both plants and animals. The losses of crop plants from fungal attacks is a serious problem worldwide and fungicides might successfully control fungal diseases. However, fungicide application have a environmental cost. Nowadays little is known about the PTI response to fungal pathogen in grapevine and therefore is deemed important to use the available genetic resources and genomic data to explore the grape immunity and characterize the involved genes and proteins. In this scenario, the functional genomics and the progressive biotechnologies represent powerful tools to establish protective strategies. The main goal of this research was the molecular investigation of the mechanisms by which grapevines resist infection by the non-adapted powdery mildew through the characterization of the grape homologous genes involved in the immune response. The approach used consist of a classification of the defense response in two parts: the perception of chitin in the epidermal cells apoplast and the intracellular signal transduction. Using *Arabidopsis* as model, the lysin motif (LysM)-containing proteins were chosen as chitin receptor, and the MAPK and WRKY gene families as key-genes in the signal transduction. The LysM domain was initially identified in bacterial enzymes involved in binding and degrading the bacterial cell wall component peptidoglycan (Joris *et al.,* 1992; Steen *et al.,* 2003; Buist *et al.,* 2008), which is structurally similar to chitin. In rice (*Oryza sativa*), CEBiP (for chitin elicitor-binding protein) was shown to be important in the activation of plant innate immunity upon chitin addition (Kaku *et al.,* 2006). This protein has an extracellular domain containing two LysMs and a single transmembrane domain. The analysis of mutants identified AtLYK1/CERK1 (for LysM-containing receptor-like kinase1/chitin elicitor receptor kinase1) as the primary PRR for chitin recognition in *Arabidopsis* (Miya *et al.,* 2007; Wan *et al.,* 2008; Shimizu *et al.,* 2010) among five characterized AtLYKs: LYK1/CERK1 and LYK2 to LYK5 (Zhang *et al.,*

2007; Wan *et al.,* 2008a). The grapevine genome encodes twelve LysM domain (Zhang *et al.,* 2009) and three of them (i.e. *VvLYK*1, -2, and -3) contain proteins, similar to *AtLYK*1. In this research, a functional analysis of each grape candidate was carried out transforming the *A. thaliana cerk1* mutant. Our data suggested that VvLYK1, VvLYK2 and VvLYK3 can play a different role in chitin signaling in grapevine. We demonstrated that a total resistance to powdery mildew is re-established in the *A. thaliana cerk1* mutant only from *VvLYK*1, but not from *VvLYK*2 and *VvLYK*3. Further experiments also suggested an inactive *VvLYK*2 and an active but with unknown function, *VvLYK*3. Published data in *Arabidopsis* and rice showed that various LysM receptor proteins can be involved in chitin recognition and signal transduction in different manner. However, there are still many unanswered questions regarding the exact composition of the respective receptor complexes, other auxiliary proteins, and the mechanism of signaling and other components of the signaling cascade leading ultimately to enhanced disease resistance (Tanaka *et al.,* 2013). In *Arabidopsis*, is clear that some LysM receptor proteins have affinity for chitin-like molecules, but the biological/biochemical function of the other *Arabidopsis* LysM receptor proteins remains enigmatic (i.e., *AtLYK*2, *AtLYK*3, *AtLYK*5 and *AtLYP1*). Based on our results, it might be possible that *VvLYK*3 and/or *VvLYK*2 proteins bind chitin but not induce downstream signaling, for example, as a decoy receptor. Such an example has been found for plant recognition of the fungal elicitor xylanase where only one receptor (Eix2) mediates defense signaling while the other (Eix1) acts as a decoy receptor to attenuate the PAMP response (Bar *et al.,* 2010; 2011). Decoy receptors are well-known in mammals (Pan *et al.,* 1997; Mantovani *et al.,* 1996; Bergtsson *et al.,* 2002), in which a decoy receptor manipulates the signaling of a cognate one by competing for ligand binding. Such interaction results in inhibition of downstream signaling to regulate tumor proliferation and cell death. If acting as a decoy, the LysM receptor protein could attenuate the MAMP response in the extracellular matrix. Alternatively, it is still possible that *VvLYK*3 and/or *VvLYK*2 recognize different size chitin oligomers, since different oligomers could have distinct functions (Khan *et al.,* 2011; Brotman *et al.,* 2012), as shown for chitin recognition by mammalian macrophages. Further studies are required to disclose the nature of molecular functions of all *VvLYK*s receptors in grapevine innate immunity.

Concerning the intracellular signal transduction, the PTI includes the activation of mitogen-activated protein kinase (*MAPK*) and the activation of transcription factors (Ausubel, 2005). Both processes are often co-activated with other ones like accumulation of ROS, transcriptional activation of pathogenesis-related genes, synthesis of antimicrobial secondary

metabolites, cell-wall reinforcement via the oxidative cross-linking of cell-wall components and the deposition of lignins (Nurnberger *et al.,* 2004). However, most of these observations have been made in dicot plants such as *Arabidopsis*, rice and tobacco and little is known in grape. Mitogen-activated protein kinase (*MAPK*) cascades consist of kinase signaling modules that are evolutionarily conserved throughout eukaryotes (MAPK Group*,* 2002). A MAPK cascade minimally consists of three kinases: a *MAPK*, a MAPK kinase (*MAPKK*) and a MAPKK kinase (*MAPKKK*) and have important functions in regulating stress responses (Nakagami *et al.,* 2005; Pedley and Martin, 2005). In the *Arabidopsis* genome were identified 20 MAPKs, 10 MAPKKs and more than 60 MAPKKKs (MAPK Group, 2002), and other several dozens of *MAPKs* have been identified and isolated from tobacco, tomato, rice, alfalfa, maize, oat, wheat, barley, cotton, petunia and poplar as reviewed by Hyun *et al.,* 2010. Recently, Hyun *et al. (*2010) conducted a genome-wide analysis of *MAPK* genes and identified 12 putative *MAPK* genes by means of *in silico* analysis of the grapevine genome sequence. Among them *VvMAPK*3 and *VvMAPK*6 are homologs of the two *Arabidopsis MAPKs*, *AtMPK3* and *AtMPK6*, involved in chitin signaling (Wan *et al.,* 2004). Nowadays, only Wang *et al. (*2014b) provided a expression analysis of all *VvMAPK*s in different grapevine tissues and organs at different developmental stages, and in leaf tissues challenged by *E. necator*, salicylic acid, ethylene, hydrogen peroxide and drought. They identified several *VvMPKs* candidate genes which might be involved in grapevine growth and development and in biotic and abiotic responses for further functional characterization. In this research, *VvMAPK*3 and *VvMAPK*6 seemed to be not involved in plant-microbe response showing no expression differences in powdery mildew infection and chitin treatment time courses. Our data confirm the findings of Wang *et al. (*2014b), who identified *VvMAPK*1 and 10 as the most prominent induction following *E. necator* infection, being expressed two fold more than that in the control, at 48 and 12 h post-inoculation, while *VvMAPK*2, *VvMAPK*3, *VvMAPK*6 and *VvMAPK*8 showed the lowest expression during the experiment with fold-changes lower than 1. Furthermore, Wang *et al. (*2014) surveyed the grapevine MAPK gene family and found that VvMPK6 increased its expression level in response to all the stress-related treatments rather than powdery mildew infection. This suggested that in the grapevine this gene might be involved in response to abiotic stresses. Overall, our findings suggest a different specialization of the *VvMAPK*s in response to biotic and/or abiotic stress. For these *MAPKs* and their homologues in other species, the available information is still scanty. Investigations of their possible involvement in grapevine resistance to biotic or abiotic

stresses will be possible only through future functional genomics experiments of gain/loss of function in grapevine.

Chitin is perceived by LYKs receptors that initiate signaling via intracellular *MAPK* cascades, this stimulates the induction of unknown *WRKY* transcriptional activators and repressors. In grapevine, a total of 59 *VvWRKY* genes were identified (Wang *et al.,* 2014), but their specialization and regulation of biological processes is still unknown. In this research, a functional study of two candidates of this important class of transcriptional regulators have been conducted to identify the role of *VvWRKY* TFs in plant defense signaling. Considering *Arabidopsis* as model, *AtWRKY*33 have been proved to be a potential downstream target based on its gene activation (Kim and Zhang, 2004; Wan *et al.,* 2004), its high expression after treatment with PAMPs (Zheng *et al.,* 2006) or infection by pathogens (Lippok *et al.,* 2007; Qiu *et al.,* 2008). For this reason an *in silico* research of *AtWRKY*33 homologs was carried out in grapevine, tobacco, parsley and rice. We were able to identify two grape homologs, *VvWRKY16* and *VvWRKY24*. The transcriptomic databases generated in previous studies of grapevine subjected to biotic and abiotic stresses, together with our qRT-PCR analysis, allowed us to identify *VvWRKY*24 gene involved in biotic stress response, but exclude *VvWRKY*16. Indeed, the *VvWRKY*24 had the strongest up-regulation in response to powdery mildew infection and chitin treatment on Cabernet Sauvignon. The effect of *E. necator* infection on *VvWRKY*s response appeared to be much stronger in a susceptible variety than in a resistant one, because the overall changes in the global transcriptome are generally lower in the resistant genotypes (Wang *et al.,* 2014). The up regulation of *VvWRKY*24 have been reported by Fung *et al. (*2008), Wang *et al. (*2014) and Guo *et al. (*2013) as a powdery mildew specific response. Indeed, Guo *et al. (*2013) reports that the change in expression profile of *VvWRKY*24 is not significant in various biotic treatments.

The possible interactions between two or more genes and regulatory mechanisms remain to be resolved, for this reason a over-expression and silencing transformation in Shiraz was begun. Because classical genetic improvement by convetional breeding is a very long process for grapevine, alternative strategies such as genetic engineering are being investigated to improve several traits, such as resistance (Coutos-Thévenot *et al.,* 2001, Maghuly 2006, Spielmann *et al.,* 2000, Yamamoto *et al.,* 2000). However, V. vinifera is recalcitrant to genetic transformation. The first report proving this recalcitrance was given by Mullins *et al.* (1990). The authors used petiole explants of Cabernet Sauvignon and Chardonnay and were unable to obtain transformants. Probably the reason why have to be correlated to the inefficient selection techniques rather than the *Agrobacterium* infectivity or gene integration. Further

studies highlighted that this grapevine inaptitude to *Agrobacterium*-mediated transformation is attributed to the necrogenesis response of its tissues after bacteria inoculation. This hypersensitivity, a stress elicited response where infected cells are killed at the site of inoculation, was assumed to be the reason for avirulence of the wide host range *Agrobacterium* to grapevine (Pu and Goodman, 1992, 1993). Later on, Perl *et al. (*1996) working with *V. vinifera* cv. Superior Seedless observed that necrosis was not induced during or after the co-cultivation process but observed 48 hours after transfer of calli to bacterial free medium with antibiotics. They found that oxidation caused due to elevated peroxidase levels was the likely cause of necrosis as peroxidase activity correlated with the onset of browning. Since the first experiment of grapevine genetic transformation, many achievements have been made to set up the best conditions. Recently, Torregrosa *et al,* (2015) described a grapevine transformation system that meets three peculiar criteria: (1) the production of highly regenerative transformable tissue, (2) optimal co-cultivation conditions for both grapevine tissue and *Agrobacterium*, and (3) an efficient selection regime for transgenic plant regeneration. Nowadays, few paper reports the stable genetic transformation of transcription factors, among them Marchivè *et al.* (2013) successfully overexpressed *VvWRKY*1 in grapevine cell culture inducing a global transcriptional reprogramming which clearly enhanced resistance to downy mildew. Following the Torregrosa et al. (2015) advices, an efficient transformation system have been performed in this research using Shiraz calli as explants. However, the grape recalcitrance induced an elongation of the expected transformation time from few months to nearly one year. Indeed, after approximatly eight months few positive embryos have been detected in overexpression transformation. This plant material represent the starting-point for future functional studies on *VvWRKY24* role in chitin perception and/or grapevine defense activation after fungal attack. On the counterpart, the knock-out genetic transformation, did not give any positive embryos. These results is probably correlated with the silencing of a transcription factor, which is usually involved in more than one biological pathway. Sometimes, the overexpression or silencing of regulation factor can be lethal.

## 3.5 Conclusion

In conclusion, a complete structural ad functional study have been conducted in grapevine to identify the genes involved in chitin perception and signal transduction. Using *Arabidopsis* as model species, we identified and functionally studied the candidate grape genes. In particular, *Atcerk1* showed three homologous in grape, *VvLYK1*, *VvLYK2* and *VvLYK3* and our findings suggested a different role in chitin signaling of these candidates. Indeed, we demonstrated that a total resistance to powdery mildew was re-established in the *A. thaliana cerk1* mutant only by *VvLYK*1. Further experiments also suggested an inactivation of *VvLYK*2 and an active but unknown function of *VvLYK*3. Based on the results obtained here, we can speculate that additional auxiliary proteins can be involved in this process and that the *VvLYK* candidates are pathogen-specific, as *VvLYK1* for powdery mildew. Alternatively, it is still possible that *VvLYK*3 and/or *VvLYK*2 recognize different size chitin oligomers, since it is possible that different oligomers could have distinct functions.

The plant basal immunity includes the activation of mitogen-activated protein kinase (MAPK) and transcription factors (WRKY). In this research, *VvMAPK*3 and *VvMAPK*6 did not seem to be involved in biotic response showing no expression differences in powdery mildew infection and chitin treatment time courses. The expression data of two grape WRKY candidates, *VvWRKY16* and *VvWRKY24,* suggested *VvWRKY*24 as the positive regulation factor of grape defense to powdery mildew, but excluded *VvWRKY*16. This is the first comprehensive experimental survey of the grapevine PTI response, which provides insights into *VvLYK*, *VvMAPK* and *VvWRKY* potential roles in plant defense activation.

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# General conclusions

The common grapevine, *V. vinifera* subsp. *sativa* L. (2n=6x=38) is one of the major fruit crops worldwide in terms of economic value and cultivated area. Italy, together with France, is one of the most important producers with more than 450 varieties registered. In the panorama of Italian viticulture and oenology, Campania region is characterized by a rich biodiversity, which collect a distinctive number of varieties including very ancient ones (more than one hundred years old). This peculiar condition is the consequence of historical, social, geographical and cultural elements, such as the region orography, the fragmentation of land tenures, the volcanic origin of the soil and the traditional cutting asexual propagation coupled with promiscuously planted grapevines in a single vineyard. The main variety is Aglianico, the undisputed star of Southern Italy red grapes and the grape behind the region's two most famous and respected red wines: Taurasi and Aglianico del Taburno. Until recently, this incredible wealth of native varieties has hardly been studied; unbelievable as it may seem, as recently as in 200, only ten varieties from Campania were listed in Italy's National Registry. In the last few years grape, as several other crops, enjoyed the use of recent genomics tools and resources. They represent now essential ingredients not only for germplasm valorization, but also for the identification of genes and QTL of interests, for understanding the genetics of complex traits, and for the development of efficient molecular markers . In addition, the genome sequence of *V. vinifera* was published in 2007, rushing in new era of grape functional and comparative genomics. In this environmental and scientific context the research activities described in the previous chapters have been carried out. One major objective was pursued in the frame of the project SALVE (Safeguarding of the plant biodiversity of Campania) funded by Campania region. It was related to Campania grape germplasm genetic authentication using microsatellite and retrotransposon-based markers in a large collection of 62 grapevines, mostly native. Data suggested that a genetic variability is still present in the germplasm collection studied and the restricted number of synonymous and homonymous found confirm the uniqueness of Campania genotypes. In our effort, we were able to identify 19 grape-specific alleles, that represent useful tools for many purposes such as traceability in germplasm, typicity preservation and varietal identification. Even if specific sequences have been identified, these molecular data cover only part of the grapevine genome, meaning that even when all the DNA sequences we analyzed look totally identical, it might turn out that genetic differences are present in other part of the genome and they might be more widespread than we believe. Indeed, the accessions sharing the same SSR/REMAP

profile must be evaluated further before being eliminated from standard grapevine collections, because they might not be redundant at all. Our molecular findings represent a peculiar starting point for whole-genome profiling of varieties native of Campania region. Within the collection analyzed, Aglianico del Taburno is a model for astringency and the strong resveratrol content confer high positive nutriceutical properties to their grapes and wines. For this reason, a transcriptomic analysis of key genes of the phenyl-propanoid pathway were carried out in different tissues of the berry during the fruit maturation. This study was correlated with the chemical analysis of total phenols, flavans, anthocyanins and tannins detected in skin and berry using a spectrophotometric assay. Anthocyanin analysis revealed the highest amount of these molecules in ripening berry skin and the lowest in seed. Correlating the transcriptomic and chemical data, the key-genes able to control production of poly-phenolic compounds in berry tissues have been determined. In order to investigate the relation between defense compounds, as polyphenols, and abiotic stresses, an expression analysis have been carried out on Aglianico del Taburno leaves stressed with wounding. The results provided evidence that an increase of antioxidant compounds occurs after wounding stress, and that phenyl-propanoid genes expression might be regulated from a transcription factor, *VvMyB14;* during the whole treatment.

Poly-phenolic compounds are key-molecules of grapevine defence and in particular of PTI (PAMP-Triggered Immunity). Indeed, they are produced as defense compounds after pathogen recognition. Nowadays, much has been written about defense mechanisms and chitin perception in *Arabidopsis*, but little is known in grapevine. Considering powdey mildew as the most destructive disease of grapevines world-wide, an intense structural and functional study of PTI-involved grape genes has been conducted. Grapevines are highly resistant to many the powdery mildew of other species, called non-adapted (e.g *E. cichoracearum*). Using *Arabidopsis* as model species, we identified and functionally studied the grape genes involved in the perception of chitin in the epidermal cells apoplast, as LysM-RLK, and in the intracellular signal transduction. Our findings suggest that three candidates, *VvLYK1*, *VvLYK2* and *VvLYK3,* can play a different role in chitin signaling in *V. vinifera*. We demonstrated that a total resistance to powdery mildew was re-established in the *A. thaliana cerk1* mutant only by *VvLYK*1, but not by *VvLYK*2 and *VvLYK*3. Further experiments also suggested an inactive *VvLYK*2 gene and an active but unknown function of *VvLYK*3. There are still many unanswered questions regarding the exact composition of the receptor complexes activated in the epidermal cells after powdery attack. Based on the results obtained here, we can speculate that additional auxiliary proteins can be involved in this process and

that the *VvLYK* candidates are pathogen-specific, as *VvLYK1* for powdery mildew. Alternatively, it is still possible that *VvLYK*3 and/or *VvLYK*2 recognize different size chitin oligomers, since it is possible that different oligomers could have distinct functions.

Regarding the signal transduction pathways, we found *VvMAPK*3 and *VvMAPK*6 to be not involved in biotic response, since they both did not show expression differences in powdery mildew infection and chitin treatment time courses. The transcriptomic data generated in this study allowed us to identify *VvWRKY*24 as the positive regulation factor of grape defense to powdery mildew, but excluded *VvWRKY*16. Indeed, the *VvWRKY*24 had the strongest up-regulation in response to powdery mildew infection and chitin treatment. To better understand the possible interactions between two or more genes and regulatory mechanisms, an over-expression and silencing transformation in Shiraz have been begun. The transformations carried out gave rise to few positive embryos after ten months from co-cultivation with *Agrobacterium* . This result is strictly correlated with the known recalcitrance of grapevine to transformation. Further studies will allow progress to be made on this point, such as the investigation of positive transformed embryos behavior after powdery mildew infection or chitin treatment. As far as we know, this represents the first complete structural and functional genomics study in grapevine aimed at identifying the major genes involved in chitin perception and signal transduction.