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CORSO DI DOTTORATO DI RICERCA IN SCIENZE DELLE PRODUZIONI VEGETALI

CICLO: XXIX

Molecular studies for disclosing the genetic identity of local cultivars in olive and understanding the stilbene synthase pathway in grapevine

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This thesis work is dedicated to
my parents, all my siblings, my dearest wife,
my lovely daughter Razan and my son Omar.

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

January 31st, 2017 Ibrahim Samir Ali Farag Hmmam

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Riassunto

L'olivo e la vite sono l'oggetto della dissertazione, condotta da un punto di vista genetico e genomico, illustrata nel presente lavoro. L'olivo (*Olea europaea* L.) è uno degli alberi da frutto più antichi dell'intera area Mediterranea. Nonostante la crescente importanza economica di questa specie e la sempre più ampia diffusione di oli extra-vergine di oliva monovarietali, pochi sono gli studi riguardanti la caratterizzazione genetica degli alberi di olivo tipici della regione Veneto. Poiché la composizione e le proprietà sensoriali degli oli extra-vergine di oliva sono influenzate principalmente dal genotipo, la caratterizzazione di cultivar autoctone di olivo rappresenta una fase cruciale per la produzione, la commercializzazione e quindi l'autenticazione di oli d'oliva di alta qualità. A questo scopo, alcuni loci di marcatori microsatelliti mappati in varie regioni del genoma sono stati analizzati così da distinguere, sulla base di polimorfismi legati al DNA nucleare, diverse varietà di olivo coltivate in Veneto, dal lago di Garda fino a colli Trevigiani, passando per i colli Euganei, valutando così in modo sistematico l'intero germoplasma della regione. L'obiettivo finale del presente lavoro è stato risolvere alcuni casi omonimia e sinonimia nonché garantire l'autenticità di cultivar di olivo e dei loro cloni. Un totale di 204 accessioni prive di una chiara assegnazione varietale e 25 varietà di referenza sono state analizzate saggiando 10 marcatori microsatelliti di loci nucleari altamente polimorfici. Un'analisi della struttura genetica della popolazione è stata eseguita al fine di definire il numero di gruppi e la loro diversità genetica. Sulla base dei coefficienti di similarità genetica, una cospicua parte delle accessioni (60 campioni) si è dimostrata geneticamente identica alla varietà di referenza "Casaliva" mentre altri genotipi sono risultati geneticamente identici a varietà nazionali e locali tra cui "Miniol" (21 campioni), "Grignano" (20 campioni), "Tonda di Villa" (19 campioni), "Leccino" e "Capolga" (17 campioni ciascuna), "Gargnà", "Compostaro" e "Oblica" (9 campioni ciascuna), "Rossanel" (8 campioni), "Fort" e "Baia" (5 campioni ciascuna), "Grappolo" e "Favarol" (3 campioni ciascuna), con supporto statistico di bootstrap superiore all'85%. Soltanto sei campioni, due per ciascuna cultivar, si sono rivelati attribuibili a "Moraiolo", "Carnica" and "Maurino". L'analisi della struttura genetica della popolazione analizzata ha evidenziato di essere costituita da 15 sottogruppi. Merita sottolineare che più del 98% delle accessioni ha mostrato una netta appartenenza ad uno di questi sottogruppi e solo 13 accessioni hanno rivelato una discendenza mista, con valori di

appartenenza ad uno dei sottogruppi inferiore al 70%. La quota totale di eterozigosi osservata è risultato pari a Ho = 91,6% (st. dev. 1,9%) mentre l'eterozigosi media attesa è risultata pari a He = 74,1% (st. dev. 2,9%). L'indice di diversità genetica di Nei è risultato pari a uHe=74,3% mentre l'indice di Shannon sulla diversità fenotipica dei profili molecolari è risultato pari a I=1.612. Tutti i coefficienti di inbreeding di Wright si sono rivelati negativi e, in media, uguali a -0,863 (Fis) e -0,159 (Fit). Nel complesso i marcatori microsatelliti impiegati in questo studio si sono dimostrati estremamente efficienti nella risoluzione dei casi di omonimia, sinonimia ed errata identificazione consentendo così la prima vera analisi sistematica del germoplasma di olivo della regione Veneto finalizzata alla valorizzazione delle varietà locali più adatte alla produzione di oli di oliva monovarietali.

Il secondo capitolo del presente lavoro si è focalizzato sulla pianta della vite ed in particolare sugli stilbeni, un gruppo di composti secondari appartenenti alla famiglia dei fenilpropanoidi accumulati in un numero limitato di piante (72) tra cui vite. Generalmente questa classe di fenilpropanoidi è accumulata nelle foglie, nelle radici e nei frutti in risposta a stress abiotici e biotici quali ferita, esposizione a raggi UV-C, infezione da patogeni e trattamenti chimici. Negli ultimi decenni l'interesse verso gli stilbeni è andato progressivamente aumentando, non solo in virtù del ruolo fondamentale che assumono nella difesa della pianta da stress ambientali, ma anche per le loro proprietà farmacologiche. La via metabolica dedita alla produzione di questi composti rappresenta una diramazione della pathway biosintetica che porta alla produzione dei fenilpropanoidi e può essere considerata un'estensione della via metabolica che flavonoidi. La stilbene sintasi è l'enzima chiave nella produzione di resveratrolo, composto alla base della biosintesi degli stilbeni complessi. Recentemente abbiamo identificato e caratterizzato funzionalmente due fattori di trascrizione di tipo R2R3-MYB che, in vite, sono direttamente coinvolti nella regolazione della via metabolica degli stilbeni. Questi fattori di trascrizione, denominati VvMYB14 e VvMYB15, co-esprimono con le *VvSTS* in diverse condizioni di stress (stress meccanico, radiazioni UV-C, attacco fungino) e in alcune fasi di sviluppo del seme e della bacca. In saggi reporter, MYB14 e MYB15 hanno mostrato un coinvolgimento diretto nell'attivazione dei promotori dei geni *VvSTS* e l'espressione ectopica di VvMYB15 in hairy root ha rivelato un incremento nell'espressione dei geni *VvSTS* nonché l'accumulo di stilbeni glicosilati (piceidi) *in planta*. Tuttavia, nonostante i risultati incoraggianti, diverse sono le questioni rimaste insolute in

relazione a questa via biosintetica. Partendo dal presupposto che geni coinvolti in processi affini o strettamente correlati possono esibire, in condizioni sperimentali controllate, profili di espressione simili, alcune analisi di co-espressione, eseguite su alcuni dataset di espressione in vite, hanno permesso di identificare fattori di trascrizione candidati, appartenenti alla famiglia WRKY, che potrebbero essere coinvolti nella regolazione della via biosintetica degli stilbeni. Questi geni hanno mostrato valori di correlazione, in termini di co-espressione, persino più alti di quelli osservati per VvMYB14 e VvMYB15 e si sono rivelati marcatamente indotti a seguito degli stessi stress che portano all'induzione di *VvSTS* e dei MYB14/15. Le analisi di correlazione su database pubblici, la validazione della co-espressione dei WRKY candidati e VvSTSs in diverse cinetiche di stress e la caratterizzazione funzionale tramite saggio luciferasi in colture cellulari di vite hanno permesso di aggiungere nuovi tasselli ai meccanismi regolatori che stanno alla base della pathway biosintetica che porta alla sintesi degli stilbeni.

Summary

This study has been focused on two main fruit trees, olive and grapevine, from the genetic and genomic point of view. Olive (*Olea europaea* L.) is one of the oldest tree crops of the Mediterranean regions. In spite of the increasing economic importance and wider diffusion of monovarietal virgin olive oils, very few studies have focused on the genetic characterization of olive cultivars locally grown in the Veneto region, North-Eastern Italy. Since the genotype mainly affects the composition and sensory properties of extra virgin olive oils, the characterization of autochthonous olive cultivars is a key step for the production, marketing and traceability of high quality olive oils. Here we have performed a survey of mapped SSR marker loci for the analysis of nuclear DNA polymorphisms to distinguish olive cultivars and cultivar groups within the entire olive cultivation area in Veneto, from the Garda Lake to the Euganean and Trevisan hills, in order to obtain a systematic examination of the Veneto regional olive germplasm patrimony. The final aim of the study was to resolve cases of homonymy and synonymy, and to warrant the genetic authenticity of olive cultivars and of their clones in case of mislabelling. A total of 204 unknown olive tree accessions along with 25 olive reference cultivars were subjected to molecular analyses using ten highly polymorphic nuclear SSR markers. The overall molecular data were used for the computation of genetic similarity and diversity statistics, for the construction of ordination dendrograms and the determination of centroids of local accessions, along with reference cultivars. A genetic structure analysis of the olive population as a whole was also performed for the definition of cultivar groups. Based on multi-locus SSR genotypes and pair-wise SM coefficients, we found that on one hand most of the olive samples displayed genetic identity with the reference cultivars "Casaliva" (60 samples) while several others showed identity with different national and local varieties: "Miniol" (21 samples), "Grignano" (20 samples), "Tonda di Villa" (19 samples), "Leccino" and "Capolga" (17 samples each), "Gargnà", "Compostaro" and "Oblica" (9 samples each), "Rossanel" (8 samples), "Fort" and "Baia" (5 samples each), "Grappolo" and "Favarol" (3 samples each) having bootstrap values greater than 85%. As few as 2 samples were attributable to "Moraiolo", "Carnica" and "Maurino" each. The uppermost hierarchical level of population structure suggested a clear maximum of cultivar groups for K=15. It is worth noting that more than 98% of individual trees revealed a pure ancestry and only 13 accessions were admixed

showing a membership to the associated cultivar group <70%. The total value of observed heterozygosity was equal to Ho=91.6% (st. dev. 1.9%). and the mean expected heterozygosity was He=74.1% (st. dev. 2.9%). Moreover, the Nei's unbiased genetic diversity was as equal to uHe=74.3%, whereas the total estimate of the Shannon's information index of phenotypic diversity was equal to I=1.612. The fixation index, equal to Fst=0.247, revealed that only less than 25% of the genetic variation was found among cultivar groups and that approximately as much as 75% of the total polymorphism was scored among trees within cultivar groups. The narrow genetic differentiation among subgroups over the investigated multi-locus marker loci was confirmed by the gene flow estimate that was as low as Nm=0.412. It is worth mentioning that all Wright's inbreeding coefficients were negative and on average equal to Fis=–0.863 and Fit=–0.159, indicating a significant excess of heterozygosity for the olive accessions of the Veneto germplasm. Overall, the SSR markers used in this study have shown their efficacy to distinguish cases of homonymy, synonymy and mislabelling, providing the first systematic analysis of olive germplasm for horticultural valorisation of Veneto cultivars in the regional areas most suited for typical monovarietal virgin olive oil productions.

For what concerns grapevine, plant stilbenes are a small group of phenylpropanoid (PP) compounds that have been detected in a limited number of unrelated plant species of at least 72 including grapevine. Plants accumulate it in leaves, roots and fruits in response to both biotic and abiotic stresses such as mechanical wounding, UV-C exposure, pathogen infection, and chemicals treatment. In the last decade stilbenes had raised the interest of many scientists not only because of their role in the plant protection against environmental stresses, but also because of their pharmacological properties. The biosynthetic pathway leading to their production is a side branch of the general PP biosynthetic pathway and can be considered as an extension of the flavonoid pathway. Stilbene synthase (STS) is the key enzyme leading to the biosynthesis of resveratrol which is the basic unit of the biosynthesis of plant stilbenes. Recently we reported the identification and functional characterization of two grapevine R2R3-MYB transcription factors (TFs), which appear to regulate the stilbene biosynthetic pathway. These TFs, designated VvMYB14 and VvMYB15, strongly co-express with VvSTS genes under a range of stress-induced conditions and in developmentally regulated tissues, including leaves exposed to wounding treatment, UV-C irradiation and downy

mildew infection, and several stages of seed and berry development. In transient gene reporter assays, these VvMYB TFs were demonstrated to specifically activate the promoters of VvSTS genes, and the ectopic expression of VvMYB15 in grapevine hairy roots resulted in increased VvSTSs expression and in the accumulation of glycosylated stilbenes in planta. However, despite these significant advances, many question remain to be answered regarding this grapevine biosynthetic pathway. Based on the notion that genes involved in similar or related processes may exhibit similar expression patterns over a range of experimental conditions, we performed a co-expression network analysis on different grapevine gene expression datasets identifying candidate TFs belonging to the WRKY family possibly involved in the regulation of the stilbene biosynthetic pathway. These genes show co-expression correlation values even higher than those observed for VvMYB14/15 and are strongly induced in response to the same abiotic stress treatments which lead to VvSTS induction. The expression of candidate WRKY TFs, namely WRKY03, WRKY43 and WRKY53 together with the expression of the two MYBs already characterized (MYB14 and MYB15) and VvSTSs genes was monitored in different stress conditions, including wounding and UV-C treatment, in order to confirm data obtained from co-expression analyses in whole transcriptome datasets. Results confirmed that there is a strong correlation between the expression of candidate WRKY genes and VvSTSs suggesting a role for these TFs in the regulation of the stilbene pathway. In order to confirm this hypothesis, we performed several functional studies. Amongst them, of particular interest are those results obtained from dual reporter luciferase assays. These assays were aimed at measuring the activity of the WRKY TFs on the VvSTS promoters by mean of transfection experiments in grapevine liquid cell cultures. Interestingly our results indicate a role for VvWRKY43 and WRKY53 in the regulation of VvSTSs. Nevertheless, these TFs seem to act exclusively in combination with MYB14 and MYB15, since, whenever co-transfected alone, they did not show any significant effect on the VvSTS promoter. Differing from WRKY43 and WRKY53, WRKY03 did not show any effect on the VvSTS promoter activity neither alone nor in combination with R2R3-MYB TFs, but was demonstrated to induce the promoter of VvMYB14. Then, our results indicate that WRKY03 probably act upstream R2R3-MYB factors in the regulation of the grapevine STS pathway whereas WRKY43 and WRKY53 have a role as co-factors with VvMYB14 and VvMYB15 TFs.

Abbreviations

°C	degrees Celcius	L/ml/µl	litre/millilitre/microlitre
4CL	4-coumarate CoA ligase	LB	Luria-Bertani Broth
aa	amino acid	min	minute(s)
ABA	abscisic acid	mRNA	messenger RNA
AFLP	Amplified Fragment Length Polymorphism	MUSCLE	MUltiple Sequence Comparison by Log- Expectation
AI	aliphatic index	MW	molecular weight
BC	before Christ	NCBI	National Center for Biotechnology Information
bHLH	basic helix-loop-helix	NJ	Neighbor Joining
bp	base pair	N_m	Gene flow
C4H	cinnamate 4-hydroxylase	OD	optical density
CaMV	Cauliflower Mosaic Virus	ORF	open reading frame
cDNA	complementary DNA	p	promoter
CHS(s)	chalcone synthase(s)	PAL	phenylalanine ammonia-lyase
COS	Centre for Organismal Studies	PCoA	Principal Coordinate Analyses
CRIBI	Centro di Ricerca Interdipartimentale Per Ie Biotrcnologie Innovative	PCR	Polymerase chain reaction
CSIRO	Commonwealth Scientific and Industrial Research Organisation	pI	isoelectric point
CTAB	cetyl trimethylammonium bromide	PIC	polymorphic information content
DBD	DNA binding domain	PP	phenylpropanoid
dH_2O	Distilled water	RAPD	Random Amplified Polymorphic DNA
DNA	deoxyribonucleic acid	RNA	ribonucleic acid
dNTP	dinucleotide triphosphate	RNS	reactive nitrogen species
E. coli	*Escherichia coli*	ROS	reactive oxygen species
EDTA	ethylenediaminetetraacetic acid	rpm	revolutions per minute
EF	elongation factor	RT-PCR	real-time-polymerase chain reaction
EU	European Union	s	second(s)
EVOO	extra virgin olive oil	SE	standard error
FAO	Food and Agriculture Organization	SNP(s)	single nucleotide polymorphism (s)
FC	fold change	SSR	Simple Sequence Repeat
F_{st}	fixation index	st. dev.	standard deviation
g	relative centrifugal force	STS(s)	Stilbene synthase(s)
g; mg; µg	gram(s); milligram(s); microgram(s)	TAE	Tris Acetate EDTA
GA	gibberellic acid	TAIR	The Arabidopsis Information Resource
GCN(s)	gene co-expression network(s)	TAL	tyrosine ammonia lyase
GRAVY	grand average of hydropathicity	TE	tris-EDTA
h	hour	TF(s)	transcription factor(s)
H_e	expected heterozygosity	TFDB	transcription factor database
H_o	observed heterozygosity	Tm	temperature of DNA dissociation (melt)
IASMA	Istituto Agrario di S. Michele all'Adige	UPGMA	unweighted pair group method
IBBR	Institute of Biosciences and Bioresources	UV	ultraviolet light
II	instability index	V	volt
IOOC	International Olive Oil Council	v	volume
ISSR	inter-simple sequence repeat	v/v	volume per volume
JA	Jasmonic Acid	w/v	weight per volume
KDa	Kilo Dalton	WOGBC	Worldwide Olive Germplasm Bank of Córdoba

Chapter 1: Veneto olive germplasm: disclosing the genetic identity of locally grown cultivars suited for typical virgin oil productions

Introduction

Olive (*Olea europaea* subsp. *europaea*, 2n=2x=46) is one of the most economically important widely tree fruit crops of the Mediterranean area, where about 805 million olive trees are grown, giving 98% of world olives production (Gomes et al., 2012). The olive tree products represent a significant share of the agricultural economy of the southern European countries. The EU, in fact, is the global leader of olive production, accounting for almost 70% of the total world output and the main net exporter towards non-producing areas such as North America. In terms of overall production, Spain is the biggest producer in the world within the period 2010-2014, followed by Italy and Greece (FAO STAT 2016).

The Mediterranean olive growing countries possess a rich diversity of olive cultivars, including Spain (133 cultivars), France (88 cultivars), Greece (52 cultivars), and Turkey (45 cultivars). Among them, Italy counts on about 600 cultivars, which represents half of the olive germplasm (Bartolini et al., 1998).

Olive grows through the typical Mediterranean climate and its spread to northern latitudes is limited by low winter temperatures (lower than −8.3°C, Ponti et al., 2014). In the coming decades, the olive tree should face the greatest climatic change that has been recorded since its spread into the Mediterranean Basin, and its cultivated area is expected to be reshaped to the predicted future climate (Moriondo et al., 2013). For this reason, regions actually at the northern limit of olive cultivation as Veneto (highest latitude at 45°58′48″ N), represent important cradles of variability because there have settled and adapted genotypes able to withstand extreme weather conditions. The cultivated olive stands out from all the other fruit species for the great varietal heritage still preserved in culture (Lazović et al., 2016). More than 1,200 cultivars have been described (Bartolini et al., 1998) and many others are still waiting to be recognized and characterized (Mousavi et al., 2014).

Several countries have established their national olive germplasm to manage *in situ* olive genetic resources for conservation purposes and eventual use in subsequent breeding programs. The main reason to establish *in situ* or *ex situ* germplasm collections is to conserve

all genetic resources of a plant species over the years, regardless of agronomic traits featured by varieties (i.e., yield, oil quality, and adaptive traits related to biotic and abiotic stresses). The recent concept of establishing modern orchard based on specific features leads to significant reduction in the number of cultivated varieties and consequently determines the erosion of the germplasm. Establishing modern orchard based on specific agronomic traits can be clearly seen in the olive-growing countries. For instance, over the last two decades in Spain the "Arbequina" and "Picual" varieties have been massively cultivated in Andalusia and Catalonia, respectively (Belaj et al., 2010). A similar case might be represented by Portugal, with the main cultivar "Galega" grown in about 80% of the olive groves (Gemas et al., 2004), and in Morocco where "Picholine Marocaine" is the dominant cultivar all over the country (Khadari et al., 2008).

In this context, the Worldwide Olive Germplasm Bank of Córdoba, Spain (WOGBC), represents the first major attempt to conserve and characterize the most important cultivars from all olive growing countries is worth mentioning. WOGBC was initiated by FAO-INIA in 1970, with the contribution of the International Olive Oil Council (Caballero et al., 2006). It includes Spanish cultivars that were collected by Barranco and Rallo (2000a) and varieties originating from other Mediterranean countries. Currently, WOGBC is one of the world's largest collections of olive germplasm as it contains 499 accessions from 21 countries (Caballero et al., 2006) and represents the main Mediterranean cultivar variability. It is worth mentioning that only 30 genotypes currently conserved in this collection are of Italian origin (Trujillo et al., 2014). In 2003, a second world olive germplasm bank was established at the experimental orchard of Tessaout [National Institute of Agronomic Research, Marrakech, Morocco; (Marrakech)]. This bank contains olive cultivars from other collections, such as the WOGBC, as well as local genetic resources (Haouane et al., 2011).

In the past, the identification of olive cultivars was based exclusively on morphological and agronomical traits, tree growth habit, resistance to pathogens and pests, fruit and oil composition and phenological parameters (e.g., flowering time, fruit set, etc.) (Grasso et al., 2016; Leon et al., 2016). However, morphological and phenological descriptors have shown major drawbacks because they provide a small number of polymorphisms and are under environment influence (Ayed et al., 2015; Gomes et al., 2012). Therefore, identification of olive varieties based on phenotypic traits has lead in the past to several confusions (Corrado

et al., 2009; Rotondi et al., 2011). On the contrary, molecular markers have been successfully exploited for the characterization of the olive germplasm and represent very accurate tools to screen, characterize and assess genetic identity of cultivars and to determine genetic diversity and relationships among cultivars (Diez et al., 2015; El Bakkali et al., 2013; Hannachi et al., 2008), with applications in breeding programs and germplasm collection management (Belaj et al., 2012; De la Rosa et al., 2013). For instance, different molecular techniques based on DNA markers, such as Random Amplified Polymorphism DNA (RAPD), Inter-Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR), were recently adopted to better characterize and evaluate the Greek germplasm (Linos et al., 2014). Also, OWGB Cordoba has served for many studies using morphological descriptors and molecular markers such as RAPD and SSR markers (Belaj et al., 2003, 2004; Trujillo et al., 2014). Similar approaches were used to investigate the germplasm residing in France (Khadari et al., 2003), Spain (Trujillo et al., 2014), Italy (Cipriani et al., 2002; Muzzalupo et al., 2009, 2014), Tunisia (Taamalli et al., 2006), Morocco (Charafi et al., 2008) and the United States (Koehmstedt et al., 2011). Microsatellite (simple sequence repeat, SSR) markers represent one of the most popular marker systems for olive DNA genotyping, since they are highly informative, reproducible within and transferable among laboratories, and are thus ideal for developing fingerprinting studies (Baldoni et al., 2009; Gomes et al., 2009, Haouane et al., 2011; Muzzalupo et al., 2009).

A great deal of research has been devoted to apply SSR markers for the characterization of olive trees accessions due to the high degree of polymorphism, co-dominant inheritance and simplicity of detection of a high number of marker alleles per locus (Ayed et al., 2016; Díez et al., 2016). In the past, some authors have tested a number of SSR markers on a number of cultivars grown in different regions in order to assess the distribution of variability, establish relationships among varietal populations, infer the origin of cultivars (Barazani et al., 2014; Sakar et al., 2016; Mackay et al., 2008), allocating olive cultivars to their geographic population of origin (Sarri et al., 2006; Díez et al., 2015).

The systematic and unequivocal identification of locally grown varieties represents a key step towards the evaluation, characterization and exploitation of autochthonous locally distributed varieties. In the present study, we have collected and molecularly characterized a large set of olive trees grown in the Veneto region by using a selection of highly polymorphic

SSR markers. This information will help to notice an important gene pool well adapted to difficult climatic growing conditions, to understand its origin, to better exploit this germplasm source, and to warrant the genetic authenticity of local olive cultivars and olive oils.

Aims of the present study

The increasing interest in the production of monovarietal virgin olive oils poses the need for an in depth and unequivocal identification of locally grown varieties both for the uncovering of cases of synonymy and homonymy, and the identification of genotypes which may be potentially interesting for the production of virgin olive oils with unique compositional characteristics. The systematic and unequivocal identification of locally grown germplasm represents a key step towards the evaluation of new varieties for production and traceability of monovarietal virgin olive oils. For this purpose, we have genetically identified and characterized a large collection of known and unknown olive plants grown in the Veneto region by using a selection of highly polymorphic SSR markers.

The specific objectives of this study were:

I. To perform a survey of mapped SSR marker loci for the analysis of nuclear DNA polymorphisms to distinguish olive cultivars and cultivar groups within the entire olive cultivation area in Veneto, from the Garda Lake to the Euganean and Trevisan hills, in order to obtain a systematic examination of the Veneto regional olive germplasm patrimony.

II. To verify the presence of autochthonous varieties in one of the northernmost areas of olive cultivation, to ascertain their possible development along the history and to solve eventual cases of homonymy and synonymy.

These objectives were achieved through using different independent approaches:

a) Calculation of genetic similarity in all possible pair-wise combinations and also with the reference cultivars.
b) Implementation of principal coordinate analyses (PCoA) for the definition of bidimentional centroids.
c) Determination of the genetic structure of the population as a whole by STRUCTURE analysis and comparing also with the Mediterranean cultivars.
d) Calculation of genetic identity and diversity statistics within and among cultivar groups.

Materials and methods

Materials

Plant materials

The area of olive cultivation of the Veneto region (north-eastern Italy), from the Garda lake to the hilly areas of Verona, Vicenza, Padova and Treviso (Figure 1.1), was deeply prospected during 2014 and 2015 seasons by experienced staff in order to collect plant material from trees whose estimated age was over 60 years, in order of gathering only local plants and avoid any recent introduction (Table 1.1). A number of olive samples (25, indicated as VISF) were also collected from the olive cultivar collection of the Institute of Horticulture, Verona.

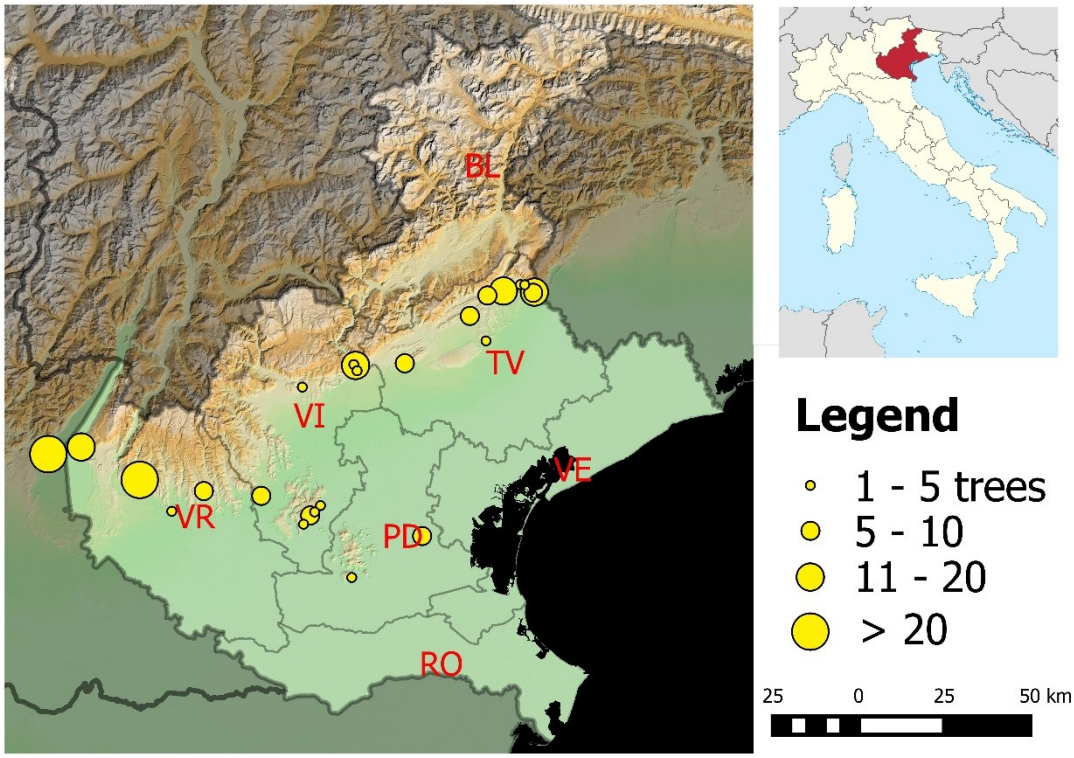

Figure 1.1 – Map of the sampling sites of the unknown Veneto olive accessions evaluated in this study.

Table 1.1 – Comprehensive list of the 204 unknown olive accessions and respective collection sites in Veneto (North-Eastern Italy), analyzed in this work along with 25 selected reference cultivars. The sites of collection are listed and the corresponding Veneto provinces are reported in brackets: BS (Brescia), TV (Treviso), PD (Padova), VI (Vicenza), VR (Verona); ISF: Institute of Fruticulture, Verona.

NO	Samples ID	Collection site	NO	Samples ID	Collection site	NO	Samples ID	Collection site
1	ASO1	Asolo (TV)	51	TDB1	Torri del Benaco (VR)	101	SOL6	Pieve di Soligo (TV)
2	ASO2	Asolo (TV)	52	TDB2	Torri del Benaco (VR)	102	SOL7	Pieve di Soligo (TV)
3	ASO7	Asolo (TV)	53	TDB3	Torri del Benaco (VR)	103	SOL8	Pieve di Soligo (TV)
4	ASO8	Asolo (TV)	54	TDB4	Torri del Benaco (VR)	104	SUS1	Susegana (TV)
5	ASO9	Asolo (TV)	55	TDB5	Torri del Benaco (VR)	105	SUS2	Susegana (TV)
6	ASO12	Asolo (TV)	56	TDB6	Torri del Benaco (VR)	106	TAR1	Tarzo (TV)
7	ASO13	Asolo (TV)	57	TDB7	Torri del Benaco (VR)	107	TAR2	Tarzo (TV)
8	ASO14	Asolo (TV)	58	TDB8	Torri del Benaco (VR)	108	TAR3	Tarzo (TV)
9	ASO15	Asolo (TV)	59	TDB9	Torri del Benaco (VR)	109	TAR4	Tarzo (TV)
10	ASO16	Asolo (TV)	60	TDB10	Torri del Benaco (VR)	110	TAR5	Tarzo (TV)
11	COR1	Cordignano (TV)	61	TDB11	Torri del Benaco (VR)	111	TAR6	Tarzo (TV)
12	COR2	Cordignano (TV)	62	TDB12	Torri del Benaco (VR)	112	TAR7	Tarzo (TV)
13	COR3	Cordignano (TV)	63	TDB13	Torri del Benaco (VR)	113	TAR8	Tarzo (TV)
14	COR4	Cordignano (TV)	64	TDB14	Torri del Benaco (VR)	114	TAR9	Tarzo (TV)
15	COR5	Cordignano (TV)	65	TDB15	Torri del Benaco (VR)	115	PAR3	PARMA
16	COR6	Cordignano (TV)	66	TDB16	Torri del Benaco (VR)	116	NAN1	Nanto (VI)
17	COR7	Cordignano (TV)	67	TDB17	Torri del Benaco (VR)	117	MOS3	Mossano (VI)
18	COR8	Cordignano (TV)	68	TDB18	Torri del Benaco (VR)	118	MOS1	Mossano (VI)
19	COR9	Cordignano (TV)	69	TDB19	Torri del Benaco (VR)	119	SDM1	Selva di Montebello (VI)
20	COR10	Cordignano (TV)	70	MEZ1	Mezzane (VR)	120	SDM2	Selva di Montebello (VI)
21	COR11	Cordignano (TV)	71	MEZ2	Mezzane (VR)	121	TDV2	Toara di Villaga (VI)
22	COR12	Cordignano (TV)	72	MEZ3	Mezzane (VR)	122	SDM3	Selva di Montebello (VI)
23	PAR1	PARMA	73	MEZ4	Mezzane (VR)	123	BAR3	Barbarano (VI)
24	FRE1	Fregona (TV)	74	MEZ5	Mezzane (VR)	124	MOS2	Mossano (VI)
25	MON1	Sarmede (TV)	75	MEZ6	Mezzane (VR)	125	BAR5	Barbarano (VI)
26	MON5	Sarmede (TV)	76	MEZ7	Mezzane (VR)	126	SDM4	Selva di Montebello (VI)
27	MON7	Sarmede (TV)	77	PDG1	Pove del grappa (VI)	127	MOS4	Mossano (VI)
28	VISF1	Verona ISF (VR)	78	PDG2	Pove del grappa (VI)	128	SDM5	Selva di Montebello (VI)
29	VISF2	Verona ISF (VR)	79	BAS2	Marostica (VI)	129	BAR2	Barbarano (VI)
30	VISF3	Verona ISF (VR)	80	BAS3	Marostica (VI)	130	BAR6	Barbarano (VI)
31	VISF4	Verona ISF (VR)	81	LUG1	Lugo (VI)	131	TDV1	Toara di Villaga (VI)
32	VISF5	Verona ISF (VR)	82	LUG2	Lugo (VI)	132	SDM6	Selva di Montebello (VI)
33	VISF6	Verona ISF (VR)	83	LUG3	Lugo (VI)	133	MOS5	Mossano (VI)
34	VISF7	Verona ISF (VR)	84	BAS1	Bassano (VI)	134	NAN3	Nanto (VI)
35	VISF8	Verona ISF (VR)	85	PDG3	Pove del Grappa (VI)	135	TDV4	Toara di Villaga (VI)
36	VISF9	Verona ISF (VR)	86	PDG4	Pove del Grappa (VI)	136	SDM7	Selva di Montebello (VI)
37	VISF10	Verona ISF (VR)	87	PDG5	Pove del Grappa (VI)	137	BAR4	Barbarano (VI)
38	VISF11	Verona ISF (VR)	88	PDG6	Pove del Grappa (VI)	138	NAN2	Nanto (VI)
39	VISF12	Verona ISF (VR)	89	PDG7	Pove del Grappa (VI)	139	BAR1	Barbarano (VI)
40	VISF13	Verona ISF (VR)	90	PDG8	Marsan (VI)	140	SFB1	San Felice del Benaco (BS)
41	LEG1	Legnaro (PD)	91	PDG9	Marsan (VI)	141	SFB2	San Felice del Benaco (BS)
42	LEG2	Legnaro (PD)	92	PDG10	Marostica (VI)	142	SFB3	San Felice del Benaco (BS)
43	LEG3	Legnaro (PD)	93	PAR2	PARMA	143	SFB4	San Felice del Benaco (BS)
44	LEG4	Legnaro (PD)	94	SAR1	Sarmede (TV)	144	SFB5	San Felice del Benaco (BS)
45	LEG5	Legnaro (PD)	95	SAR2	Sarmede (TV)	145	SFB6	San Felice del Benaco (BS)
46	LEG6	Legnaro (PD)	96	SOL1	Pieve di Soligo (TV)	146	SFB7	San Felice del Benaco (BS)
47	PAD1	Baone (Padova)	97	SOL2	Pieve di Soligo (TV)	147	VISF14	Verona ISF (VR)
48	PAD2	Baone (Padova)	98	SOL3	Pieve di Soligo (TV)	148	SFB8	San Felice del Benaco (BS)
49	PAD3	Baone (Padova)	99	SOL4	Pieve di Soligo (TV)	149	VGO1	Verona (VR)
50	PAD4	Baone (Padova)	100	SOL5	Pieve di Soligo (TV)	150	SFB9	San Felice del Benaco (BS)

Continued

NO	Samples ID	Collection site	NO	Samples ID	Collection site	NO	Samples ID	Collection site
151	VISF15	Verona ISF (VR)	178	SFB23	San Felice del Benaco (BS)	205	BIAN_Ref	BIANCHERA_Reference
152	VISF16	Verona ISF (VR)	179	VISF28	Verona ISF (VR)	206	CAPO_Ref	CAPOLGA_Reference
153	VISF17	Verona ISF (VR)	180	PDG11	Pove del Grappa (VI)	207	COLO_Ref	COLOMBINA_Reference
154	VISF18	Verona ISF (VR)	181	PDG12	Pove del Grappa (VI)	208	CRNI_Ref	CRNICA_Reference
155	SFB10	San Felice del Benaco (BS)	182	CRO1	Sarmede (TV)	209	FAVA_Ref	FAVAROL_Reference
156	VISF19	Verona ISF (VR)	183	CRO2	Sarmede (TV)	210	FRAN_Ref	FRANTOIO[1]_Reference
157	SFB11	San Felice del Benaco (BS)	184	SAR3	Sarmede (TV)	211	GARG_Ref	GARGNA_Reference
158	SFB12	San Felice del Benaco (BS)	185	SAR4	Sarmede (TV)	212	GHIA_Ref	GHIACCIOLO_Reference
159	SFB13	San Felice del Benaco (BS)	186	SAR5	Sarmede (TV)	213	GRAP_Ref	GRAPPOLO_Reference
160	SFB14	San Felice del Benaco (BS)	187	SAR6	Sarmede (TV)	214	GRPP_Ref	GRAPPUDA_Reference
161	SFB15	San Felice del Benaco (BS)	188	SOL9	Solagna (VI)	215	LECC_Ref	LECCINO_Reference
162	VISF20	Verona ISF (VR)	189	SOL10	Solagna (VI)	216	MAUR_Ref	MAURINO_Reference
163	VISF21	Verona ISF (VR)	190	VIV1	Vittorio Veneto (TV)	217	MINI_Ref	MINIOL_Reference
164	VGO2	Verona (VR)	191	VIV2	Vittorio Veneto (TV)	218	MORA_Ref	MORAIOLO_Reference
165	VISF22	Verona ISF (VR)	192	VIV3	Vittorio Veneto (TV)	219	OBLI_Ref	OBLICA_Reference
166	SFB16	San Felice del Benaco (BS)	193	VIV4	Vittorio Veneto (TV)	220	OLIV_Ref	OLIVELLO_Reference
167	SFB17	San Felice del Benaco (BS)	194	VIV5	Vittorio Veneto (TV)	221	PIGN_Ref	PIGNOLA_Reference
168	VISF23	Verona ISF (VR)	195	VIV6	Vittorio Veneto (TV)	222	POSO_Ref	POSOLELLA_Reference
169	SFB18	San Felice del Benaco (BS)	196	VIV7	Vittorio Veneto (TV)	223	ROSS_Ref	ROSSANEL_Reference
170	VISF24	Verona ISF (VR)	197	VIV8	Vittorio Veneto (TV)	224	TDIV_Ref	TONDA DI VILLA_Reference
171	VISF25	Verona ISF (VR)	198	VIV9	Vittorio Veneto (TV)	225	FORT_Ref	FORT_Reference
172	VISF26	Verona ISF (VR)	199	VIV10	Vittorio Veneto (TV)	226	BAIA_Ref	BAIA_Reference
173	SFB19	San Felice del Benaco (BS)	200	VIV11	Vittorio Veneto (TV)	227	COMP_Ref	COMPOSTARO_Reference
174	SFB20	San Felice del Benaco (BS)	201	VIV12	Vittorio Veneto (TV)	228	GRIG_Ref	GRIGNANO_Reference
175	VISF27	Verona ISF (VR)	202	VIV13	Vittorio Veneto (TV)			
176	SFB21	San Felice del Benaco (BS)	203	VIV15	Vittorio Veneto (TV)			
177	SFB22	San Felice del Benaco (BS)	204	VIV16	Vittorio Veneto (TV)			

[1]The FRANTOIO-like variety widely cultivated in the Veneto region is locally known as CASALIVA.

Methods

Genomic DNA extraction

Young leaves were harvested from 204 single trees representing previously uncharacterized olive accessions. Total DNA was extracted from 100 mg of fresh leaves using CTAB method (Angiolillo et al., 1999), with slight modifications. Plant tissues were manually ground using mortar and pestle, liquid nitrogen and 600 µl of preheated CTAB buffer were added to each sample with 1.2 µl β-mercaptoethanol. Samples were incubated in water bath at 60°C for 60 min, inverting the tubes vigorously every 15 min. After incubation, 600 µl of chloroform/isoamyl alcohol (24:1) were added and mixed by inverting tubes. Next, the tubes were centrifuged at 10,000 rpm for 15 min and the upper aqueous phase was transferred into 1.5 ml tubes. Two µl RNase (20 mg/ml stock) were added and incubated at 37°C for 15 min. After that 400 µl of cold isopropanol were added and mixed by inverting tubes, followed by centrifugation at maximum speed for 20 min. The supernatant was discarded and the pellet was washed twice using 400 µl of 70% cold ethanol then centrifuged

at maximum speed for 10 min. The supernatant was decanted and pellet was air dried. DNA was finally resuspended in 50 µl of sterile water. The integrity of extracted DNA was estimated by electrophoresis on a 1% agarose/1×TAE gel containing 1X Sybr Safe DNA stain (Life Technologies). Purity and quantity of DNA extracts were assessed by means of the NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific).

SSR analysis

Ten highly polymorphic SSR markers were chosen on the basis of their effectiveness of discrimination (Baldoni et al., 2009) and the availability of a wide dataset of molecular profiles for the most important olive varieties built by the Institute of Biosciences and Bioresources (CNR, Perugia-Italy) to use as reference database: DCA3, DCA5, DCA9, DCA16, DCA18 (Sefc et al., 2000), EMO90 (De la Rosa et al., 2002), GAPU71b, GAPU101, GAPU103A (Carriero et al., 2002) and UDO-043 (Cipriani et al., 2002).

PCR amplifications were performed in a reaction volume of 25 µl containing 25 ng of template DNA, 10x PCR buffer, 2,5 mM of each dNTP, 10 pmol of each primer (forward primer labeled with FAM, NED, PET or VIC fluorescent dyes) and 2 U of Perfect Taq DNA Polymerase (5 PRIME, Eppendorf). Amplifications were performed on the thermal cycler PCR System 9600 (Applied Biosystems, Foster City, CA, USA), using the following cycling conditions: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing temperatures as suggested by the authors for 30 s and 72°C for 25 s, followed by a final elongation step at 72°C for 30 min. The resulting PCR products were first visualized by 2% agarose gel electrophoresis and then loaded onto an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Output data were analyzed using GeneMapper 3.7 (Applied Biosystems).

Data analysis

The overall molecular data were used for computation of genetic similarity and diversity statistics. Frequency analyses were performed on a total of 204 olive genotypes from Veneto. Intra-population genetic statistics such as observed number of alleles (Na), effective number of alleles (Ne), observed (Ho) and expected (He) heterozygosity estimates and fixation index (F), were calculated using GenAlEx 6.5 (Peakall and Smouse, 2013).

The SSR data have further been analyzed by STRUCTURE 2.3.4 software (Pritchard et al., 2009), running 100 replicates MCMCs with a burn-in period of 100,000 followed by a sampling period of 100,000 for 500,000 iterations, applied for each K. The range of possible number of clusters (K) was from 1 to 10, considering independent alleles and admixture of individuals. Bayesian analysis divided sampled individuals into a number of clusters (K) and the most likely value of K was estimated using delta K (Evanno et al., 2005), performed by STRUCTURE Harvester (Earl, 2012). Molecular phylogenetic analyses were performed by MEGA6 (Tamura et al., 2013) using the Neighbor Joining (NJ) method. The unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm from Sneath and Sokal (1973) was used to generate circular dendrograms based on Nei's (1973) genetic distances among the subgroups of accession as well as to group and plot centroids according to the Principal Coordinates Analysis (PCoA), as reported by Rohlf (1998). The overall molecular data were also used for computation of genetic similarity and identity estimates in all possible pairwise combinations using the simple matching coefficient for the construction of UPGMA dendrograms and the determination of centroids of single accessions, including the reference cultivars.

Results

A total of 204 unknown olive tree accessions were selected from different geographical places of Veneto region and subjected to molecular analyses using the ten highest polymorphic nuclear SSR markers available for this species. The accessions were identified based on historical searches, interview with growers and field technical assistants, and only old plants of at least 50 years of age were finally selected for analysis.

The SSR genotypic data obtained by molecular analyses for each accession were compared with the database of SSR markers available at the IBBR-CNR of Perugia, including a comprehensive several Italian varieties, the most important varieties of the Mediterranean countries and other accessions of potential interest from other areas, for a total of 273 olive genotypes. In addition, a selection of 25 locally grown reference cultivars, maintained in different repositories of the Istituto Sperimentale di Frutticoltura di Verona, were included in the study in order to provide a better coverage of references for varieties which may be exclusively located in Veneto region.

Relationships among accessions and cultivar groups are shown in the obtained NJ tree (Figure 1.2). We found that most of the olive samples displayed genetic identity with some reference cultivars and could be unequivocally assigned to them: “Casaliva” (60 samples), “Miniol” (21 samples), “Grignano” (20 samples), “Tonda di Villa” (19 samples), “Leccino” and “Capolga” (17 samples each), “Gargnà” and “Compostaro” (9 samples each), “Rossanel” and “Oblica” (8 samples), “Fort” and “Baia” (5 samples each), “Grappolo” and “Favarol” (3 samples each). Nine samples revealed genetic similarity and resulted to be closely related to the two reference cultivars “Tonda di Villa” and “Oblica” having bootstrap values greater than 85%, while as few as 2 samples each were attributable to “Moraiolo”, “Crnica” and “Maurino”. Moreover, the examination of the UPGMA dendrogram based on all the sampled trees (data not shown) allowed classifying the genotypes into 15 main distinct cultivar subgroups including the reference cultivars with a highly degree of similarity found ranging from 85% to full identity of 100%. Finally, 16 samples (labelled as TAR8, ASO13, PAD2, TB18, LUG2, SFB14, MOS4, MON 7, TAR5, PAR 3, COR10, SFB8, SFB22, VISF27, and VISF13, TDB10, revealed a unique genetic pattern and did not match with any of the reference cultivars considered in this study (Figure 1.2). Genetic relationships and variations of the main subgroups were confirmed by constructing a dendrogram using an unbiased

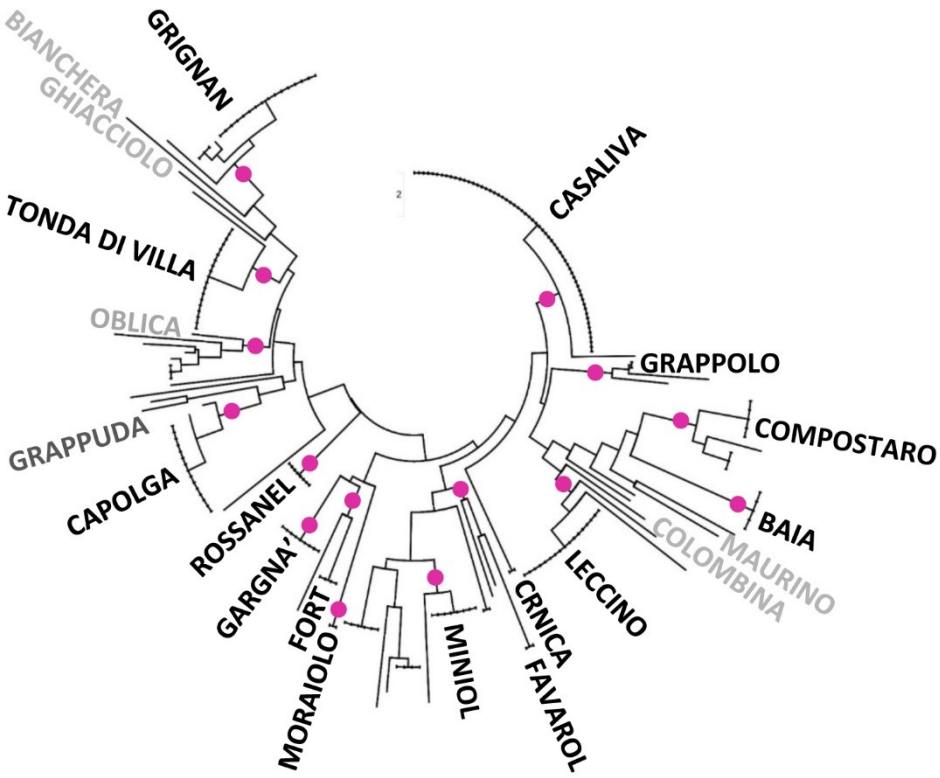

Figure 1.2 – Neighbor-Joining circular tree depicting the genetic relationships between the 229 olive accessions from the Veneto region based on SSR markers. The olive reference genotypes/varieties are reported for the main cultivar groups.

genetic distance symmetrical matrix (Figure 1.3, panel A) and by defining centroids according to a principal coordinate analysis (Figure 1.3, panel B) based on the whole set of monomorphic and polymorphic SSR marker alleles. In particular, the ordination data showed that the first two principal axes explained about 43% of the total genetic variation among groups, with about 32% associated to the first component and 11% associated to the second component.

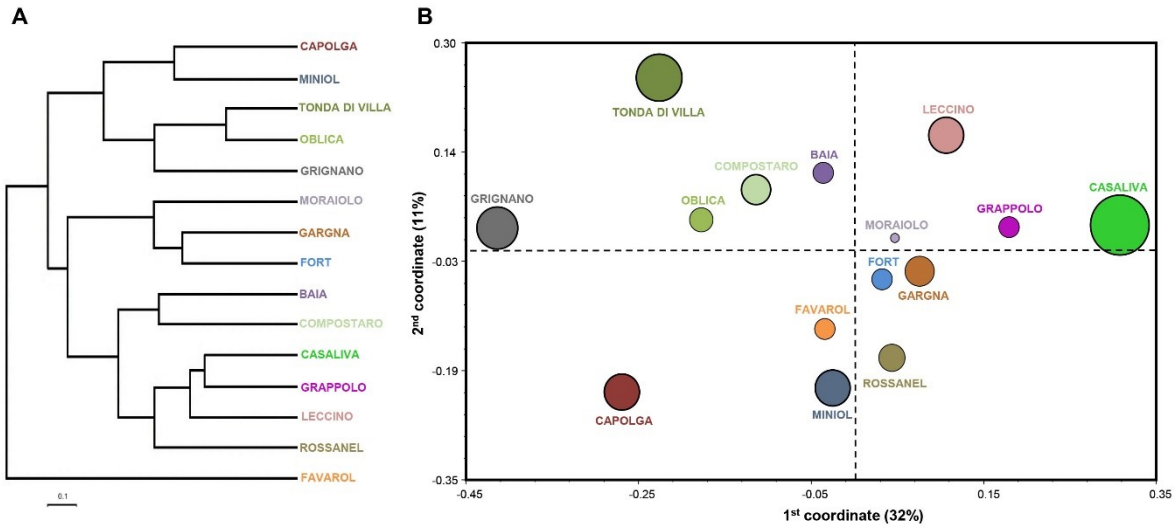

Figure 1.3 – A) UPGMA dendrogram constructed using Nei's (1978) unbiased genetic distances calculated for the 15 olive cultivar groups; B) Bidimensional centroids deriving from Principal Coordinate Analysis (PCoA) of the olive cultivar groups plotted on the basis of the first two principal coordinates. Circle size of each centroid is proportionally related to the number of accessions of each cultivar group.

The whole data set, including 229 Veneto trees and 273 Mediterranean cultivars, was subjected to genetic structure analysis on the basis of all SSR marker alleles for further definition of the cultivar groups, using the model-based (Bayesian) clustering algorithm implemented in the STRUCTURE software generation of the best ΔK. When Veneto trees were analyzed alone, the existence of 15 distinct genotypes was hypothesized for the olive germplasm cultivated in this region (Figure 1.4). Together with the Mediterranean cultivars, for a total of 502 accessions, the uppermost hierarchal level of the whole population structure suggested a clear peak of the log probability of the data at $K = 3$. These distinct clusters were found sharing a proportion of membership associated with the cultivar group ranging from 70% to 100% (Figure 1.5).

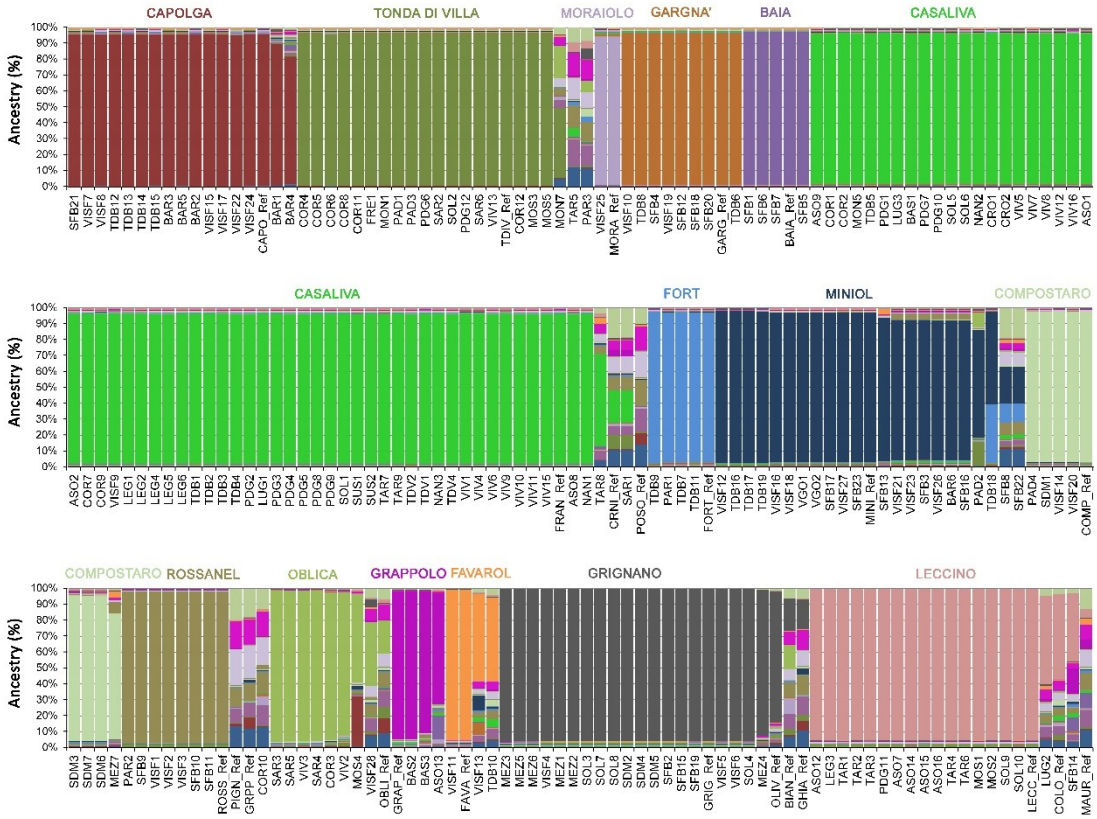

Figure 1.4 – Population structure of the 229 Veneto olive accessions grouped into fifteen major genetically different clusters ($K = 15$) as defined according to individual assignment.

The genotypes from Veneto region were spread in all three Mediterranean groups but with significant differences in the percentage. In fact, about 16% and 18% of the Veneto's genotypes were placed on the cluster representing almost all western and eastern Mediterranean cultivars, respectively. As much as 66% of the Veneto's olive accessions were placed on the central Mediterranean cluster, with a few accessions that resulted intermixed among two or three populations (Figure 1.5).

The main descriptive statistics among these cultivar groups were thus determined in terms of genetic diversity including the average number of observed alleles (Na), effective number of alleles (Ne) per locus, observed (Ho) and expected (He) heterozygosity and Nei's genetic diversity equivalent to the unbiased expected heterozygosity (uHe), as reported in Table 1.2.

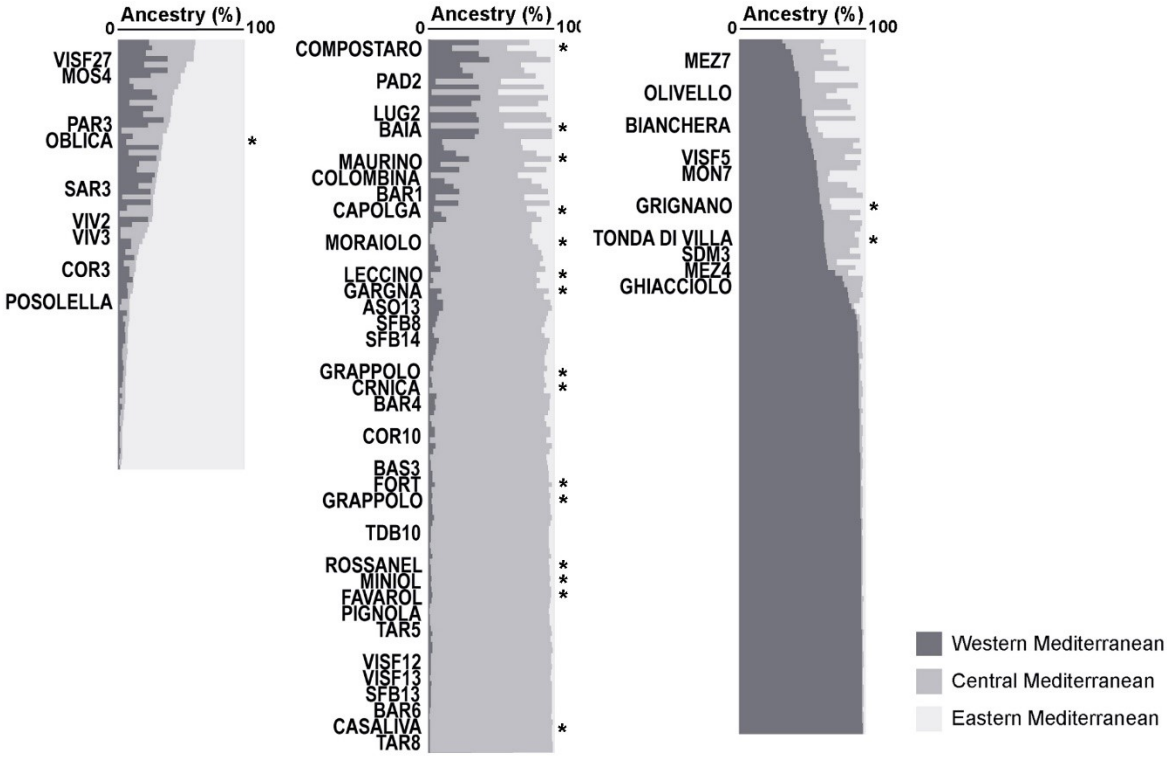

Figure 1.5 – Total population structure of the 229 Veneto olive accessions and 273 Mediterranean cultivars grouped into three major clusters ($K = 3$). The reference accessions of the main olive cultivar groups are marked by an asterisk.

It is worth nothing that the calculated PIC values for the individual marker loci ranged from 0.473 (DCA05) to 0.802 (DCA03), with an average value equal to 0.696 (data not shown).

A total of 110 marker alleles were detected across the assayed SSR loci with an average number of observed and effective alleles Na=10.800 and Ne=4.254, respectively (Table 1.2) The overall value of observed heterozygosity was very high, equal to Ho=91.6% (st. dev. 1.9%), and the mean expected heterozygosity was He=74.1% (st. dev. 2.9%). Moreover, the Nei's unbiased genetic diversity was as equal to uHe=74.3%, whereas the total estimate of the Shannon's information index of phenotypic diversity was equal to I=1.612 (Table 1.2). The fixation index, equal to Fst=0.247, revealed that only less than 25% of the genetic variation was found among cultivar groups and that approximately as much as 75% of the total polymorphism was scored among trees within cultivar groups. The narrow genetic

differentiation among subgroups over the investigated multi-locus marker loci was confirmed by the gene flow estimate that was as low as Nm=0.412. It is worth mentioning that all Wright's inbreeding coefficients were negative and on average equal to Fis=–0.863 and Fit=–0.159, indicating a significant excess of heterozygosity for the olive accessions of the Veneto germplasm.

Table 1.2 – Descriptive statistics of the ten marker loci analyzed in the Veneto olive accessions, including the average number of observed alleles (Na) and effective Number of alleles (Ne) per locus, estimates of Shannon's information index of phenotypic diversity (I), observed heterozygosity (Ho), expected heterozygosity (He), coefficients of Nei's genetic diversity corresponding to the unbiased expected heterozygosity (uHe) and Wright's inbreeding coefficients or fixation index (Fst) estimates. The overall values and standard deviations are also reported for the population as a whole.

Locus ID	Sample size	Na	Ne	I	Ho	He	uHe	Fst
DCA03	229	11.000	5.914	1.962	0.983	0.831	0.833	0.182
DCA05	229	8.000	2.247	1.005	0.777	0.555	0.556	0.400
DCA09	229	17.000	5.130	1.897	0.904	0.805	0.807	0.123
DCA016	227	11.000	4.341	1.722	0.872	0.770	0.771	0.133
DCA018	229	8.000	2.886	1.253	0.891	0.653	0.655	0.363
EMO90	229	5.000	3.302	1.290	0.961	0.697	0.699	0.378
GAPU71B	229	10.000	3.146	1.350	0.948	0.682	0.684	0.389
GAPU101	229	12.000	4.906	1.857	0.921	0.796	0.798	0.157
GAPU103	227	12.000	5.028	1.896	0.934	0.801	0.803	0.166
UDO-43	229	14.000	5.634	1.888	0.969	0.823	0.824	0.179
Mean	**228,6**	**10.800**	**4.254**	**1.612**	**0.916**	**0.741**	**0.743**	**0.247**
St. Dev.	**0,267**	**1.062**	**0.401**	**0.111**	**0.019**	**0.029**	**0.029**	**0.037**

A summary of the main descriptive statistics of genetic diversity computed for the single cultivar groups (*i.e.*, distinct genotypes) are reported in Table 1.3.

The unbiased measures of genetic identity and genetic diversity among the fifteen olive cultivar groups in all pair-wise comparisons were also calculated (Table 1.4). We found that

Table 1.3 – Descriptive statistics of the fifteen Veneto olive cultivar groups, including the average number of observed alleles (Na) and effective number of alleles (Ne) per locus, estimates of Shannon's information index of phenotypic diversity (I), values of observed heterozygosity (Ho) and expected heterozygosity (He), coefficients of Nei's genetic diversity corresponding to the unbiased expected heterozygosity (uHe). The overall values and standard deviations are also reported for each cultivar group.

Population ID	Size (No.)	Na	Ne	I	H_o	H_e	uH_e
Cultivar group "CAPOLGA"	17	2.200	1.920	0.660	0.910	0.480	0.470
St. Dev.		0.630	0.250	0.090	0.280	0.110	0.110
Cultivar group "TONDA DI VILLA"	19	2.500	1.960	0.690	0.910	0.480	0.470
St. Dev.		0.530	0.320	0.210	0.300	0.150	0.150
Cultivar group "MORAIOLO"	2	1.800	1.800	0.550	0.800	0.530	0.400
St. Dev.		0.420	0.420	0.290	0.420	0.280	0.210
Cultivar group "GARGNA'"	9	2.000	2.000	0.690	1.000	0.530	0.500
St. Dev.		0.000	0.000	0.000	0.000	0.000	0.000
Cultivar group "BAIA"	5	1.600	1.600	0.420	0.600	0.330	0.300
St. Dev.		0.520	0.520	0.360	0.520	0.290	0.260
Cultivar group "CASALIVA"	60	2.400	2.010	0.710	1.000	0.510	0.500
St. Dev.		0.700	0.020	0.030	0.000	0.010	0.010
Cultivar group "FORT"	5	1.800	1.800	0.550	0.800	0.440	0.400
St. Dev.		0.420	0.420	0.290	0.420	0.230	0.210
Cultivar group "MINIOL"	21	2.800	2.160	0.820	0.800	0.510	0.500
St. Dev.		0.790	0.500	0.320	0.310	0.190	0.180
Cultivar group "COMPOSTARO"	9	2.600	2.320	0.830	0.900	0.550	0.520
St. Dev.		0.840	0.740	0.360	0.320	0.210	0.200
Cultivar group "ROSSANEL"	8	1.800	1.800	0.550	0.800	0.430	0.400
St. Dev.		0.420	0.420	0.290	0.420	0.220	0.210
Cultivar group "OBLICA"	8	3.400	2.450	0.970	0.940	0.620	0.580
St. Dev.		1.170	0.530	0.250	0.120	0.080	0.070
Cultivar group "GRAPPOLO"	3	2.500	2.240	0.780	0.850	0.570	0.500
St. Dev.		1.180	0.820	0.400	0.320	0.230	0.200
Cultivar group "FAVAROL"	3	1.900	1.900	0.620	0.900	0.600	0.450
St. Dev.		0.320	0.320	0.220	0.320	0.210	0.160
Cultivar group "GRIGNANO"	20	4.800	2.380	1.000	0.990	0.590	0.570
St. Dev.		1.480	0.310	0.190	0.030	0.050	0.050
Cultivar group "LECCINO"	17	2.700	1.990	0.710	0.890	0.490	0.470
St. Dev.		0.820	0.340	0.230	0.300	0.150	0.150

the cultivar group “Tonda di Villa” was closely related to the cultivar group “Oblica”, with a coefficient of genetic identity equal to 76%, followed by “Casaliva” and “Grappolo”, showing a 72% of genetic identity. The lowest value of genetic identity was scored between the cultivar groups “Casaliva” and “Grignano” (20%).

Table 1.4 – Matrix of the Nei’s (1978) unbiased measures of genetic identity (above diagonal) and genetic diversity (below diagonal) between all pair-wise comparisons of cultivar groups.

Population ID	CAPOLGA	TONDA DI VILLA	MORAIOLO	GARGNA’	BAIA	CASALIVA	FORT	MINIOL	COMPOSTARO	ROSSANEL	OBLICA	GRAPPOLO	FAVAROL	GRIGNANO	LECCINO
CAPOLGA	****	0.515	0.283	0.349	0.294	0.344	0.410	0.622	0.500	0.579	0.582	0.279	0.346	0.569	0.338
TONDA DI VILLA	0.663	****	0.414	0.390	0.447	0.429	0.403	0.352	0.604	0.393	0.763	0.429	0.294	0.565	0.414
MORAIOLO	1.261	0.883	****	0.584	0.369	0.480	0.624	0.287	0.348	0.443	0.288	0.544	0.243	0.225	0.374
GARGNA’	1.053	0.941	0.538	****	0.434	0.559	0.660	0.403	0.315	0.421	0.410	0.432	0.309	0.246	0.451
BAIA	1.224	0.804	0.997	0.836	****	0.430	0.437	0.334	0.595	0.355	0.524	0.562	0.390	0.383	0.623
CASALIVA	1.067	0.848	0.734	0.583	0.845	****	0.559	0.536	0.457	0.602	0.350	0.723	0.409	0.203	0.682
FORT	0.891	0.910	0.471	0.416	0.828	0.582	****	0.389	0.321	0.386	0.466	0.407	0.330	0.318	0.459
MINIOL	0.475	1.044	1.248	0.908	1.098	0.623	0.943	****	0.560	0.526	0.421	0.458	0.452	0.449	0.423
COMPOSTARO	0.694	0.505	1.056	1.154	0.519	0.784	1.137	0.581	****	0.552	0.569	0.545	0.403	0.552	0.626
ROSSANEL	0.546	0.934	0.815	0.864	1.036	0.508	0.952	0.643	0.594	****	0.315	0.631	0.468	0.324	0.532
OBLICA	0.541	0.270	1.247	0.892	0.646	1.050	0.763	0.864	0.565	1.155	****	0.367	0.255	0.594	0.444
GRAPPOLO	1.275	0.846	0.608	0.840	0.576	0.324	0.898	0.781	0.608	0.461	1.004	****	0.327	0.304	0.654
FAVAROL	1.061	1.225	1.413	1.175	0.941	0.894	1.109	0.795	0.909	0.759	1.369	1.118	****	0.364	0.304
GRIGNANO	0.564	0.571	1.490	1.404	0.959	1.596	1.147	0.802	0.595	1.127	0.521	1.191	1.012	****	0.381
LECCINO	1.086	0.883	0.983	0.797	0.474	0.383	0.780	0.861	0.468	0.632	0.813	0.425	1.191	0.964	****

Discussion

The increasing market of monovarietal virgin olive oils reflects the recent attention paid by consumers to high quality extra virgin olive oils (EVOOs), due to their distinct sensory and nutraceutical properties. Olive oil composition is highly dependent on genetic aspects (varietal genotype) and on the interaction of the genotype with the environment where olive trees are grown and olive drupes are ripened. These effects are particularly evident in the northern areas of olive tree cultivation as Veneto region.

Previous studies showed that EVOO from cultivar "Grignano", a variety endemically cultivated in the area surrounding the Garda Lake and Verona, is characterized by aroma profiles which distinguish this olive oil from most Italian and European olive oils and have highly appreciated sensory characters (Ferasin et al., 2010; Monteleone et al., 2012; Vezzaro et al., 2012). In order to further explore the potential biodiversity of olive genotypes present within the Veneto region for the subsequent valorisation of monovarietal olive oils with unique distinguished characters, we have undertaken an in-depth and as-systematic-as possible genetic analysis of the Veneto region olive germplasm. Besides being an essential step towards the unequivocal identification of locally grown varieties to uncover cases of synonymy and homonymy, this very detailed survey of the Veneto olive genotypes also represents the way to the identification of genotypes with potentially interesting characters for the production of virgin olive oils with unique compositional features. A complete genetic description of the Veneto germplasm will also set a corner stone for traceability of monovarietal olive oils.

Recently, big efforts have been made in order to characterize the global olive resources available worldwide (Trujillo et al., 2014). A substantial re-organization of olive germplasm collections has been achieved on the basis of molecular analyses in most of the olive-growing countries (Baldoni and Belaj, 2010), including Italy (Marra et al., 2013, Muzzalupo et al., 2014; Las Casas et al., 2014), Greece (Linos et al., 2014) and Spain (Trujillo et al., 2014; Marti et al., 2015). However, out of the 499 accessions of the World Olive Germplasm Bank of Cordoba, Spain (WOGBC), verified by Trujillo et al. (2014) using 33 SSR markers, only 36 were of Italian origin.

In the current comprehensive study, we established the first systematic analysis of olive the Veneto olive germplasm. The results were compared with the database of SSR markers

available in CNR-Perugia, which represents the most comprehensive collection of all known Italian varieties and of the most important varieties of the Mediterranean countries and other accessions of potential interest from other areas, for a total of 840 genotypes.

Interestingly, the SSR method used in this study was able to uniquely recognize and unambiguously differentiate each of the 204 cultivated olive accessions under study and confirmed the high efficiency of the SSR markers in that aspect. The overall molecular data were processed to obtain the 15 most consistent clusters or cultivar groupings and to assess the genetic identity of genotypes as belonging to regional cultivars, and their putative admixed or exchanged origin by using different independent approaches: i) calculation of simple matching coefficients of genetic similarity; ii) implementation of principal coordinate analyses (PCoA) for the definition of bidimensional centroids; iii) determination of the genetic structure of the population as a whole by STRUCTURE analysis; and iv) calculation of genetic identity and diversity statistics within and among cultivar groups.

The biggest cluster was found for “Casaliva”, which included 60 accessions that could not be genetically distinguished from the reference cultivar “Casaliva”, the most widespread cultivar in Italy. Similarly, 17 accessions were assigned to the group of “Leccino”. The remaining 127 accessions could be assigned to other fourteen clusters, each of them unequivocally identified on the basis of known locally cultivated olive cultivars used as references. This evidence shows that many locally grown cultivars, namely “Tonda di Villa”, “Compostaro”, “Fort”, “Rossanel”, “Favarol” and “Grignano”, have been spread widely over different Veneto provinces by clonal propagation (Albertini et al., 2011) through cuttings or grafting methods. In detail, the cultivar “Grignano”, endemic in the area surrounding Verona and the Garda Lake, was found to be present in several accessions in Treviso and Verona provinces, and it appeared highly similar to the cultivar “Olivello”. Differently, “Tonda di Villa” was found almost exclusively located in eastern Veneto (Treviso) and within a cluster distinct from all cultivars present in the database. Similarly, several plants widespread throughout the Veneto area resulted to belong to clusters of varieties exclusively grown in the region, such as “Gargnà”, “Miniol”, “Fort”, “Compostaro”, “Rossanel” and “Favarol”. Besides, the presence in the eastern part of Veneto of accessions with high similarity with the cultivar “Oblica” and of one accession with the cultivar “Crnica” supports a past introduction of Slovenian cultivars through its close border. Interestingly, nine accessions

revealed genetic similarity and to be closely related, but not identical, to two reference cultivars “Tonda di Villa” and “Oblica”, and having bootstrap values of genetic similarity greater than 85%. Our results show that the unique genetic distance pattern of a few accessions collected from six different collection sites in Veneto region, designated ASO13, COR10, LUG2, TAR5, TAR8, and VISF13, did not match with any of the reference cultivars of SSR olive database. Also in terms of their genotypic structure, these accessions displayed to have an admixed genetic background and showed a membership to the associated cultivar group lower than 70%. These accessions may probably represent new putative hybrid progenies or introgressions of Veneto cultivars or could be local ancient cultivars distributed in the Italian regions with accumulated somatic mutations which may produce intra-cultivar variation, as already reported for other olive genotypes (Baali-Cherif and Besnard 2005; Marra et al., 2013; Diez et al., 2015; Banilas et al., 2003; Baldoni et al., 2006; Belaj et al., 2007; Belaj et al., 2010; Besnard et al., 2013). Furthermore, not only these accessions revealed high level of admixture haplotype but also there were four accessions, designated MON7, PAR3, TDB10 and VISF27, which represented novel and peculiar olive genotypes probably maintained through asexual propagation methods. Consequently, these admixture accessions may represent a valuable resource of genetic variation for future breeding programs of local olive oil cultivars and for establishing the Veneto olive germplasm collection.

The PIC value of the SSR marker loci used in this study was very high and this finding confirmed that all chosen loci are highly variable, informative and suitable for systemic individual cultivar identification and cultivar group discrimination in olive. Statistically, a total of 110 marker alleles over ten SSR genomic loci utilised in this study were produced with an average number of observed alleles per locus n_a=9. This number is comparable with the 135 marker alleles with an average of 9.6 per locus published by Lopes et al. (2004), and 67 marker alleles with an average 8.4 found among Sicilian accessions reported by Las Casas et al. (2014). Similarly, 75 marker alleles with an average of 6.8 were reported for olive cultivars in Southern Italy by Muzzalupo et al. (2009), and higher than 104 marker alleles with average 3.6 reported by Cipriani et al. (2002). The higher number of markers alleles, equal to 159 with an average of 13.2, noticed by Sarri et al. (2006), may be explained by the

higher number of accessions considered, coming from fourteen Mediterranean countries, and different multi-locus SSR markers.

The observed heterozygosity values of each cultivar group were significant compared with the expected values and Nei's genetic diversity, equivalent to the expected heterozygosity, indicating that the significant excess of heterozygous loci in the olive genome and the Veneto olive populations considered may exhibit a high level of outbreeding. This is also confirmed by the negative values of Wright's inbreeding coefficients. The total value of observed heterozygosity for Veneto olive cultivar groups appeared very high (H_o=91.9%) in comparison to the previously reported ones by works on Italian accessions, *e.g.* 65% by Sarri et al. (2006), 62% by Marti et al. (2015), 60% by Muzzalupo et al. (2014), suggesting a remarkably large genetic variation amongst the Veneto germplasm.

Nowadays, 79 olive collections were descripted and they are located in 24 countries which include about 1,200 cultivars with more than 3,000 different names (Bartolini et al., 1998), underlying the importance attributed to the maintenance of olive tree biodiversity. A large portion of this olive germplasm includes cases of homonymy (*i.e.*, the same name for different cultivars) synonymy (*i.e.*, different names for the same cultivar) and toponyms, extremely frequent among and within olive-growing countries (Barranco et al., 2000b).

Conclusions

Olive (*Olea europaea* L.,) is one of the most economically important widely grown tree fruit crops in the countries of the Mediterranean basin. The fine genotipization of the olive germplasm, eventually throughout the acquisition of new genetic information, is required to better characterize cultivars of great interest from an economic and agricultural point of view. Moreover, conservation of genetic resources is essential to preserve adaptive characters and to use interesting genes for breeding.

Our work represents a first comprehensive and systematic molecular study of the Veneto olive germplasm collection, from the western side of Garda Lake to the Euganean and eastern Trevisan hills. This contributes to the correct assignment of cultivars grown in Veneto within the general molecular database of the national Italian olive cultivars germplasm. We also provide the genetic information about the autochthonous Veneto olive cultivars, along with molecular tools for their genetic authentication, which will represent not only a significant resource for future breeding programs, but also an irreplaceable bank of locally adapted materials for economic valorisation.

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Chapter 2: New insights into the regulation of the stilbene biosynthetic pathway in grapevine

Introduction

Grapevine and its genome

Grapevine (*Vitis vinifera* L.) represents one of the most important economic crop species in the world, with a world production approaching 89 million of tons and a harvest area of over 7 million hectares (FAO STAT 2016). The numerous uses of grapes have made it one of the top crop species in many Mediterranean countries such as Italy, France, Spain, but also in the rest of the world, with well-established productive realities such as Australia, South Africa and New Zealand.

V. vinifera L. includes the cultivated subspecies *vinifera* (*V. vinifera* subsp. *vinifera*) and the wild subspecies s*ylvestris* (*V. vinifera* subsp. *Sylvestris*). Both species are inter-fertile and the morphological differences between them are probably the result of a prolonged domestications process. One of the most obvious morphological distinction between wild and cultivated grapevine is related to the reproductive organs, with wild grapevine being substantially dioecious (male and female plants), and cultivated grapevine being monoecious, carrying self-fertile hermaphrodite flowers (This et al., 2006). The domestication and cultivation of grapevine probably started in the geographical area between the Black Sea and Iran during the period from the fourth and the seventh millennium before Christ (BC). Its cultivation was then subjected to a slow and continuous diffusion, firstly in close regions such as Mesopotamia and Egypt, then within the Mediterranean basin and lastly over the entire Europe during the Roman époque. In Italy, the oldest affirmations of grapevine cultivation and development goes back to the IXth century BC (Terral et al. 2010). According to Lacombe et al. (2011), the number of grapevine varieties included in the Italian National Catalogue in 2010 is up to 548 varieties, its production reached more than 8 million tons, and the area harvested achieved over 702.100 hectares in 2013 according to the Food and Agriculture Organization of the United Nations (FAOSTAT 2016). http://faostat3.fao.org/download/Q/QC/E.

In the last decade, the will to increase the economic competitiveness linked to grapevine cultivation in terms of quality and productivity, and the need to adapt it to the new geographical areas depletion, climate changes, the advent of new diseases and the market demands, led to a remarkable increase of physiology and pathology studies on this species, together with the development of tools and genetic resources aimed at its improvement.

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 (IASMA; Velasco et al., 2007). The French-Italian sequencing was obtained by the selection of the PN40024 line, a particular clone characterized by a high degree of homozygosity (approximately 84%) and obtained through multiple auto-fecundation cycles in order to by-pass the high heterozygosis that characterize grapevine. The most updated genome sequence available on line is the 12X v2 prediction hosted at CRIBI (http://genomes.cribi.unipd.it/grape/) (Vitulo et al. 2014). This version improved the previous one with 2,258 new coding genes. To date, the actual number of gene predictions within the PN40024 genome is equal to 31922.

The availability of a well-annotates genome led the grapevine research to quickly step up with an increasing number of studies on gene function and regulation in this species.

Abiotic stress and plant responses

Abiotic stresses are defined as environmental conditions that reduce growth and yield beneath ideal levels (Wang et al., 2003). According to Cramer et al. (2011) there is a vast range of abiotic stresses affecting plants including those ones related to the water status (e.g. drought and flooding), temperature (e.g. cold, chilling, freezing and heating), light (e.g. ultraviolet UV-C, high or low), chemical soil content (e.g. salinity, presence of heavy metals, mineral deficiency or low fertility, mineral toxicity, acidity, presence of air pollutants, etc.) and others (e.g. wind, wounding etc.). These stresses are rarely experienced singularly, but they often occur in combination. Consequently, plants have evolved different signalling pathways to actively and dynamically respond to different environmental stimuli. One example is represented by the primary signal related to Reactive Oxygen Species (ROS) (Noctor and Foyer 1998; Grene, 2002; Kawano, 2003; Laloi et al., 2004; Mittler et al., 2004; Cramer et al., 2011; Mittler et al., 2011; Molassiotis and Fotopoulos, 2011; Ben Rejeb et al.,

2014). ROS, such as H_2O_2, are substances continuously produced in the plants under normal conditions (Finkel and Holbrook, 2000). Abiotic stress factors can perturb the balance between both ROS production and their scavenging, leading to an excessive production of these components that can lead to damage as well as death of plant because ROS accumulation causes oxidative stress (Apel and Hirt, 2004). During the course of evolution, plants were able to achieve a high degree of control over ROS accumulation, but now ROS is a key signalling molecule in plants. For example, under cold stress, ROS activate mitogen protein kinases (MAPK) which are imperative for cold acclimation in Arabidopsis (Teige et al., 2004).

Another signal acting together with ROS is represented by Reactive Nitrogen Species (RNS) which play an important role in enzyme activity and gene regulation in response to stresses (Mittler et al., 2011).

Also hormones, such as abscisic acid (ABA), Jasmonic acid (JA), gibberellic (GA) and ethylene play an important role in response to abiotic stress; for example, ABA controls the stomatal opening and closure regulating the plant water and ions status. (Abe et al., 1997; Pastori et al., 2002 ; Cao et al., 2011; Wang et al., 2011; Zhang et al., 2011a ; Deinlein et al., 2014; Ferrandino and Lovisolo, 2014).

Grapevine secondary metabolism provides an important line of defense against abiotic stress. For instance, the synthesis of major class of phenolic compounds “phytoalexins”. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathway in plants (Randhir et al., 2004). Phenolic compounds play an important role in growth and reproduction, providing protection against pathogens and predators (Bravo, 1998), besides contributing towards the colour and sensory characteristics of fruits and vegetables (Alasalvar et al., 2001). Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Bravo, 1998). Despite this structural diversity, the group of compounds are often referred to as “polyphenols”. Most naturally occurring phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (Harborne 1989; Harborne et al., 1999; Shahidi et al., 1995).

Phytoalexins are low molecular weight, lipophilic, antimicrobial component that accumulate quickly at the site of incompatible plant-pathogen interaction, and act transiently as toxins which are readily degraded or polymerized by extracellular peroxidases (Barz et al., 1990a). The accumulation of phytoalexins, along with lignification, suberisation, callose formation and the production of agglutinins and inhibitors of extracellular microbial hydrolases, gives off an impression of being a piece of a multi-component response mechanism associated with disease resistance and wound repair as well. (Kuć, 1984; Kuć and Rush 1985 and Barz et al., 1990b). Not only phytoalexins have biological activity on an extensive variety of pathogens being accumulated in plants as a response to biotic stress, but they also involved in a wide-range response to many abiotic stresses. Most phytoalexins produced by the plants as secondary metabolites belong to six isoflavonoid classes: isoflavones, isoflavanones, pterocarpans, pterocarpenes, isoflavans and coumestans and small number of legumes produce non-isoflavonoid phytoalexins such as furanoacetylenes and stilbenes (Jeandet et al., 2014). The majority of phytoalexins found in different plant families are phenolic compounds emanates from phenylpropanoid pathway which is one of the most important plant secondary metabolism pathway and it is involved in the synthesis of a wide variety of important natural products from plants including flavonoids, lignin, coumarin, and particularly resveratrol and stilbenes in the *Vitaceae* lineage (Langcake and Pryce, 1976; Vogt, 2010 and Jeandet et al., 2010, 2013).

Stilbene: definition, role and biosynthesis

Stilbenes are small group of phenylpropanoid compounds (PP) produced and accumulated in a relatively small number of unrelated plant species (approximately 72 plant species) including grapevine (Langcake and Pryce, 1976). Plants accumulate these compounds in leaves, roots and fruits in response to both biotic and abiotic stresses such as mechanical wounding, UV-C exposure, pathogen infection, and chemicals treatment (Dixon and Paiva 1995; Bais et al., 2000; Chiron et al., 2000; Pezet et al., 2003; Yu et al., 2005; Parage et al., 2012; Vannozzi et al., 2012). In the last decade stilbenes had raised the interest of many scientists not only because of their role in the plant protection against environmental stresses, but also because of their pharmacological properties, such as the prevention and protection from cardiovascular and neurodegenerative diseases, cancer, and diabete (Morales et al.,

2000; Baur et al., 2006; Yu et al., 2008; Jeandet et al., 2014). Most plant stilbenes, including those ones detected in grapevine (Langcake and Pryce, 1976), are derivatives of the basic unit trans-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene). The term "Resveratrol" was first mentioned in a Japanese article in 1939 by M. Takaota, who isolated this compound from the poisonous medicinal *Veratrum album*, var. grandiflorum. The name probably come from the fact that this compound is a resorcinol derivative coming from a *Veratrum* species (Takaota, 1939). The biosynthetic pathway leading to the biosynthesis of resveratrol is a side branch of the general phenylpropanoid pathway and can be considered as an extension of the flavonoid one (Vannozzi et al., 2012).

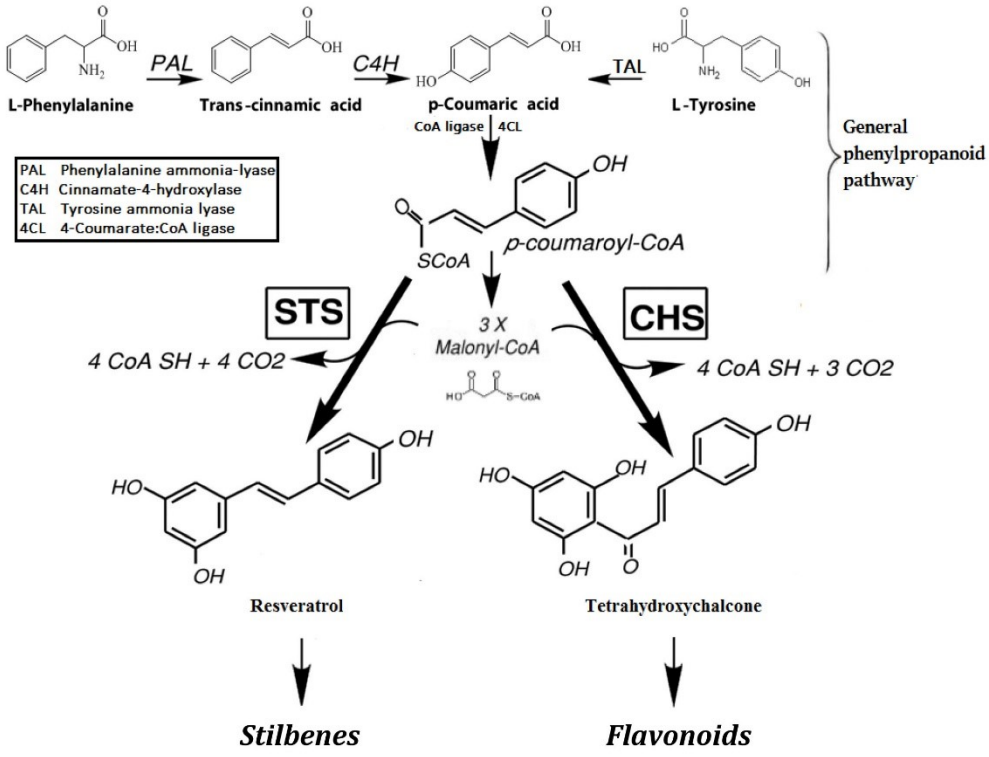

Figure 2.1 – Schematic view of general phenylpropanoid metabolism including stilbenes and flavonoids pathway.

The general phenylpropanoid pathway starts with phenylalanine (Figure 2.1). The first enzyme of the pathway, phenylalanine ammonia lyase (PAL), catalyses the deamination of phenylalanine to produce cinnamic acid, which acts as the precursor for all phenylpropanoid compounds (Calabrese et al., 2004; MacDonald and D'Cunha, 2007). Cinnamic acid 4-

hydroxylases (C4H) catalyses the introduction of a hydroxyl group at the *para* position of the phenyl ring of cinnamic acid, giving rise to coumaric acid (Munro et al., 2007; Rosler et al., 1997); the final thioester-activation step of the general phenylpropanoid pathway is catalysed by the enzyme *p*-coumaroyl: CoA ligase (4CL), a member of the ubiquitous AMP-producing adenylating enzymes superfamily (AAE). The carboxyl group of *p*-coumaric acid is activated by the formation of a thioester bond with CoA to produce *p*-coumaroyl-CoA (Dixon and Paiva 1995; Ferrer, 1999; Kodan et al., 2002; Emiliani et al., 2009; Jeandet et al., 2010 and Jeandet et al., 2014).

In some specific cases, the use of tyrosine as beginning substrates reduces the number of steps of the general phenylpropanoid pathway from three to two (Hwang et al., 2003). However, in all plants characterized to date, the *p*-coumaroyl end product of the phenylpropanoid pathway represents the substrate entering the flavonoid pathway. Chalcone synthases (CHSs; Jez et al., 2000, 2001) is the key enzymes responsible for the biosynthesis of all flavonoids in plants. On the other hand, stilbene synthase (STS) is the key enzyme leading to the production of resveratrol (Stilbene). Although representing the two key enzymes for the two competing flavonoid and stilbene pathways, the two enzymes, which compete for the same substrate, e.g. the *p*-coumaroyl-CoA, are very close in terms of primary structure. As a matter of fact, stilbene synthase (STS) is a member of the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs; Chong et al. 2009).

STSs are classified into two types: (i) *p*-coumaroyl-CoA specific type, such as resveratrol synthase or (ii) Cinnamoyl-CoA-specific type such as pynosylvin synthase, depending on their preferred starter molecule (Figure 2.2). Previous studies clearly reported that the first type occurs mainly in angiosperms such as peanut (Schnöpper et al.,1988), grapevine (Melchior and Kindl, 1991) and sorghum (Yu et al., 2005), while the latter type is typically found in gymnosperms such as *P. sylvestris* (Fliegmann et al., 1992), *P. strobus* (Raiber et al., 1995), and *P. densiflora* (Kodan et al., 2002).

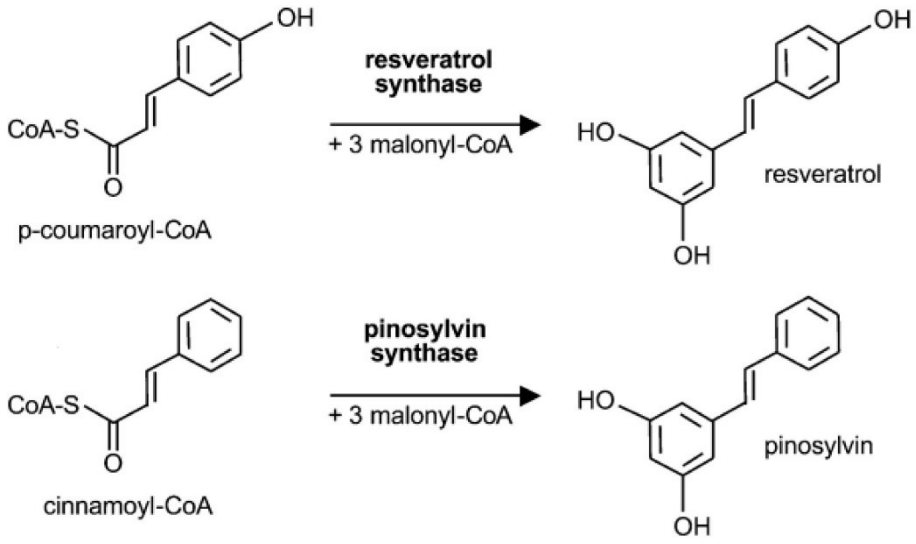

Figure 2.2 – An example of STS and STS-relevant enzymes (modified from Chong et al., 2009).

The first STS protein was firstly extracted and purified in 1984 from a suspension of peanut (*Arachis hypogaea*) cell cultures subjected to stress (Schnöpper and Kindl, 1984). Likely to CHSs, STSs are proteins of approximately 390 amino-acid residues in length, depending on the species considered. A preserved cysteine residue, located in the central section of these proteins has been shown to be fundamental for the catalytic activity of both STS and CHS enzymes and represents the binding site for the *p*-coumaroyl-CoA starting substrate (Lanz et al., 1991). The region around this active site is well conserved and can be used as mark design for CHS and STS. STS proteins demonstrate a high level of similarity based on sequence homology, sharing a proportion of identity level ranging from 75 to 90% with CHS (Ferrer et al., 2008). Moreover, the phylogenetic analysis of 34 CHS and four *STS* gene sequences demonstrated that *STS* genes might have developed from *CHS* genes several times in the course of evolution (Tropf et al., 1994). This hypothesis was reinforced by the observation that, two *STS* genes in peanut revealed extensive homology to *CHS* throughout the coding region, the position of the single intron was conserved in both genes (Schröder et al., 1988). *STS* genes have been identified and cloned from peanut (*A. hipogaea*) and Eastern white pine (*P. strobus*) (Schröder et al., 1988 and Raiber et al., 1995). Then, from scots pine (*P. sylvestris*), which has a small multigene family of at least five pynosylvin synthase genes

(*PST1, PST2, PST3, PST4 and PST5*) (Preising-Müller et al., 1999) and Japanese red pine (*P. densiflora*) which have three members (*PdSTS1, PdSTS2 and PdSTS3*) (Kodan et al., 2002). Only one *STS* gene has been isolated from Japanese knotweed (Liu et al., 2011), and the sequencing of sorghum genome has shown that *SbSTS1* was the only *STS* gene in this plant monocotyledon species (Yu et al., 2005; Paterson et al., 2009). In grapevine, a recent analysis of the STS multigenic family based on both the PN40024 and PN ENTAV 115 genomes (Jaillon et al., 2007; Velasco et al., 2007) led to the identification of 48 putative *VvSTS* gene sequences, with at least thirty-three full length sequences encoding *VvSTS* genes identified (Vannozzi et al., 2012). These ones, based on predicted amino acid sequences, cluster in 3 principal groups designated A, B and C. The majority of *VvSTS* genes cluster in groups B and C and are located on chr16 whereas the few gene family members in group A are found on chr10 (Vannozzi et al., 2012).

The transcriptional regulation of stilbene synthases

A number of different factors are required in regulation and control of the transcription process. Amongst them, transcription factors (TFs) represent one of the main ones. These proteins are able to recognise specific sites in the promoter of target genes in a sequence-specific manner and to regulate the frequency of initiation of transcription (Stracke et al., 2001). TFs are proteins, which came in numerous shapes and sizes, and can be divided into a number of functional classes. (i) activators and repressors (ii) co-activators or co-repressors. (iii) general transcription factors and (iv) architectural transcription factors (Singh, 1998). DNA-binding domains is a distinct feature of TFs that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. Some transcription factors bind to a DNA promoter sequence near the transcription start site and help forming the transcription initiation complex. Other transcription factors bind to regulatory sequences, such as enhancer sequences, and can either stimulate or repress transcription of the related genes. These regulatory sequences can be thousands of base pairs upstream or downstream from the gene being transcribed. In eukaryotes, gene expression is often regulated by multi-protein complexes. Based on similarities in the DNA binding-domain, TFs are grouped into different families such as MYBs, helix-loop-helix, zinc finger which is the shortest domains, helix-turn-helix, leucine zipper, scissors, MADS cassettes, etc. (Du, 2009).

In recent years, a growing number of TFs involved in the regulation of flavonol, lignin, or anthocyanin biosynthesis have been isolated, most of which belonging to the MYB TFs family. MYB proteins are one of the most plentiful classes of TFs in plants, the majority of which belong to the R2R3-MYB family, so called because of the two adjacent sequence repeats within the binding domain (Dubos et al., 2010). R2R3-MYB TFs are thought to descend from an R1R2R3-MYB gene ancestor, by the loss of the sequences encoding the R1 repeat and subsequent expansion of the gene family. The R2R3-MYB regulatory genes constitute a large family of more than 100 members in higher plant species including Arabidopsis (Stracke et al., 2001), maize (Du et al., 2012a), soybean (Du et al., 2012b), apple (Cao et al., 2013) and pear (Feng et al., 2015). For instance, in Arabidopsis (*Arabidopsis thaliana*) the analysis of the complete genome sequence led to the identification of 126 genes belonging to the R2R3-MYB family (Stracke et al., 2001). A similar analysis of the pear *(Pyrus bretschneideri Rehd.)* genome sequence led to the identification of 184 R2R3-MYB genes (Feng et al., 2015).

The first survey of R2R3-MYB family members in grapevine identified 108 genes based on the annotated genome sequence of the homologous PN40024 genotype of *V. vinifera* cv. Pinot noir (Matus et al., 2008). Wong et al. (2016) represent the latest research on R2R3-MYB transcription factors (TFs) in grapevine. They identified 134 genes using microarray and RNA-Seq data and these genes were systematically named according to the Super-Nomenclature Committee.

The study of plant R2R3-MYBs started with the characterization of the first gene encoding a transcription factor from maize (*Zea mays*) *COLORED1* (*ZmC1 or ZmPl1*) (Paz-Ares et al., 1987) which regulates genes encoding enzymes of the anthocyanin biosynthetic pathway. The R2R3-MYB gene family has been shown to be involved in several physiological and biochemical processes such as the regulation of secondary metabolism (Nesi et al., 2001; Baudry et al., 2004), control of cell morphogenesis (Lee and Schiefelbein, 1999, 2001; Higginson et al., 2003), meristem formation and floral and seed development (Shin et al., 2002; Steiner-Lange, 2003) and the control of the cell cycle (Ito et al., 2001; Araki et al., 2004). Also, R2R3-MYB TFs were also suggested to be involved in the response of plant cells to biotic and abiotic stress (Abe et al., 2003; Denekamp and Smeekens, 2003; Nagaoka and Takano, 2003; He et al., 2016). R2R3-MYB TFs family is a common denominator in the

regulation of phenylpropanoid-derived compound (Lai et al., 2013). In grapevine (*Vitis vinifera* L.), represent one of the most studied plants according to the study of R2R3-MYB TFs involvement in the flavonoid biosynthetic pathway, MYB TFs MYB5a and MYB5b (Deluc et al., 2006, 2008) and *VvMYBPA1* (Bogs et al., 2007) demonstrated to modulate several branches of the flavonoid pathway, regulating structural genes, such as LEUCOANTHOCYANIDIN REDUCTASE (LAR), ANTHOCYANIDIN REDUCTASE (ANR), FLAVONOID 39-HYDROXYLASE, and CHALCONE ISOMERASE leading to the production of proanthocyanidins (PAs). Another study on MYB TFs reported that MYBA1 and MYBA2 regulate UDP-Glc: FLAVONOID-3-0-GLUCOSYLTRANSFERASE (UFGT), which encodes an enzyme responsible for the conversion of anthocyanidins to anthocyanins (Figure 2.3) (Kobayashi et al., 2002; Walker et al., 2007). Recently, Wong et al., (2016) found that MYB108B co-expressed with *ANTHOCYANIN GLUTATHIONE S-TRANSFERASE 4* (*GST4*), the gene involved in anthocyanin transport into vacuoles.

For what concern STS pathway in grapevine, Höll et al. (2013) illustrated two TFs belong to R2R3-MYB–type TFs, so-called *VvMYB14* and *VvMYB15*, play an important role in the transcriptional regulation of the stilbene biosynthetic pathway in grapevine under normal and stressed conditions, transcript analyses showed a strong correlation between the expression

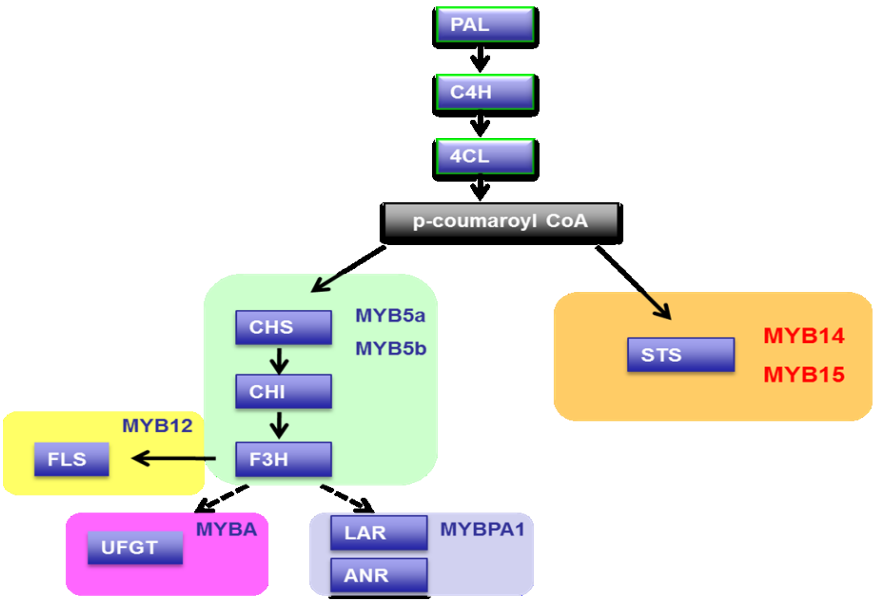

Figure 2.3 – Schematic representation of the branch pathways for flavonoid and stilbene production and the R2R3-MYB TFs known to be involved in their regulation, Höll et al. (2013).

of *VvMYB14* and *VvMYB15* and selected members of the STS gene family and the subsequent accumulation of stilbenes (Figure 2.3). Then, Wong et al. (2016) confirmed these results using network co-expression analysis (GCN). The GCN results showed that the resveratrol-related *VviMYB14* and *VviMYB15* TFs share common co-expressed *VvSTS* genes with the uncharacterized *VviMYB13*.

The WRKY transcription factors family

Together with R2R3-MYB TFs, WRKY proteins represent another family of transcriptional regulators playing crucial roles in the response to both abiotic and biotic stresses being also central components of the innate plant immune system (Eulgem et al., 2000; Lai et al., 2008; Chen et al., 2012; Bakshi and Oelmüller, 2014; Gao et al., 2014). Genome-wide studies of the WRKY gene family have provided a description of this gene family in many plant species including *Arabidopsis* (Eulgem et al., 2000), rice (*Oryza sativa;* Ross et al., 2007), *Brachypodium distachyon* (Tripathi et al., 2012), soybean (*Glycine max*; Yu et al., 2016), cotton (*G. raimondii* and *hirsutum;* Dou et al., 2014), *Medicago truncatula* (Song and Nan, 2014), shrub willow (*Salix suchowensis*; Bi et al., 2016), Peach (*Prunus persica*; Gu et al., 2016), grapevine (*Vitis vinifera* L.; Wang et al., 2014); white pear (*Pyrus bretschneideri*; Huang et al., 2015) and bean (*Phaseolus vulgaris*; Wang et al., 2016).

The term "WRKY" derives from the highly conserved WRKYGQK motif within the N-terminal region of the DNA binding domain (DBD). This region, which is about 60 to 70 amino acid in length, is able to recognize a particular *cis* element (TTGACC/T) within the DNA promoter of target genes, called W-box (Ulker and Somssich 2004; Rushton et al., 2010; Chen et al., 2012; Bakshi and Oelmüller, 2014 and Gao et al., 2014). The C-terminal region of the WRKY DBD contains either a Cys2-His2 or Cys2-His-Cys zinc-finger motif (Figure 2.4; Eulgem et al., 2000; Zhang and Wang, 2005; Chen et al., 2012). Based on the number of WRKY domains and the pattern of the zinc-finger motif, the WRKY TFs have been divided into three main groups (I, II and III). Group I WRKYs typically contain two WRKY domains whereas group II and group III members only contain one WRKY domain (Eulgem et al., 2000; Yamasaki et al., 2005). Moreover, up to five subgroups (IIa, IIb, IIc, IId, and IIe) are recognized in the group II WRKY TFs (Eulgem et al., 2000).

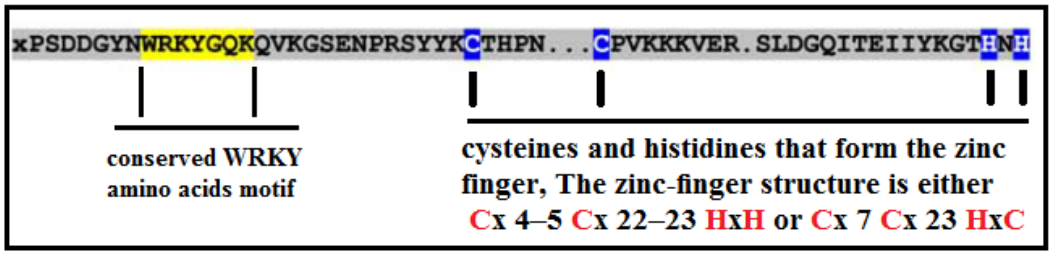

Figure 2.4 – Simple representation of WRKY domain in plants.

Since the first WRKY gene was cloned and characterized from sweet potato (Ishiguro & Nakamura, 1994), many studies have been performed in several higher plants. To date, numerous WRKY proteins have been described to be associated with the response to biotic and abiotic stresses such as wounding, chemical treatments, UV radiation, pests, pathogens, drought, salt, heat shock, freezing, cold adaptation and nutrient deficiency (Chen et al., 2009, 2010a, 2012; Hara et al., 2000; Skibbe et al., 2008; Naoumkina et al., 2008; Phukan et al., 2016; Dang et al., 2013; Johnson *et al*., 2002; Liu et al., 2014; Mao et al., 2011; Göhre et al., 2012; Okay et al., 2014; Shi et al., 2014; Wang et al., 2014; Yu et al., 2016). Moreover, WRKY TFs were also demonstrated to be involved in the regulation of developmental processes, such as germination, embryogenesis, seed development and leaf senescence (Jiang and Yu, 2009; Lagace and Matton, 2004; Robatzek and Somssich, 2001; Zhang et al., 2011b).

Finally, WRKY TFs have a key role in many processes of hormone-mediated signal transduction including the signalling pathways related to gibberellic acid (GA) (Zhang et al., 2004), abscisic acid (ABA) (Zou et al., 2004), salicylic acid (Li et al., 2004; Du and Chen, 2008) and Jasmonic Acid (JA) (Li et al., 2004; Marchive et al., 2013; Schluttenhofer et al., 2014). To give an example in grapevine, Marchive et al. (2013) showed that the overexpression of *VvWRKY1* in the transgenic grapevines (*35S::VvWRKY1*) induces expression of JA pathway-related genes and confers higher tolerance to the downy mildew (*Plasmopara viticola*). Furthermore, under abiotic stresses WRKY genes also act as activators of abscisic acid (ABA) signalling-pathway, which represents a key signal to regulate plant growth, developmental processes and plant responses to abiotic and biotic stress (Rushton et al., 2012). For example, some important ABA responsive genes, such as *AtABF4*, *AtABI4*, *AtABI5*, *AtDREB1A*, *AtMYB2* and *AtRAB18*, have been demonstrated to be bound by *AtAD1A* (*AtWRKY40*) through direct interaction with the W-box sequence

upstream of their promoters (Shang et al., 2010). Another function of WRKYs (*WRKY6* and *WRKY75*) in response to different type of stress which is phosphate (Pi) limitation in the soil was illustrated by Nilsson et al. (2010). Hence, we can conclude that WRKY TFs involved in various kinds of stresses and play important roles in the regulation of transcriptional reprogramming linked with plant stress responses.

In grapevine, Wang et al. (2014) identified 59 full-length WRKY genes based on the 12X V1 prediction of the PN40024 genome. Genes were named as *VvWRKY1* to *VvWRKY59* based on their chromosome location according to (Vannozzi et al., 2012). Based on this study the *VvWRKY* family genes were classified into three main groups, and within the same groups and subgroups VvWRKY revealed high degree of similarity in terms of both exon-intron structure and WRKY motif compositions. *VvWRKY* genes play important roles in grapevine growth and development as indicated by their spatial and temporal expression profiles. Group II-a and Group III genes exhibited different patterns of expression in response to different stresses. For instance, *VvWRKY* genes belonging to Group II-a and Group III were expressed in response to drought stress, and represent good candidate genes for exploring the role of *WRKYs* in grapevine stress response pathways.

Aims of the present study

The present study was aimed at extending the actual knowledge on the regulation of stilbenes biosynthesis in grapevine. As previously reported, recently Höll et al. (2013) identified two transcription factors belonging to R2R3-MYB group, namely VvMYB14 and VvMYB15, which are involved in the regulation of the STS pathway. Nevertheless, many questions still remain to be answered concerning the transcriptional regulation of *VvSTS* genes in grapevine, including the existence of other TFs than R2R3-MYBs involved in the regulation of this pathway.

The main goal of this study was to identify and characterize other TFs than R2R3-MYBs (VvMYB14 and VvMYB15) potentially involved in the regulation of the stilbene synthase biosynthetic pathway in grapevine.

The specific objectives described in this study were achieved thorough the following work packages:

I. Co-expression analyses on whole transcriptome datasets to identify TFs other than MYB14 and MYB15 highly correlated with *VvSTS* upon both stressed and unstressed conditions;

II. Real time expression analyses on candidate WRKY TFs, together with *VvSTS* and *MYB14* and *MYB15* genes upon different abiotic stresses;

III. Gene reporter assay to investigate the ability of candidate VvWRKY TFs to regulate the promoter of selected *VvSTS* genes;

IV. Gene reporter assay to investigate the ability of candidate VvWRKY TFs to regulate the promoter of VvMYB14 and VvMYB15 TFs.

Materials and methods

Materials

General solutions and growth media

All chemicals used were analytical or molecular biology grade and obtained from Sigma. Solutions and growth media used in this project are listed Table 2.1. Solutions were prepared with nanopure and autoclaved water when appropriate. Restriction endonucleases were obtained from New England Biolabs®, Fermentas® or Roche®. The Sources of any other supplies used in this project are indicated in the "relevant methods" section.

Table 2.1 – General solutions and growth media. Antibiotics were added according to the purpose of tissue culture.

Solution	Composition
Gel Loading Dye, Blue (6X)	2.5% Ficoll®-400, 11mM EDTA, 3.3mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue, pH 8.0
50X TAE Buffer/L	242gr Tris base, 57,1 ml Glacial acetic acid , 100 mlEDTA 0,5 M (pH 8) and ddH20 up to 1 liter
TE Buffer (pH 7.6)	10 mM Tris-HCl, 1 mM EDTA (pH 8)
LB (liquid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, (pH 7)
LB Agar (Solid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.2% (w/v) bacto-agar (pH 7)
SOC medium	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM, MgCl2, 10 mM MgSO4, 20 mM glucose
Grape Cormier (GC) medium	Gamborg B5 with minimal organics (Sigma medium G5893)3.2 g/l, sucrose 30.0 g/l, casein hydrolysate 0.25 g/l, kinetin 0.93 g/l, NAA 0.54 g/l, pH 5,8 (1N KOH), Agar 0.8%

Oligonucleotide primers

All oligonucleotide primers used in this study were obtained from Sigma-Aldrich (Milan, Italy) or Invitrogen S.r.l. (Milan, Italy) and their sequences are reported in Table 2.2. Primers were designed using Primer3 Plus software (Untergasser et al., 2007; http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Bacterial strains

All cloning procedures were performed using *Escherichia coli* chemically competent cells "One Shot® TOP10" (Invitrogen ™ by life technology) or electro competent cells obtained in the lab starting from the same commercial strain. Propagation of empty gateway vectors was performed using the One Shot® ccdB Survival™ 2 T1R strain (Invitrogen ™).

Table 2.2 – oligonucleotide primers used in this study.

Name	Sequence	Description
EF1-Fw	CGGGCAAGAGATACCTCAAT	qRT-PCR
EF1-Rev	AGAGCCTCTCCCTCAAAAGG	qRT-PCR
VvCHS-1 Fw	GTTTGTGATAAACTATGTGCTACAG	qRT-PCR
VvCHS-1 Rev	GGTTTAGAGAGAGCAACTCCA	qRT-PCR
VvCHS-2 Fw	GAAGATGGGAATGGCTGCTG	qRT-PCR
VvCHS-2 Rev	AAGGCACAGGGACACAAAAG	qRT-PCR
VvCHS-3 Fw	TCGGCTGAGGAAGGGCTGAA	qRT-PCR
VvCHS-3 Rev	AGGGGAAAACCAAATCAGGCA	qRT-PCR
VvSTS29 Fw	GGTTTTGGACCAGGCTTGACT	qRT-PCR
VvSTS29 Rev	GAGATAAATACCTTACTCCTATTCAAC	qRT-PCR
VvSTS41 Fw	GAGTACTATTTGGTTTTGGACCT	qRT-PCR
VvSTS41 Rev	AACTCCTATTTGATACAAAACAACGT	qRT-PCR
VvSTS48 Fw	CTTGAAGGGGGAAAATGCT	qRT-PCR
VvSTS48 Rev	TTACTGCATTGAAGGGTAAACC	qRT-PCR
VvMYB14 Fw	TCTGAGGCCGGATATCAAAC	qRT-PCR
VvMYB14 Rev	GGGACGCATCAAGAGAGTGT	qRT-PCR
VvMYB15 Fw	CAAGAATGAACAGATGGAGGAG	qRT-PCR
VvMYB15 Rev	TCTGCGACTGCTGGGAAATC	qRT-PCR
VvWRKY03 Fw	GGAAGTATGGGCAAAAAGCTGTC	qRT-PCR
VvWRKY03 Rev	AAGCATAAACTTGCATCTGGCT	qRT-PCR
VvWRKY43 Fw	AGGAAGTACGGTCAAAAGGCA	qRT-PCR
VvWRKY43 Rev	GGAGGAGCTTATTAGGGAGCG	qRT-PCR
VvWRKY53 Fw	CTAATGGTGGAACCCATGCT	qRT-PCR
VvWRKY53 Rev	GCAATGCAGGAGGAAGAAAC	qRT-PCR
VvWRKY03 GW Fw	CACCATGGATAGCTTCTCCACTCTCTTTC	Cloning
VvWRKY03 GW Rev	TTAAAAGGAAGCATAAACTTGCATC	Cloning
VvWRKY43 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGAGAATTATCAGAACTTCTTCGCTTG	Cloning
VvWRKY43 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGGGAGCGTAGATTTGCATCTGATT	Cloning
VvWRKY43 seq Rev	TTCAGCTTTGGCTGATTCAA	Sequencing
VvWRKY53 GW Fw	CACCATGAACAAGGAAGAAAGAGCTA	Cloning
VvWRKY53 GW Rev	TTAACTAGTGTGCTCTCTATTTTCAGC	Cloning
VvWRKY53_seq Rev	TCAAGTATATCAACCTGGCTCCT	Sequencing

Plant material

Grapevine leaves for promoter and gene amplification and for quantitative RT-PCR analyses were obtained from plants grown under glasshouse conditions at the experimental farm "Lucio Toniolo" of the University of Padova (45° 21' N, 11° 58' E) (Legnaro, PD, Italy). *V. vinifera* cv Pinot noir (clone 115 on K5BB rootstock) canes were obtained from a certified nursery (Vitis Rauscedo, Pordenone, Italy). For expression analyses upon stress treatments leaves of similar age, based on similar size and node position, were collected from the plants and immediately stored on ice until treatment.

Methods

Preparation of electro-competent TOP10 *E. coli* cells

One tube (50 µl) of One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen™ by life technology) was streaked out on a LB-plate and incubated at 37°C overnight. The next day, a single colony was picked from the LB plate and transferred into 10 ml LB media ("Starter Culture"), and incubated overnight at 37°C on a shaker (ca. 220 rpm). The day after, 4 flasks containing x 250 ml LB medium each were inoculated with the 10 ml (distributed) of the starter culture. The bacteria were grown overnight in Erlenmeyer flasks, optimally to an OD_{600} of 0.35 - 0.4. The cultures were filled into pre-chilled (4°C) centrifuge bottles and centrifuged at 1000 *g* for 20 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 200ml ice cold H_2O and again centrifuged at 1000 *g* for 20 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 100 ml ice-cold H_2O. Two suspensions each were combined and centrifuged at 1000 *g* for 20 minutes at 4°C. After discarding the supernatant again, the pellets were resuspended in 40 ml ice-cold 10% glycerol, transferred into a 50 ml falcon tube and centrifuged as previously indicated. The supernatant was decanted and discarded and the pellets were resolved in 1 ml of 10% glycerol. The suspension was aliquoted (40µl each) into 1,5 ml screw-cap tubes and snap frozen in liquid nitrogen. The competent cells were stored at -80°C until using.

Preparation of electro-competent *Agrobacterium rhizogenes* strain (A4)

One tube of commercial competent *Agrobacterium rhizogenes* strain (A4) was streaked out on a LB-plate and incubated at 28°C overnight. After three days, a single colony was picked from the LB plate and transferred into 10ml LB ("Starter Culture"), and incubated overnight at 28°C. On day 5, 5ml of the overnight culture were transferred into each of two fresh 400ml LB flasks and grown at 28°C. When OD600 reached 0.3-0.6 the cultures were filled into pre-chilled (4°C) centrifuge bottles and centrifuged at 2000g for 10 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 200ml ice cold H_2O and again centrifuged at 2000g for 10 minutes at 4°C. The supernatant was discarded and the pellets resuspended once more in 100ml ice-cold H_2O and again centrifuged at 2000g for 10 minutes at 4°C. After the supernatant was discarded again, the pellets were resuspended in 40ml ice-cold 10% glycerol, transferred into a 50ml falcon tube and centrifuged as before. The

supernatant was decanted and discarded and the pellets were resolved in 1ml of 10% glycerol. The suspension was aliquoted (40µl each) into 1,5ml screwcap tubes and snap frozen in liquid nitrogen. The competent cells were stored at -80°C until use.

Network Gene Co-expression analyses

Two grapevine expression datasets produced by means of NimbleGen technology, the first one obtained from the *V. vinifera* cv. Corvina expression atlas and encompassing 54 samples corresponding to various tissues and organs at different developmental stages (Fasoli et al., 2012), the second one constituted by a 170 sample dataset corresponding to Corvina berries collected at 3 developmental stages (véraison, mid-ripening and harvest) in 3 consecutive years and in 11 different locations in Valpolicella (Dal Santo et al., 2013), were considered for a gene co-expression network (GCN) analysis using Cytoscape v. 3.4.0 software (Shannon et al., 2003). Co-expression analyses were performed considering the whole set of full-coding *VvSTS* genes reported in Vannozzi et al. (2012) and all the transcription factors identified in grapevine based on the Plant Transcription Factor Database (Plant TFDB; Jin et al., 2014) encompassing 1276 loci classified into 58 gene families. With the aim of reducing the complexity of gene correlation networks the Correlation coefficient threshold was set to R=|0.8| and we only considered those edges directly connecting TF to *VvSTS* nodes and not those existing between different TFs.

Phylogenetic analysis of WRKY genes in Arabidopsis, grapevine and peach

The deduced protein sequences of all 56 WRKY full-length genes identified in grapevine based on Wang et al., 2014 were downloaded from the 12X v1 prediction of the PN40024 genome available at the CRIBI (Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative) Biotech website (http://genomes.cribi.unipd.it/). Seventy-one Arabidopsis WRKY amino acids sequences were downloaded from the database of Arabidopsis transcription factors (http://planttfdb.cbi.pku.edu.cn/) and 61 peach WRKY amino acids sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov; Gu et al., 2016) and used for comparative analysis. Alignments of WRKY amino acids sequences from the three species were performed using MUSCLE (Edgar, 2004). By means of MEGA 6.0 software (Tamura et al., 2013), an unrooted phylogenetic tree was constructed. The neighbour-joining

method (NJ; Saitou et al., 1987), with bootstrap values of 1000 iterations, was utilized to conduct the phylogeny test (Tamura et al., 2013).

Phylogenetic analysis, Sequence alignments of candidate VvWRKY TFs with other WRKY transcription factors

The phylogenetic relation between candidate grapevine WRKY genes *VvWRKY03* (VIT_01s0010g03930), *VvWRKY43* (VIT_14s0068g01770) and *VvWRKY53* (VIT_17s0000g05810) and 42 WRKY TFs functionally characterized in other plant species and known to be involved in the regulation of secondary metabolic pathways or in the response to stress, was build using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and MEGA 6.0 softwares. Phylogenetic analyses were performed using the Neighbor-joining (NJ) method. Bootstrap values were calculated using 1000 iterations. The accession number of the 42 other WRKY amino acid sequences used in the phylogenetic analysis were as follows: *AaWRKY01* (FJ390842), *ATWRKY01* (NP_178565.1), *ATWRKY12* (AF404857), *ATWRKY16* (NM_180802.2), *ATWRKY18* (NM_119329.4), *ATWRKY22* (NM_116355.3), *ATWRKY23* (AY052647), *ATWRKY29* (NM_118486.6), *ATWRKY33* (AK226301), *ATWRKY40* (NM_106732.4), *ATWRKY44* (NM_129282), *ATWRKY46* (NM_130204.3), *ATWRKY50* (NM_122518.3), *ATWRKY51* (NM_125877.4), *ATWRKY52* (NM_001344604.1), *ATWRKY60* (NM_001335968.1), *CjWRKY01* (AB267401), *CrWRKY01* (HQ646368), *GaWRKY01* (AY507929), *HbWRKY01* (JF742559), *HbWRKY41* (GU372969), *MtSTP* (HM622066), *MtWRKY100577* (EU526033), *MtWRKY100630* (EU526034), *MtWRKY108715* (EU526035), *MtWRKY109669* (EU526036), *NbWRKY08* (AB445392), *OsWRKY13* (EF143611), *OsWRKY45* (AK066255), *OsWRKY53* (AB190436), *OsWRKY74* (XP_015651250.1), *OsWRKY76* (AK068337), *OsWRKY89* (AY781112), *PgWRKY01* (KR060074), *PqWRKY01* (JF508376), *PsWRKY01* (JQ775582), *PtrWRKY73* (Potri.013G153400.1), *SlWRKY73* (NM_001247873), *SpWRKY01* (AK320342), *TcWRKY01* (JQ250831), *VvWRKY01* (*AY585679*), *VvWRKY02* (AY596466).

In order to identify the variations in WRKY core domains, a multiple sequence alignment of 45 WRKY core domains of the same 45 WRKY TFs mentioned above was conducted using T-Coffee (https://tcoffee.crg.cat/apps/tcoffee/references.html; Notredame et al., 2000) and Boxshade (https://www.ch.embnet.org/). The graphic exon–intron structure of candidate

WRKY TFs was obtained from Grape Genome Database (http://genomes.cribi.unipd.it/grape/). The three-dimensional structure predictions were accomplished using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) according to Kelley et al. (2015). Length of protein sequences, molecular weight (MW), theoretical isoelectric point (pI), the protein instability index (II), aliphatic index (AI), and grand average of hydropathicity (GRAVY) of the 3 candidate WRKY proteins were calculated by an online tool provided by Expasy website (https://web.expasy.org/protparam/; Gasteiger et al., 2005).

Mechanical wounding and UV-C treatments

Leaf discs of 10 mm of diameter were punched from healthy leaves detached from glasshouse-grown *V. vinifera* cv. P. noir vines. Discs were obtained from leaves belonging to different plants and showing similar age based on size and node positions in plants, treated with different biotic and abiotic stresses and incubated upside down on 3MM moist filter paper in large Petri dishes until harvest at which point discs were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The punching of discs was considered as a wounding treatment *per se*. In a first experiment, performed for looking at the expression of selected *VvSTSs*, *R2R3-MYBs* and candidate *WRKYs* in a long term stress kinetic, discs were sampled at 0, 2, 4, 6, 8 and 10 h after treatment. In order to check the expression pattern of the same genes in longer stress kinetics, a second time course was carried out. In this case, discs were sampled at early stages post-wounding: 0, 1, 3, 6, 24 and 48 h. Control discs (0 h) corresponded to an unwounded leaf straight detached from a healthy vine. For what concerns the UV-C treatment, according to Vannozzi et al. (2012), discs were punched immediately after detaching of leaves from plants, placed on 3MM moist filter paper, abaxial side up in large square Petri dishes and irradiated at 254 nm (0.36J cm^{-3}) for 10 min, at a distance of 10 cm from the UV-C source. Efficiency of the elicitation treatments under different experimental conditions was determined histochemically evaluating the intensity of auto-fluorescence of discs mounted with the underside up in a lactic acid, glycerol and water mixture (1:1:1, v/v/v) on glass slides under long-wave UV light (365 nm). The intensity of the blue fluorescence observed was correlated with the quantity of resveratrol present in samples. Control discs were not elicited and exposed to normal light conditions. After

treatment, all samples were incubated in the dark at 22 °C. Five discs were randomly chosen from control and UV-C treatments 0, 1, 3, 6, and 24 h.

Extraction of total RNA and first strand cDNA synthesis

Fresh or frozen grapevine leaf tissues were used to extract total RNA using the Spectrum™ Plant total RNA Kit (Sigma) according to manufacturer's instructions. Tissue samples obtained from leaves were ground in liquid nitrogen and 80-110 mg of powder were used for extraction. Total RNA was quantified spectrophotometrically using Nano Drop 2000c UV-Vis Spectrophotometer (Thermo Scientific). Total RNA (1µg) was reverse transcribed by the Transcription First Strand cDNA Synthesis Kit (Roche) using oligo (dT)20 primer according to manufacturer's instructions. Before use in RT-PCRs, cDNA was diluted 1:10 in autoclaved water.

Preparation of DNA samples for sequencing

DNA were dehydrated in miniAmp® at 60 °C using thermo cycle and directly sent to BMR Genomics company (Padova, Italy) to sequence, the oligonucleotide primers used for sequencing were M13 forward or reverse and T7 depending on the specific plasmid used for cloning. Sequence chromatograms were analysed by Chromas software (Invitrogen).

Polymerase Chain Reaction (PCR)

Amplification of DNA was carried out in several condition with different Taq polymerase enzymes depending on the specific requirement. The pre-optimized reaction mix Mango Mix (Bioline®) containing Mango Taq DNA Polymerase was used generally to do colony PCR on a plasmid as a template or normal amplification from cDNA as a template, the reactions were performed in a 15 µl volume with 2 µl of cDNA or 10ng of DNA and oligonucleotide primers at a concentration between 200 and 500 nM. For proofreading amplifications Platinum® Taq DNA Polymerase High Fidelity (Invitrogen™) was used, according to manufacturer's instructions. Alternatively, *PfuUltra II* Fusion HS DNA Polymerase or KOD Hot Start Polymerase were used according to manufacturer's instructions to amplify the ORF of certain gene and the reactions were carried out in 50 µl

volume. Generally thermal cycling consisted of 1-3 min at 94 °C (1 cycle); 30 s at 94 °C, 30 s at 50-60 °C, 30-90 s at 72 °C (26-40 cycles); 10 min at 72 °C (1 cycle).

Agarose gel electrophoresis

Agarose gels 1.0 - 2.0% w/v were prepared with 1X TAE Buffer (Table 2.1), and 1X/L of SYBR Safe® DNA Gel Stain (10.000X). Before loading, DNA Gel Loading Dye, Blue (6X) (Table 2.1) was added to each sample to a final concentration of 1X. Gels were run at approximately 100 V in 1X TAE running buffer and visualized using a short wavelength UV trans-illuminator.

Purification of DNA fragments from agarose gels

Purification of specific DNA fragments from agarose gels after visualization by trans-illuminator was carried out using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 30 µl of TE buffer (Table 2.1) diluted 1:10 in sterile water.

Quantitative real-time PCR analysis

Expression analyses were carried out by quantitative RT-PCR using the "Fast SYBR® Green Master Mix" and the StepOne™ Plus Real-Time PCR System (Applied Biosystems). The samples were analysed in three technical replicates. Each 10 µl reaction contained 5 µl SYBR Green Master Mix, 0.6 µl of each primer, 1 µl cDNA and 2.8 µl H_2O. The thermal cycling conditions used were 95 °C for 10 min followed by 40 cycles of: 95 °C for 15 s, 60 °C for 1 min., and 72 °C for 30 s, followed by a melt cycle with 1 °C increments from 55 to 96 °C. The selection of reference genes in order to normalize the cDNA represents a critical step in any quantitative RT-PCR analysis. After testing its suitability, elongation factor EF1 (GenBank Accession no. AF176496) was selected for normalization of all samples analysed. The expression of each target gene was calculated relative to the expression of elongation factor in each cDNA using StepOne™ Software version 2.1 (Applied Biosystems) to calculate normalized expression values (Yuan et al., 2006), observe melt profiles, extrapolate the concentration and measure primer pairs efficiencies. All primers sequences are reported in Table 2.2.

Transformation of bacteria with recombinant plasmids

Transformations were performed using either *E. Coli* Top10 cells (One Shot®TOP10 or electro competent cells) or electro competent Agrobacterium cells (A4 strains). Regarding the One Shot® TOP10 *E. Coli* competent cell after a short incubation in ice, a mixture of chemically competent bacteria and DNA subjected to a heat shock at 42°C for 45 seconds and then placed back on ice and immediately 250µl SOC (Invitrogen) (Table 2.1) medium were added and the transformed cells were incubated at 37°C for 1h with shaking (220 rpm) and subsequently streaked out on LB agar plates with the respective antibiotic resistant, which were incubated at 37°C overnight. For what concern electro competent *E. coli* or Agrobacterium cells (A4 strains) transformations of plasmids were carried out using a Gene-pulser (Bio rad) for electroporation. The electrically competent cells were thawed on ice, the plasmid was added (1-2µg of plasmid /40 µl of electro cells), and the mixture was transferred into gene-pulser cuvettes and electroporated with 1.80kV, 125µF and 200Ω. Immediately afterwards, 800µl LB medium were added and together with the cells transferred to a 2ml Eppendorf tube. The cells were then incubated at 28°C for 1.5h with shaking (150 rpm) and subsequently streaked out on LB agar plates with the appropriate antibiotic resistant, which were incubated at 28°C overnight.

Cloning of blunt-ended-PCR products

Cloning of blunt-ended-PCR products generated with proofreading Taq polymerase (*PfuUltra II* Fusion HS DNA Polymerase or KOD Hot Start Polymerase) was carried out using the Zero Blunt® PCR cloning kit (Invitrogen™) according to the manufacturer's instructions.

Overexpression constructs for dual reporter luciferase assays

For transient expression of selected VvMYB and VvWRKY genes, the ORF (open reading frame) were amplified from cDNA obtained from UV-C irradiated *V. vinifera* cv. Pinot noir leaf discs. The amplification reaction was performed using KOD Hot Start Polymerase. The band corresponding to the expected size were purified from agarose gels and cloned directly in an entry vector of Gateway technology according to manufacturer's instructions. Reporter

constructs carrying firefly luciferase and the promoter sequences of each *VvSTS29*, *VvSTS41, VvMYB14 and VvMYB15* genes are described in Höll et al. (2013) and were kindly provided by the Centre for Organismal Studies (COS) of the University of Heidelberg. Each of *VvWRKY03*, *VvWRKY43* and *VvWRKY53* ORF was ligated into a pARt7 expression vector under the control of the 35S promoter.

Transient transfection experiments and dual luciferase assay

Dual-luciferase™ reporter assay (DLR, Promega) used in this study, modified according to Czemmel et al. (2009). This approach was chosen because already successfully used to demonstrate the interaction of R2R3-MYB TFs with promoters of genes encoding members of the flavonoid biosynthetic pathway (Bogs et al., 2007; Deluc et al., 2008; Czemmel et al., 2009), and to confirm the role of vvMYB14 and VvMYB15 in the regulation of the VvSTS genes (Höll et al., 2013). In this approach, grapevine cells were co-transfected with a plasmid carrying the ORF of candidate TFs under the control of the CaMV 35S promoter and a Dual luciferase™ reporter plasmid pGreenII-0800-35S-LUC (pLUC) carrying a promoter fragment of selected *VvSTS* genes. This allows the quantification of promoter activity by measuring firefly luciferase activity, which is under control of the selected promoter, and normalising against the *Renilla* luciferase activity, which is under control of a CaMV 35S promoter (Horstmann et al., 2004). A cell suspension of *Vitis vinifera* cv. Chardonnay and Pinot Noir petiole callus cultures maintained on Grape Cormier (GC) medium (Do and Cormier, 1991) were used to perform the transient transfection in grapevine cell cultures as described in Bogs et al., 2007 and Walker et al., 2007. Transient assays were performed with and without VvMYB TF and VvWRKY TF, always in co-presence with a VvSTS promoter, obviously from grapevine and the transfection without VvMYB TF or VvWRKY TF, the missing amount of total plasmid concentration per shot was compensated with empty pART7 vector. Next, cells were incubated for 48h in the dark, at 22°C. After that, the cells were harvested for protein extraction and ground on ice in 200 µl of Passive Lysis Buffer (PLB; Promega) and the lysate were centrifuged 1 minute at 10000 rpm. Ultimately, measurement of the luciferase activities was performed according to the Dual-luciferase™ reporter assay (DLR, Promega), where 10 µl of lysate supernatant were mixed with 25 µl LARII (containing firefly substrate beetle luciferin) and Stop & Glo® (containing *Renilla* substrate

coelenterazine). The light emission, generated by active luciferase in the lysate, was measured with a Lumat LB 9507 Luminometer (Berthold Technologies). The relative luciferase activity was calculated as the ratio between the firefly (*Photinus pyralis*) and the *Renilla reniformis* (control) luciferase. Each experiment was performed with three technical replicates, i.e. three independent bombardments per treatment. The total number of experiments was six. The first one was used for evaluating the quality of results.

Colony PCR

Single colony of transformed cells was picked from LB agar plates or cell liquid culture in order to check the insertion of a construct by colony PCR. The reaction was carried out in final volume 15µl, 7.5µl Mango-Mix, 1µl Fwd. primer and 1µl Rev. primer were mixed and added to either 2µl liquid culture and 3.5µl H_2O or 5.5µl H_2O with a colony from the LB plate and the appropriate thermos cycler program.

Plasmid Purification

Cell liquid cultures were prepared by inoculate one single colony from the confirmed master plate colonies with 3 ml LB and the appropriate antibiotic resistant, incubated overnight. The next day, 2 ml of cell liquid culture were used in order to extract and purified the plasmids with the cloned constructs from the cells using PureLinkTm Quick Plasmid Miniprep Kit (Invitrogen) according to manufacturer's instructions.

Preparation of bacterial glycerol stocks

1 ml of overnight bacterial cell liquid culture with 1 vol. of 40% (v/v) sterile glycerol were added into 1.5 ml screw cap tubes, snap-freezing in liquid nitrogen, and storing at -80 °C.

DNA ligation

All ligations were performed in 10 µl reaction volumes containing an insert: vector ratio of approximately 6:1, 10 units of T4 DNA ligase (M0202) (New England Biolabs) in the supplied buffer and incubated overnight at 16 °C or room temperature.

Butanol precipitation of ligation reactions

In order to eliminate the salt from the ligation buffer and therefore increase the efficiency of transformation, 1ml of n-butanol was added to the samples in a 1.5 ml tube and vortex for 5 seconds, the samples were centrifuged at 11.300g for 10 minutes at room temperature and the supernatant was discarded. 200µl of 70% ethanol was added and the samples were centrifuged as before then the supernatant was discarded and the DNA was dried for 10 minutes at 37°C. Subsequently the pellet was resuspended in 2-10µl sterile dH_2O.

Purification of DNA samples following enzymatic reactions

Purification and concentration of DNA samples after restriction enzyme digestion and PCR was achieved using a PureLink™ Quick Gel Extraction Kit according to the manufacturer's instructions.

Results

Candidate TFs other than R2R3-MYBs potentially involved in the *VvSTSs* regulation

With the aim of identifying other transcription factors involved in the regulation of the stilbene biosynthetic pathway in grapevine, we screened two whole transcriptome expression datasets for genes co-expressed with *VvSTSs*. Figure 2.5 reports the gene co-expression networks (GCNs) obtained from two large datasets obtained with NimbleGen microarray technology: (i) the *V. vinifera* cv. Corvina expression atlas, encompassing 54 samples corresponding to various tissues and organs at different developmental stages (Fasoli et al., 2012) (Figure 2.5 panel A) and (ii) a 170 sample dataset corresponding to *V. vinifera* cv. Corvina berries collected at 3 developmental stages (véraison, mid-ripening and harvest) in

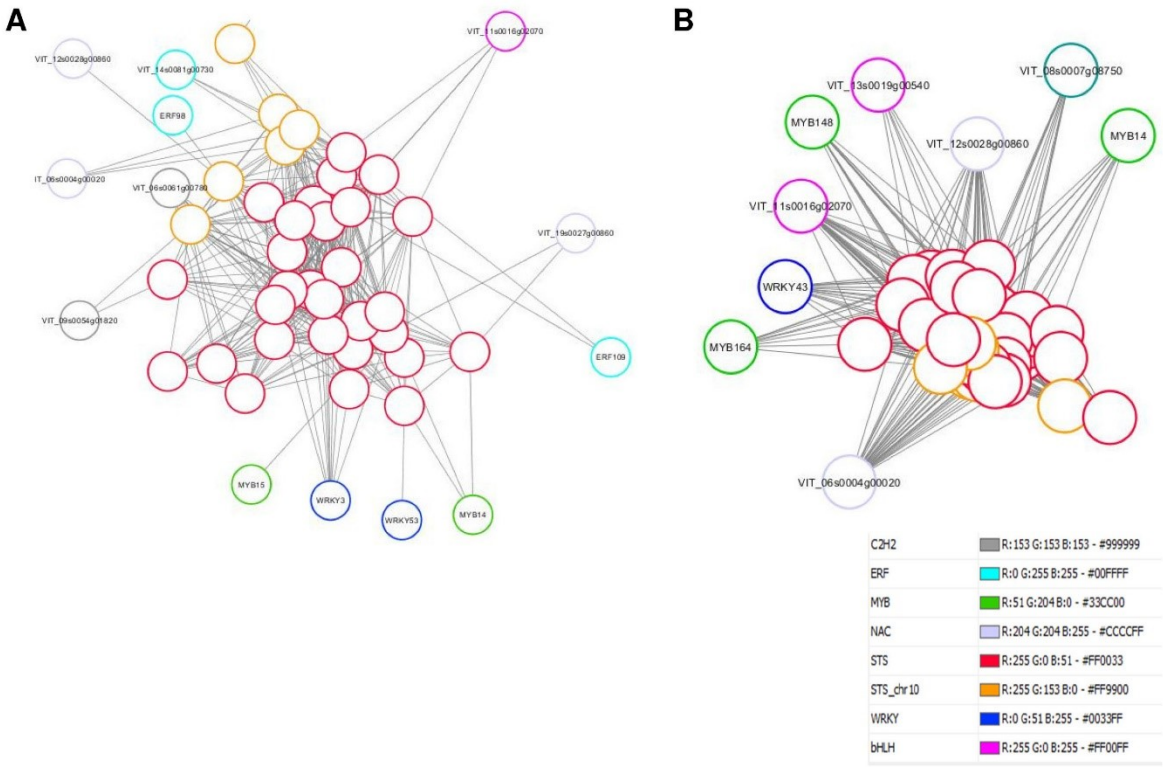

Figure 2.5 – Grapevine STS and TFs Gene co-expression networks (GCN). GCNs were constructed using the Gene expression tool of Cytoscape software with a correlation coefficient R>|0,8| and considering only the interactions between *VvSTSs* and grapevine TFs. The interactions between TFs are not represented. A) GCN obtained from the *V. vinifera* cv Corvina expression atlas (Fasoli et al., 2012) B) GCN obtained from the berry dataset produced by Dal Santo et al. (2013). Nomenclature of WRKY, MYB and ERF factors is based on Wang et al. 2014, Matus et al. (2008) and Licausi et al. (2010).

3 consecutive years and in 11 different locations in Valpolicella (Dal Santo et al., 2013) (Figure 2.5 panel B). In order to reduce the complexity of the correlation analyses we only considered grapevine TFs listed in the PlantTFDB, corresponding to 1276 loci classified into 58 families, together with the 33 full-coding *VvSTS* genes described in Vannozzi et al. (2012) and we only select those edges directly connecting TFs to *VvSTS* nodes excluding those ones existing between different TFs. The Correlation coefficient threshold was set to R=|0.8| considering the large size of the datasets and the observation that higher |R| values only identified interactions between different *VvSTS* members. Amongst those genes showing significant correlations with *VvSTSs* we identified TF belonging to 6 main TF families: R2R3-MYB, WRKY, NAC, ERF, bHLH and C2H2. As expected, looking at the at the Corvina Atlas (Figure 2.5 panel A), those TFs belonging to the R2R3-MYB family corresponded to *VvMYB14* and *VvMYB15*, already characterized and demonstrated to be involved in the transcriptional regulation of *VvSTSs* (Höll et al., 2013). *VvMYB14* was also detected in the berry dataset (Figure 2.5 panel B), together with 2 other MYBs, designated *MYB148* and *MYB164* based on the recent reclassification of R2R3-MYB family (Wong et al., 2016). Interestingly *MYB15* was not detected in the dataset corresponding to *V. vinifera* cv. Corvina berries (Figure 2.5 panel B). Amongst the others, a bHLH (VIT_01s90016g02070) and two NAC genes were represented in both GCNs and deserve further investigation. Concerning the WRKY TFs family we identified three genes, which were significantly correlated with *VvSTS* genes. Two of these, already designated as *VvWRKY03* and *VvWRKY53* by Wang et al. (2014), showed a strong correlation with *VvSTSs* within the Corvina atlas dataset (Figure 2.5 panel A) whereas a third one, designated as *VvWRKY43* (Wang et al., 2014), within the berry dataset (Figure 2.5 panel B).

Candidate WRKY genes belong to the group II WRKY family

In order to contextualize the three WRKYs identified by mean of the co-expression analyses within the full WRKY family, we analysed the whole grapevine WRKY multigenic family together with the ones from peach (*Prunus persica*) and *Arabidopsis thaliana*, considering a total number of 188 accessions. So far, WRKY TFs families have been described in many species whose genome has been sequenced. The choice to compare the WRKY family in grapevine and peach together with a model organism (Arabidopsis) was

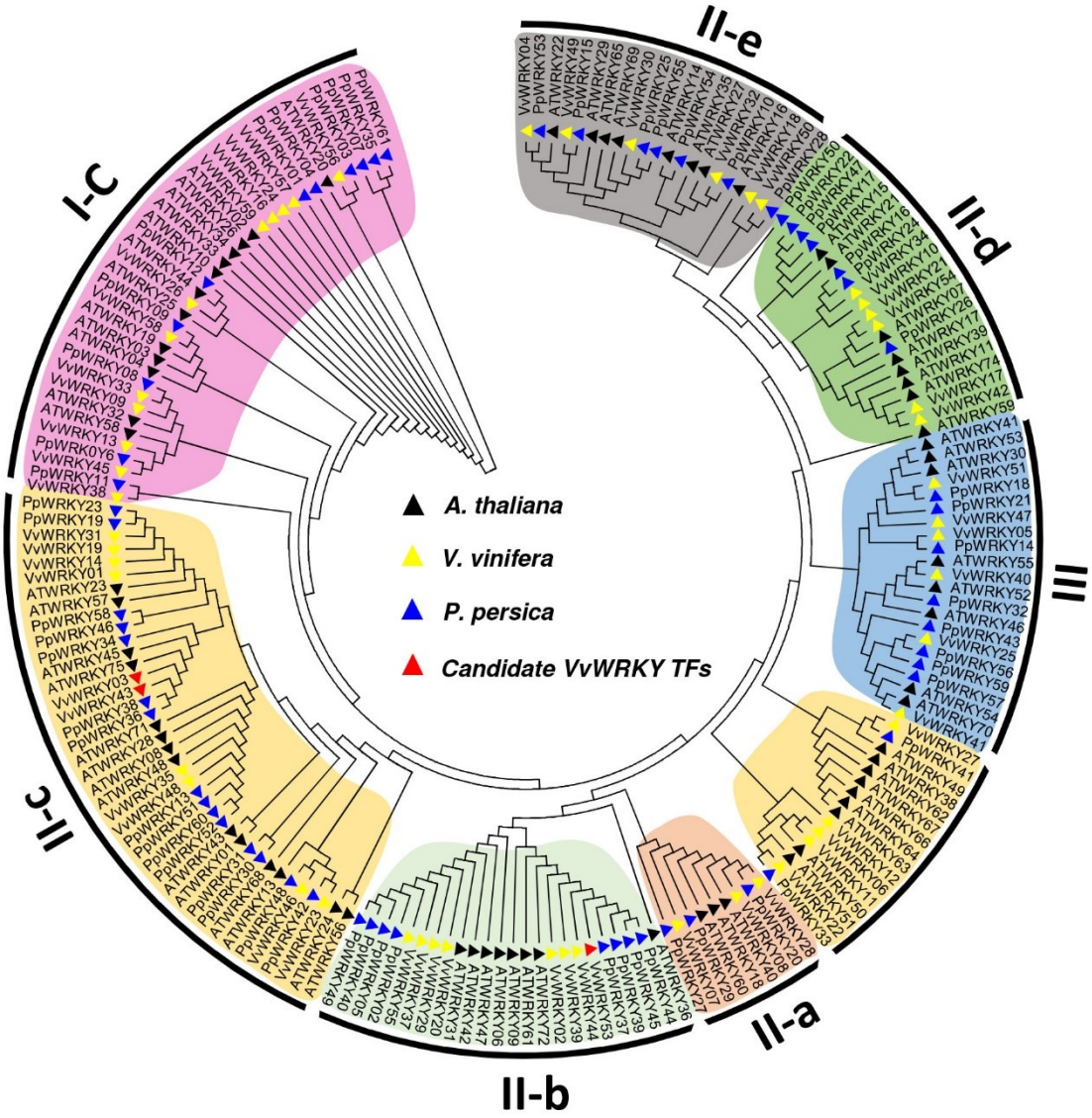

Figure 2.6 – Phylogenetic relationship and subgroup classification of WRKYs from *A. thaliana* (black triangle), *V. vinifera* (yellow triangle and red for three candidates), and *P. persica* (blue triangle). The protein sequences were aligned by Muscle and the unrooted phylogenetic tree was constructed using a Neighbor-joining method with 1000 bootstrap replicates. Each group is indicated by a specific colour.

taken in order simplify the analysis and to consider both a climacteric and a non-climacteric plant (Figure 2.6). According to literature, *WRKY* genes can be classified into three main groups (I, II and III) based on their number of WRKY domains and the pattern of their zinc finger motif (Eulgem et al., 2000). Group I WRKYs typically contain two WRKY domains whereas group II and group III members only contain one single WRKY domain. Group II can be further sub-divided within five subgroups, namely subgroups IIa, IIb, IIc, IId, and IIe. As shown in the phylogenetic tree (Figure 2.6) all the WRKY TFs analysed clustered in these three groups. WRKY TFs from different species in the same group were more similar than those from the same species in different groups. Looking at the candidate transcription factors identified from the co-expression analyses, *VvWRKY03* and *VvWRKY43* were found to cluster within subgroup II-c, whereas *VvWRKY53* clustered within subgroup II-b.

Sequence homology of candidate WRKY with other WRKY TFs

Together with the general phylogenetic analyses described below, we also performed a more detailed analysis considering only those WRKY TFs already characterized from a functional point of view. Multiple amino acid sequences of VvWRKY03, VvWRKY43 and VvWRKY53 were aligned together with 42 other WRKY TFs from other plant species. Among them, 27 WRKYs were involved in the regulation of several branches of the phenylpropanoid pathway, whereas the remaining 15 proteins were related to the plant response to both biotic and abiotic stresses. Results indicate that both *VvWRKY03* and *VvWRKY43* closely cluster with another grapevine TF, namely *VvWRKY01* (Accession number: AY585679) and with *CjWRKY01* (Accession number AB267401), while *VvWRKY53* is closely associated with *SlWRKY73* (Figure 2.7). Figure 2.8 shows the alignment detail of candidate grapevine WRKYs together with the closes WRKYs from other species. Since the complete sequences of each WRKY protein showed low levels of identity and similarity even within the same species, we further narrowed the region of analysis of the 45 WRKYs previously mentioned to only include the core WRKY domains of these proteins. To better understand, the same 45 amino acids and only the regions containing the WRKY motif and the zinc-finger motif (WRKY core domains) were used for comparison and to identify the variations in WRKY core domains by producing a Neighbor-joining phylogenetic tree (Figure 2.9 panel A) as well as multiple WRKY core domains sequences

alignment (Figure 2.9 panel B). Results revealed that, VvWRKY03 is closely associated with CjWRKY01, whereas VvWRKY43 is close to VvWRKY01. VvWRKY53 clustered with SlWRKY73 and MtWRKY100630 as previously observed using the whole protein sequence.

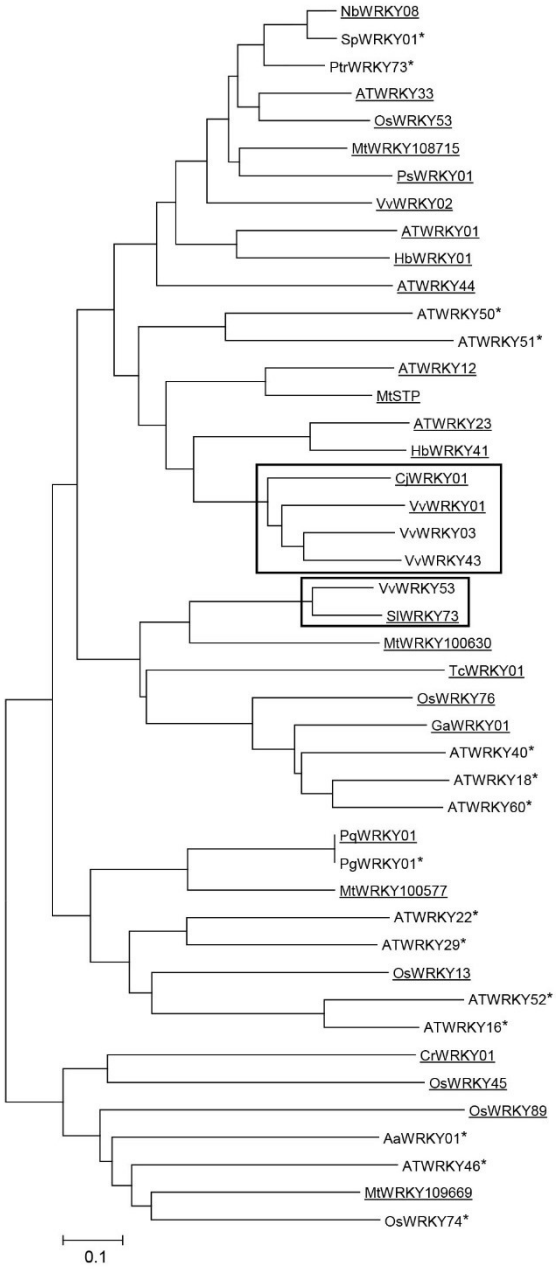

Figure 2.7 – A Neighbor-joining phylogenetic tree obtained aligning deduced amino acid sequences of VvWRKY03, VvWRKY43 and VvWRKY53 together with 42 other WRKY TFs already characterized in other plant species and involved in the regulation of the phenylpropanoid pathway (27 proteins; underlined) and in biotic and abiotic stress responses (15 proteins; marked with "*"). The WRKY deduced proteins inside the boxes were used for sequence alignment detail reported in Figures 2.8 and 2.9.

Table 2.3 The common names and gene accession numbers of 45 WRKY genes.

Species	Gene name	GeneBank	Assigned role (pathway)	Reference
A. thaliana	*AaWRKY01*	FJ390842	Artemisinin (Sesquiterpene)	Ma et al., 2009; Han et al., 2014
A. thaliana	*ATWRKY01* *	NP_178565.1	Sanguinarine, chelirubine (BIAs) and SA signaling pathway	Apuya et al.,2008; Duan et al., 2007
A. thaliana	*ATWRKY12*	AF404857	Cegulate cell wall biosynthesis and lignin deposition	Wang et al. 2010; Tang et al. 2013
A. thaliana	*ATWRKY16* *	NM_180802.2	Activation of defense related genes	Rushton et al., 2010
A. thaliana	*ATWRKY18* *	NM_119329.4	Stimulate SA-signaling and JA-signaling	Xu et al., 2006; Brotman et al., 2013
A. thaliana	*ATWRKY22* *	NM_116355.3	Regulated responses to both bacterial and fungal pathogens	Göhre et al., 2012
A. thaliana	*ATWRKY23*	AY052647	Flavonol (Phenylpropanoid)	Grunewald et al., 2012
A. thaliana	*ATWRKY29* *	NM_118486.6	Regulated responses to both bacterial and fungal pathogens	Göhre et al., 2012
A. thaliana	*ATWRKY33*	AK226301	Camalexin (Indole alkaloid)	Mao et al., 2011
A. thaliana	*ATWRKY40* *	NM_106732.4	Stimulate JA-signaling	Brotman et al., 2013
A. thaliana	*ATWRKY44*	NM_129282	Tannin, mucilage	Johnson et al., 2002
A. thaliana	*ATWRKY46* *	NM_130204.3	ABA signaling	Ding et al., 2015
A. thaliana	*ATWRKY50* *	NM_122518.3	JA signaling	Gao et al., 2011
A. thaliana	*ATWRKY51* *	NM_125877.4	JA signaling	Gao et al., 2011
A. thaliana	*ATWRKY52* *	NM_001344604.1	Activation of defense related genes	Rushton et al., 2010
A. thaliana	*ATWRKY60* *	NM_001335968.1	Salt and osmotic stress	Chen et al., 2010b
C. japonica	*CjWRKY01*	AB267401	Berberine (BIA)	Kato et al., 2007
C. roseus	*CrWRKY01*	HQ646368	Catharanthine, serpentine (TIAs)	Suttipanta et al., 2011
G. arboreum	*GaWRKY01*	AY507929	Gossypol (Sesquiterpene)	Xu et al., 2004
H. brasiliensis	*HbWRKY01*	JF742559	Rubber (Polyterpene)	Wang et al., 2013
H. brasiliensis	*HbWRKY41*	GU372969	Rubber (Polyterpene)	Zhang et al., 2012
M. truncatula	*MtSTP*	HM622066	Lignin (Phenylpropanoid)	Wang et al., 2010
M. truncatula	*MtWRKY100577*	EU526033	Rutin, kaempferol, caffeic acid, lignin	Naoumkina et al., 2008
M. truncatula	*MtWRKY100630*	EU526034	Rutin, kaempferol, caffeic acid, lignin	Naoumkina et al., 2008
M. truncatula	*MtWRKY108715*	EU526035	Rutin, kaempferol, caffeic acid, lignin	Naoumkina et al., 2008
M. truncatula	*MtWRKY109669*	EU526036	Rutin, kaempferol, caffeic acid, lignin	Naoumkina et al., 2008
N. benthamiana	*NbWRKY08*	AB445392	Capsidiol (Sesquiterpene)	Ishihama et al., 2011
O. sativa	*OsWRKY13*	EF143611	Momilactone A (Diterpene)	Qiu et al., 2008
O. sativa	*OsWRKY45*	AK066255	Momilactone A, oryzalexin, phytocassane (Diterpenes)	Akagi et al., 2014
O. sativa	*OsWRKY53*	AB190436	Momilactone A (Diterpene)	Chujo et al., 2014
O. sativa	*OsWRKY74* *	XP_015651250.1	nutrient deficiency and cold stress response	Dai et al., 2016
O. sativa	*OsWRKY76*	AK068337	Momilactone A, phytocassane, sakuranetin (Diterpenes)	Yokotani et al., 2013
O. sativa	*OsWRKY89*	AY781112	Lignin (Phenylpropanoid)	Wang et al., 2007
P. ginseng	*PgWRKY01* *	KR060074		Nuruzzaman et al., 2016
P. quinquefolius	*PqWRKY01*	JF508376	Triterpene	Sun et al., 2013
P. somniferum	*PsWRKY01* *	JQ775582	BIA pathway and stress response	Mishra et al., 2013
P. trichocarpa	*PtrWRKY73* *	Potri.013G153400.1		Duan et al., 2015
S. lycopersicum	*SlWRKY73*	NM_001247873	Monoterpenes	Spyropoulou et al., 2014
S. pimpinellifolium	*SpWRKY01* *	AK320342		Chu et al., 2015; Li et al., 2015
T. chinensis	*TcWRKY01*	JQ250831	Paclitaxel (Diterpene)	Li et al., 2013
V. vinifera	*VvWRKY01*	AY585679	JA pathway	Marchive et al., 2013
V. vinifera	*VvWRKY02*	AY596466	Lignin	Guillaumie et al., 2010
V. vinifera	*VvWRKY03*	VIT_01s0010g03930	Candidate gene in this study	
V. vinifera	*VvWRKY43*	VIT_14s0068g01770	Candidate gene in this study	
V. vinifera	*VvWRKY53*	VIT_17s0000g05810	Candidate gene in this study	

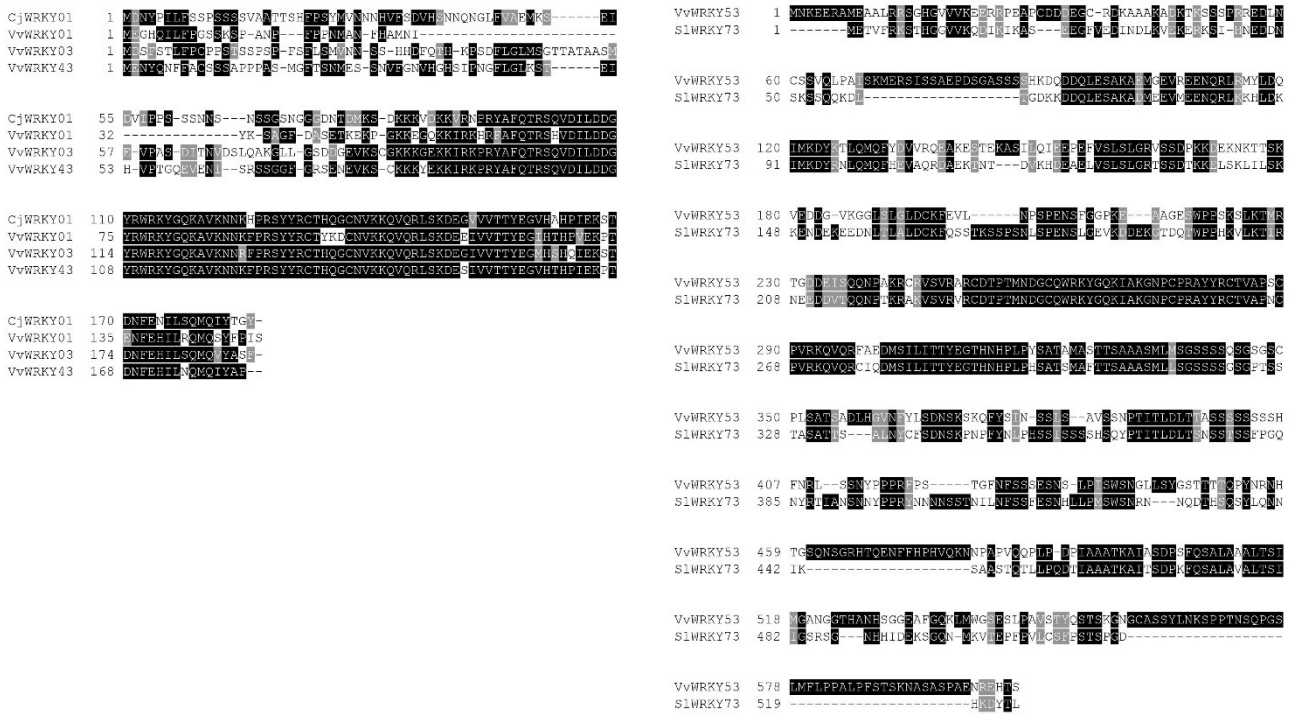

Figure 2.8 – Full-length protein sequence alignments of the closely TFs associated with candidate TFs. On the left VvWRKY03 and VvWRKY43 and its closest homologs VvWRKY01 and CjWRKY01, while on the right VvWRKY53 and its closest homologs SlWRKY73 protein. Identical residues are shown in black, conserved residues in dark grey, and similar residues in light grey.

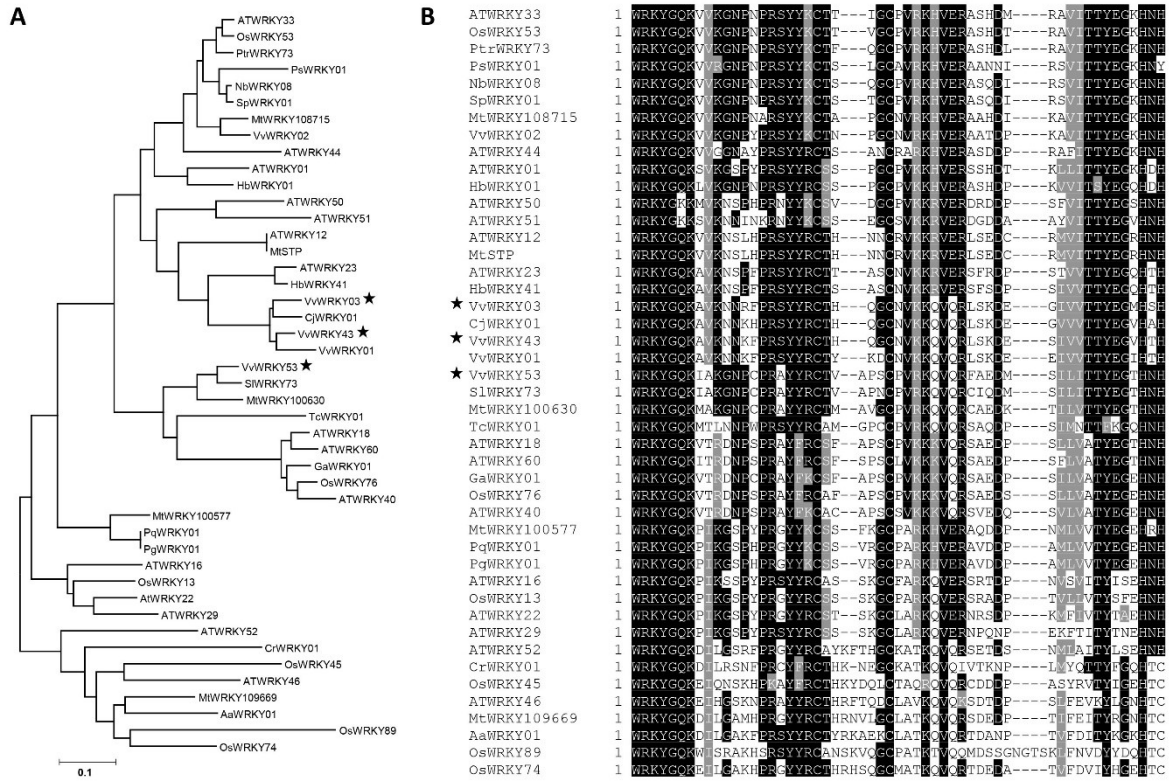

Figure 2.9 – (A) A Neighbor-joining phylogenetic tree of WRKY core domains of three candidate WRKY TFs with other closely related WRKY domains from other plant species for a total 45 WRKY core domains (B) Comparison of the amino acid sequences of WRKY core domains. Identical residues are shown in black, conserved residues in dark grey, and similar residues in light grey. The three candidate WRKYs are marked with black stars.

Gene and protein features of candidate WRKY genes

Looking at the exon-intron structures of candidate *VvWRKY* genes, both *VvWRKY03* and *VvWRKY43* shared a similar gene structure, containing one single intron (phase 2), while, *VvWRKY53* was found to possess five phase 0 introns (Figure 2.10).

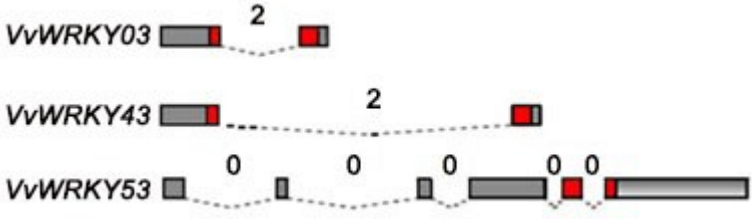

Figure 2.10 – Exon–intron composition of *VvWRKY03*, *VvWRKY43* and *VvWRKY53* candidate genes. Black lines connecting two exons represent an intron. The number upon the dashed lines indicates intron phase as described by Patthy, 2008.

Based on the phylogenetic analysis, we inferred the 3D structure of candidate grapevine WRKY genes from the protein structure of the closest Arabidopsis ones. VvWRKY03 and VvWRKY43 structures were obtained based on AtWRKY23 (Figure 2.11. panel A) whereas VvWRKY53 structure was inferred from AtWRKY18 (Figure 2.11. panel B).

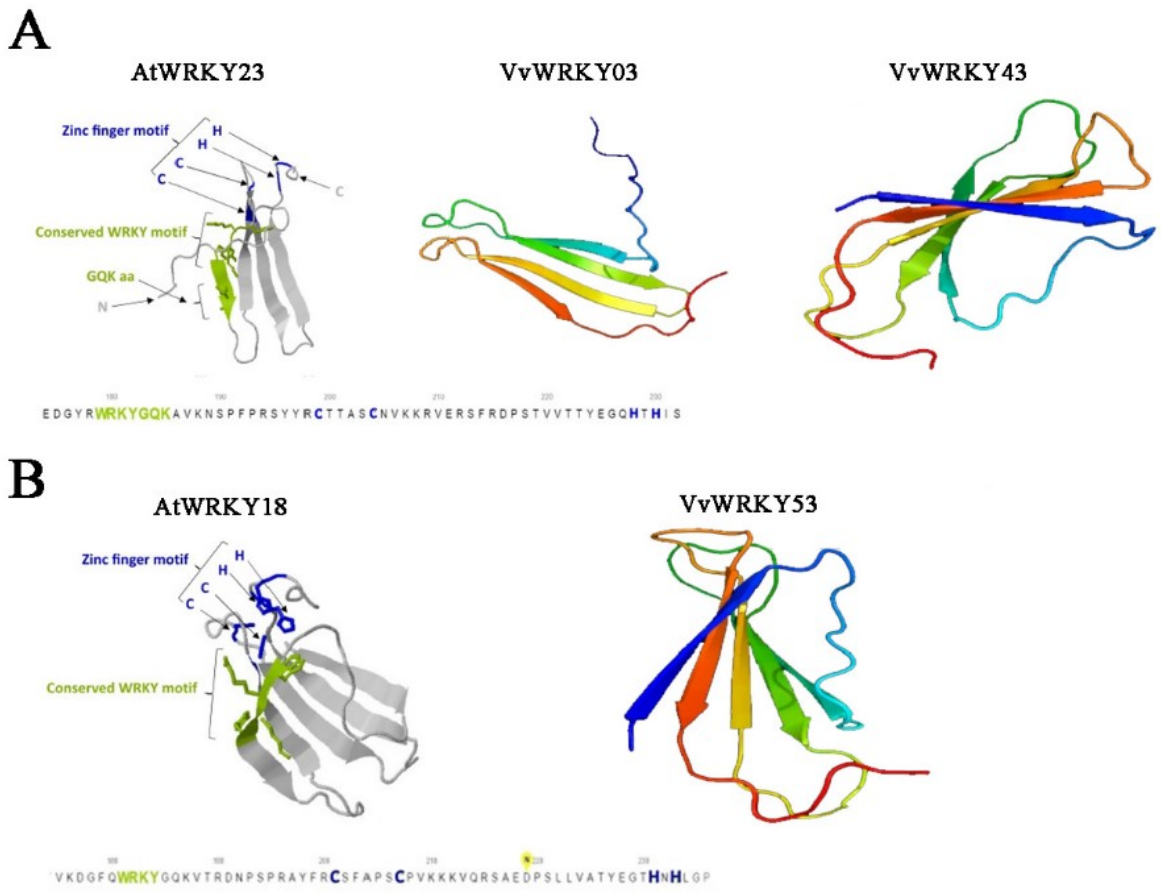

Figure 2.11 – (A) The 3D structure of VvWRKY03 and VvWRKY43 proteins based on the structure of their closest homologs ATWRKY23 and (B) the 3D VvWRKY53 protein based on AtWRKY18. The protein structures were inferred using Phyre2 software. The green region in the 3D structure indicates the location of the WRKY motif.

Together with the prediction of the three-dimensional structures of candidate WRKY genes we also performed several *in silico* analyses determining the molecular weight (MW) of predicted proteins, the theoretical isoelectric point (pI), the protein instability index (II), the

aliphatic index (AI), and the grand average of hydropathicity (GRAVY). All these parameters are listed in table 2.4.

Table 2.4 – Description of the grapevine candidate WRKY transcription factors.

					Physical and chemical parameters					
VvWRKY candidate	**PN40024 123 V1 ID**	**Group**	**Chr**	**Introns**	**Length (aa)**	**MW (kDa)**	**PI**	**II**	**AI**	**GRAVY**
VvWRKY03	VIT_01s0010g03930	II-c	1	1	189	21.26	9.13	44.76	54.13	– 0.723
VvWRKY43	VIT_14s0068g01770	II-c	14	1	182	20.79	9.41	49.81	52.42	– 0.884
VvWRKY53	VIT_17s0000g05810	II-b	17	5	605	65.19	7.55	55.2	52.66	– 0.746

Both VvWRKY43 and VvWRKY53 deduced protein showed a lower molecular weight (21.26 kDa for VvWRKY03 and 20.79 kDa for vvWRKY43) compared to VvWRKY53 (65.19 kDa). This observation is in line with the protein length which was much lower in VvWRKY03 and VvWRKY43 (189 and 182 aa, respectively) compared to vvWRKY53 (605 aa). The isoelectric point values were 9.13, 9.41 and 7.55 for VvWRK03, VvWRK43 and VvWRKY53, respectively whereas all the instability index (II) values were higher than 40, meaning that all candidate proteins were an unstable. All values of grand average of hydropathicity (GRAVY) were negative, indicating that the three candidate VvWRKY are a hydrophilic protein.

Quantitative RT-PCR analyses in grapevine leaf discs upon wounding

Gene co-expression networks (GCN) obtained from two large datasets allowed identifying three candidate TFs belonging to WRKY TF family strongly correlated with full-coding *VvSTS* genes located on Chr. 16. In order to validate and investigate this observation in more detail and upon different conditions, the transcript levels of *VvWRKY03*, *VvWRKY43* and *VvWRKY53*, together with three highly responsive Vv*STS* genes, i.e. *VvSTS29*, *VvSTS41* and *VvSTS48* and the two R2R3-MYB factors that already characterized (*VvMYB14* and *VvMYB15*) were monitored by mean of quantitative RT-PCR in wounded and UV-C treated Pinot noir leaves over a short and long term. Moreover, we also analysed the transcript level of the three *VvCHS* genes identified in grapevine in order to monitor the behaviour of key genes of the main parallel and antagonistic pathway to the stilbene one. Fold changes were calculated as a ratio between the normalised transcript level at each time point and the transcript level in healthy tissues (corresponding to the 0h time point). Overall, the results of both short and long term experiment were in agreement with what expected based on previous studies. Generally, under both wound and UV-C treatments, there was a progressive decrease in chalcone synthase (*CHS*) expression (Figures 2.12, 13 and 14) coupled to an increase in the level of VvSTSs expression. As a matter of facts, the transcript level of *VvSTS29*, *41* and *48* increased gradually over a 10 or a 48 periods in response to wound stress. Concerning the short-term wounding treatment (Figure 2.12), *VvSTS41* showed an increase in the expression level with a peak around 4 h and reaching a fold change equal to 105.84. *VvSTS48* and *VvSTS29* showed a similar pattern of induction with fold changes (FC) equal to 31.31 and 15.82, respectively, compared with the control. Looking at the expression of candidate WRKY TFs, we found that, all of them having the same pattern of expression with two peak at 4h and 8h with slightly decrease in-between at 6h. *VvWRKY03* was the highest and significant induction scored fold change values at 4 and 8h equal to 62.86 and 146.21, respectively. *VvWRKY43* recorded fold change equal to 58.85 at 4h and 132.15 at 8h. While *VvWRKY53* recorded fold change value as equal as 16.60 and 15.67 at the same two-time points. The expression pattern of the R2R3-MYB TFs (*VvMYB14* and *VvMYB15*) showed slightly different from that observed for the *VvSTS* genes, although both the R2R3-MYB TF were found to be induced upon stress as expected moreover, their induction appeared to occur before the one observed for *VvSTS* genes.

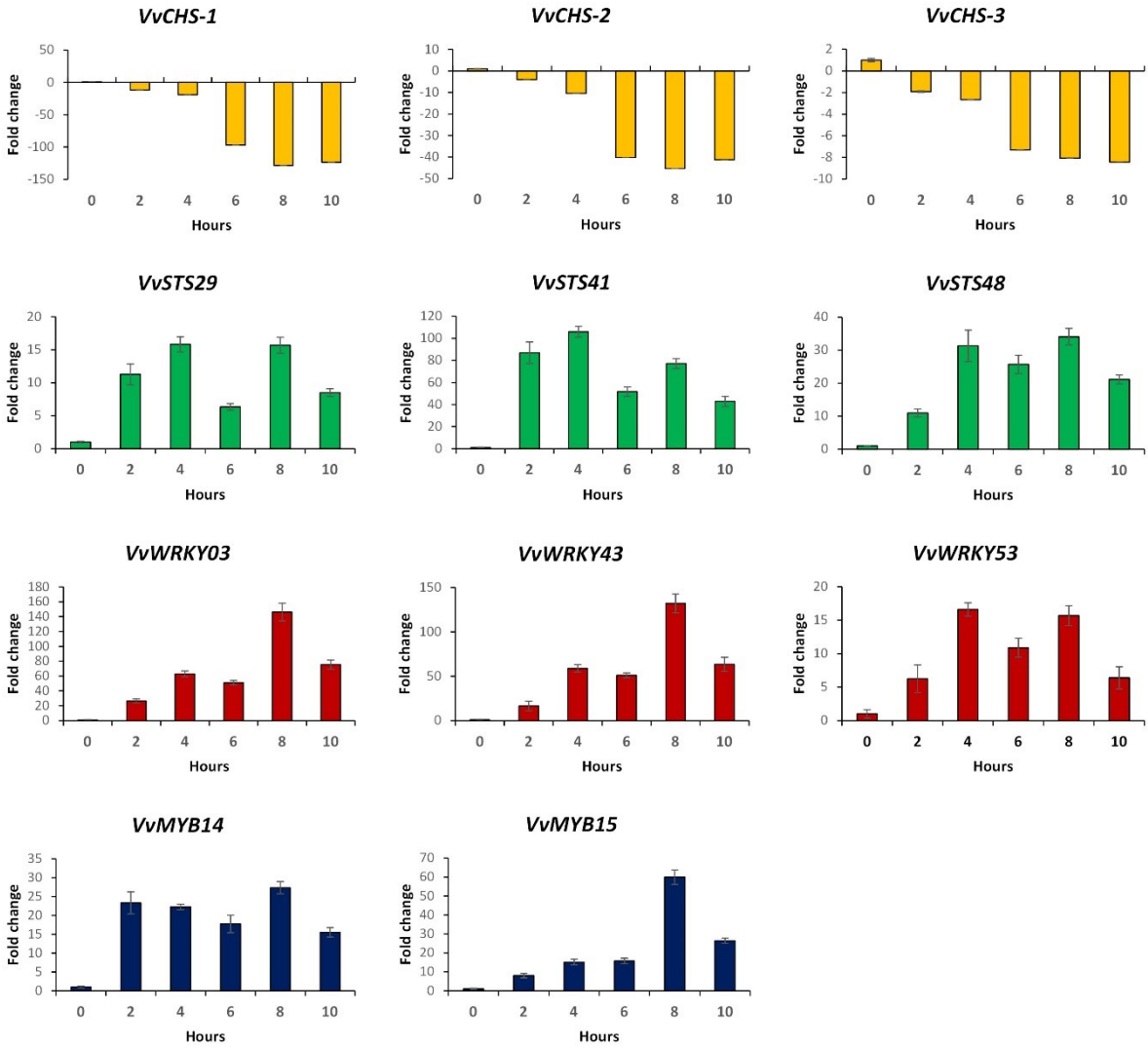

Figure 2.12 – Quantitative RT-PCR analyses of *CHS-1, CHS-2* and *CHS-3*, *VvSTS29, VvSTS41* and *VvSTS48* together with the three candidate genes belong to VvWRKY TFs and two VvMYB TFs already characterized, in wounded Pinot noir leaf discs over a 10h period. Fold changes were calculated as explained in the main text. All values were normalised to the expression of elongation factor (EF1). Error bars represent the standard error.

VvMYB14 was found to be induced since the first hour after the imposition of stress and appeared to maintain a sort of stability within all the 10 hours. While, *VvMYB15* revealed a gradual increase scored the highest value of fold change (FC=59.95) around 8h (Figure 2.12). Concerning the transcript level of long term wounding treatment (Figure 2.13), *VvSTS29* showed a gradual increment in the expression level with a peak around 48h reaching a fold

change equal to 712.26. *VvSTS41* showed a similar pattern of induction, reaching the peak of expression at 48h with a fold change equal to 1694.73 compared with the control. Similarly happened with *VvSTS48* scored fold change equal to 380.81 at 48h. Generally, the data obtained from this experiment was in line with the previous experiment upon wounding short term over the eleven investigated genes. As expected, both WRKY and R2R3-MYB TFs were induced upon wounding stress. *VvWRKY03* recorded the highest and significant induction value scored as fold change values at 6 and 48h equal to 236.08 and 330.23,

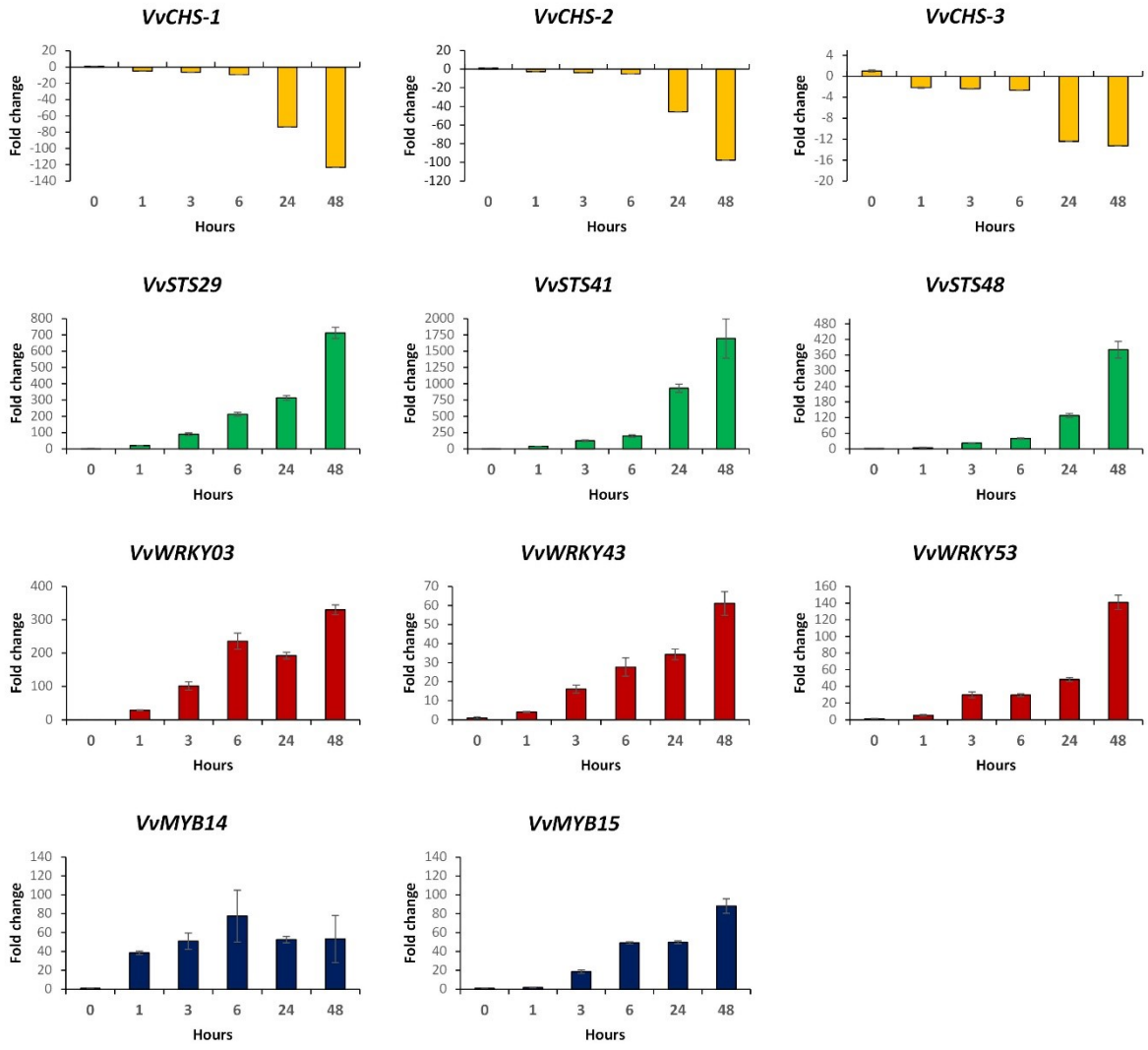

Figure 2.13 – Quantitative RT-PCR analyses of *CHS-1, CHS-2* and *CHS-3*, *VvSTS29, VvSTS41* and *VvSTS48* together with the three candidate genes belong to VvWRKY TFs and two VvMYB TFs already characterized, in wounded Pinot noir leaf discs over a 48h period. Fold changes were calculated as explained in the main text. All values were normalised to the expression of elongation factor (EF1). Error bars represent the standard error.

respectively. *VvWRKY43* showed a regular increase until the 48h peak (FC= 61.12). *VvWRKY53* has its fold change peak in expression activity at 48h (FC=141.02). *VvMYB14* was induced since the first hours after the imposition of stress. Although a peak of induction was detected at 6h, with a fold change equal to 77.43. While, *VvMYB15* reaches a first peak in expression later, at 6h (FC=49.13), followed by a slight increase at 24h (FC=50) and by a second higher peak in expression at 48h (88.22).

Quantitative RT-PCR analyses in grapevine leaf discs upon UV-C radiation

In order to confirm the expression pattern of the eleven genes previously investigated in more details under a different abiotic stress, we carried out an experiment treating plant with UV-C radiation. With this aim, Pinot noir leaf discs were exposed to UV-C radiation and sampled at 0, 1, 3, 6, and 24 hours. The stress kinetic was limited to the first 24 hours after the imposition of stress since in the following hours the UV-C irradiation led to a degradation of RNA making it difficult to perform expression analyses. Fold changes in mRNA transcript levels were calculated as the ratio between UV-C treated and untreated leaf discs sampled at the same time points. The results were in agreement with the previous results obtained in wounded leaf discs. Results in figure 2.14 showed extremely decrease in the parallel pathway of stilbenes synthase of all *VvCHS* genes when compared to the transcript level reached in wounded discs. It is worth noting that the fold changes observed upon UV-C treatment were not as high as those observed upon wounding. This is probably due to the fact that the UV-C treatment was performed on punched discs, representing a combination of the both wounding and UV-C effect. *VvSTS29* and *VvSTS48* reached their peaks of induction at 6h (FC = 39.07 and 12.29 respectively) under UV-C exposure, then showed a slight decrease in over the 24 h. *VvSTS41* showed a gradual increase reaching its peak at 24h, with scored fold change value of 40.73. *VvWRKY03* reached the maximum induction at 3h (FC=2.86), remained stable at 6h (FC=2.81) and then decreased at 24h (FC=1.94). *VvWRKY43* reached a first peak of fold change value at 3h (FC=6.93), followed by a slight decrease at 6h with fold change equal to 4.11, then a higher peak at 24h (FC=18.49). A similar trend was observed for *VvWRKY53*, which reached a first peak of expression at 3h, followed by a slight

decrease at 6h, and by a second higher peak at 24h (FC = 4.87). For what concerns the behaviour of *VvMYB14* and *VvMYB15* (Figure 2.14), *VvMYB14* showed a progressive increase in expression, reaching its peak at 24h (FC = 4.76), whereas *VvMYB15* reached the

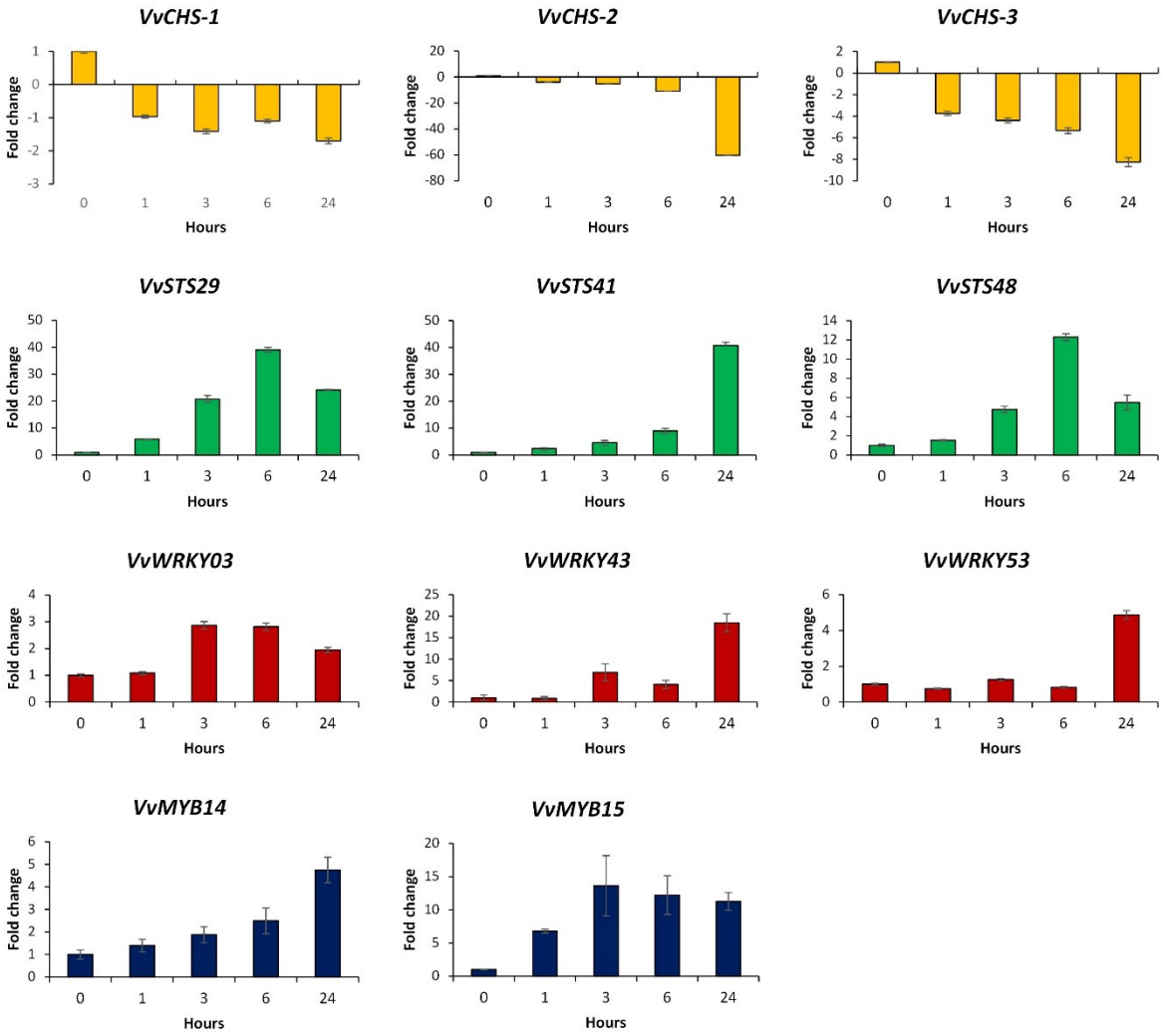

Figure 2.14 – Quantitative RT-PCR analyses of *CHS-1, CHS-2* and *CHS-3*, *VvSTS29, VvSTS41* and *VvSTS48* together with the three candidate genes belong to VvWRKY TFs and two VvMYB TFs already characterized, in Pinot noir leaf discs irradiated with UV-C over a 24h period. Fold changes were calculated as explained in the main text. All values were normalised to the expression of elongation factor (EF1). Error bars represent the standard error.

maximum expression at 3h, with a fold change of approximatively 13.63, followed by a slight decrease in the next 20 hours and a limiting FC value of approximately 11.3.

Generally, the observations obtained by both wounding and UV-C treatment, confirmed that, together with *VvMYB14* and *VvMYB15*, out candidate VvWRKY TFs may be potentially involved in the regulation of the stilbene pathway, at least in term of co-expression patterns.

Functional characterization of regulation of VvSTSs transcription using dual luciferase assay

In order to confirm from a functional point of view the observation obtained by both GCN analysis on whole transcriptome datasets and quantitative RT-PCR analyses, which indicated that there is a strong correlation between the expression pattern of stilbene synthase genes and three WRKY TFs, *VvWRKY03*, *WRKY53* and *WRKY43*, a functional approach was used to investigate the effect of selected *VvWRKY* genes on the induction of *VvSTS* promoters.

Role of candidate VvWRKY TFs in the regulation of *VvSTS29* and *VvSTS41* promoters

Results obtained by Dual reporter luciferase assays clearly confirmed that both VvMYB14 and VvMYB15 TFs are involved in the regulation of *VvSTSs* genes. Looking at the independent action of VvMYB14 and VvMYB15 in the *VvSTS29*p and *VvSTS41*p activity, grapevine cell suspensions expressing *VvSTS29*p constructs showed statistically significant increment of approximately 34.7 fold of expression level when co-transformed with VvMYB14 in comparison to control and 21.3 fold when co-transformed with VvMYB15. Something similar was observed for *VvSTS41* promoter (FC= 4.1 with *VvMYB14*; FC=13.7 with *VvMYB15*). These findings clearly confirmed the observation by Höll et al. (2013). It is worthy also to mention that the average values of each bombardment represents three independent experiments performed with each *VvSTS* promoter. The co-bombard of VvWRKY03 along with VvMYB TFs ones showed low values in luciferase activity and consequently activity of *VvSTS*s promoters. *VvWRKY03* showed a fold change in the *VvSTS29p* activity of approximately 11 when co-transformed with VvMYB14 and of 15 when co-transformed with VvMYB15, in comparison to control (Figure 2.15). A similar

trend was observed with *VvSTS41p* activity: VvWRKY03 showed about 3 of fold induction activity with VvMYB14 and 6 with VvMYB15 comparing with the control.

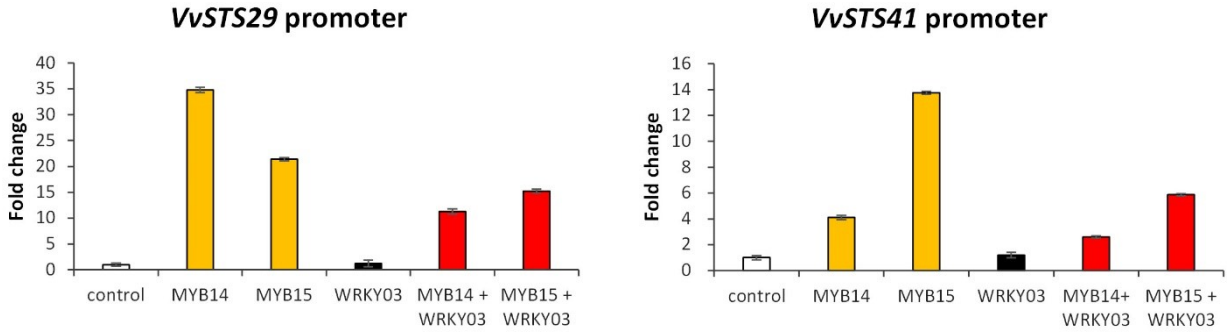

Figure 2.15 – Dual luciferase™ reporter assays showing the effect of *VvMYB14*, *VvMYB15* R2R3-MYB TF as well as their co-bombarded along with *VvWRKY03* in the activation of *VvSTS29* and *VvSTS41* promoters. The figure shows a graphical representation of data obtained from one representative experiment out of at least three independent ones with error bars indicating SE.

Concerning the role of VvWRKY43 TF illustrated in figure 2.16, the data showed that VvWRKY43 play an important role in the activation of *VvSTS* promoter when co-bombarded along with the R2R3-MYB one. As regard to *VvSTS29p* activity, VvWRKY43 showed significant value of fold change was equal to 109.3 when co-bombarded with VvMYB14 and of 55 of fold induction when co-bombarded with VvMYB15. Also as to its effect on *VvSTS41p*, VvWRKY43 shows a fold induction equal to 10 with VvMYB14 and of 14 with VvMYB15. Data in figure 2.17 indicated that, VvWRKY53 showed significant effect on the

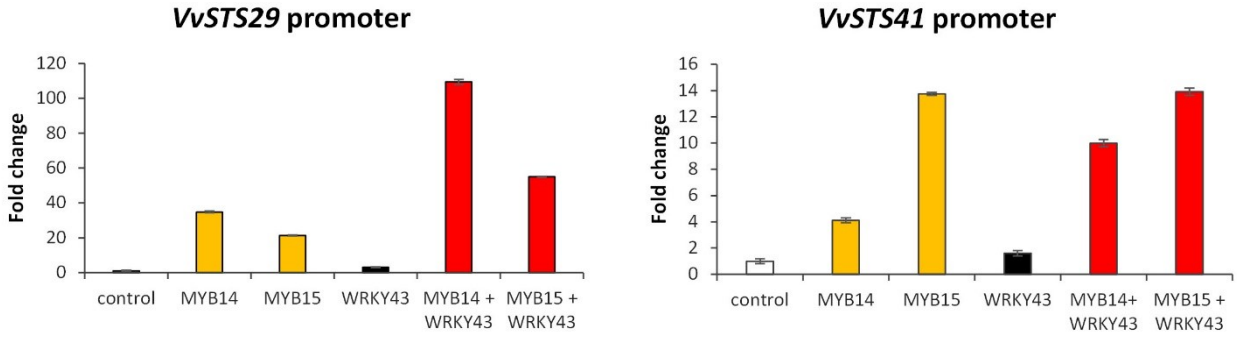

Figure 2.16 – Dual luciferase™ reporter assays showing the effect of *VvMYB14*, *VvMYB15* R2R3-MYB TF as well as their co-bombarded along with *VvWRKY43* in the activation of *VvSTS29* and *VvSTS41* promoters. The figure shows a graphical representation of data obtained from one representative experiment out of at least three independent ones with error bars indicating SE.

activation of *VvSTS* promoters. Concerning VvSTS29p the fold induction was equal to 62.9 and 49.6 when co-transformed with VvMYB14 and VvMYB15 respectively. Also, as regards to *VvSTS41p*, VvWRKY53 showed a fold induction activity of approximately 7.5 and 22 when co-transformed with VvMYB14 and VvMYB15 respectively.

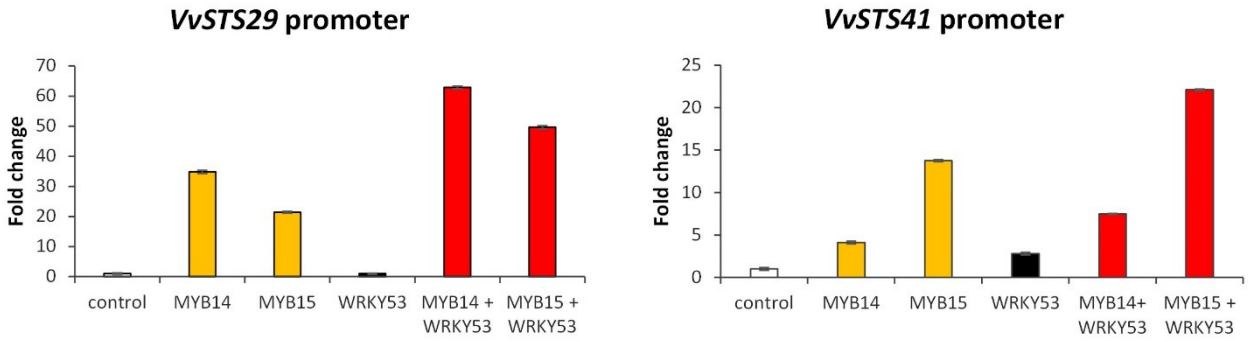

Figure 2.17 – Dual luciferase™ reporter assays showing the effect of *VvMYB14*, *VvMYB15* R2R3-MYB TF as well as their co-bombarded along with *VvWRKY53* in the activation of *VvSTS29* and *VvSTS41* promoters. The figure shows a graphical representation of data obtained from one representative experiment out of at least three independent ones with error bars indicating SE.

From these results obtained by Dual-luciferase™ reporter assay, it can be concluded that, whereas the VvWRKY TFs alone were not able to determine any change in the activity of *VvSTS* promoters compared to control and to co-bombardments performed with the two R2R3-MYB TFs, they appear to have an impact whenever co-transformed in combination with the R2R3-MYB TFs. Both VvWRKY43 and VvWRKY53 TFs appeared to act synergistically with VvMYB14 and VvMYB15, leading to an increase in the VvSTS promoter activity more than VvWRKY03.

This observation raises the question as to whether (i) these WRKY TFs act upstream (or downstream) the MYB TFs in the regulation of VvSTS or whether; (ii) they interact together with MYBs at the protein level, as observed in the regulation of other structural genes of the parallel flavonoid pathway.

Role of VvWRKY TFs in the regulation of *VvMYB14* and *VvMYB15* promoters

In order to investigate both these hypotheses we also investigated the effect of several WRKY TFs on the promoter activity of both *VvMYB14p* and *VvMYB15p* (Figure 2.18). Although we could not observe any significant effect of most of WRKY TFs on the *VvMYB15* promoter, preliminary data show a significant increase in the *VvMYB14* promoter activity when co-bombarded with VvWRKY03 suggesting this effect could act upstream in the regulation of the VvSTS pathway.

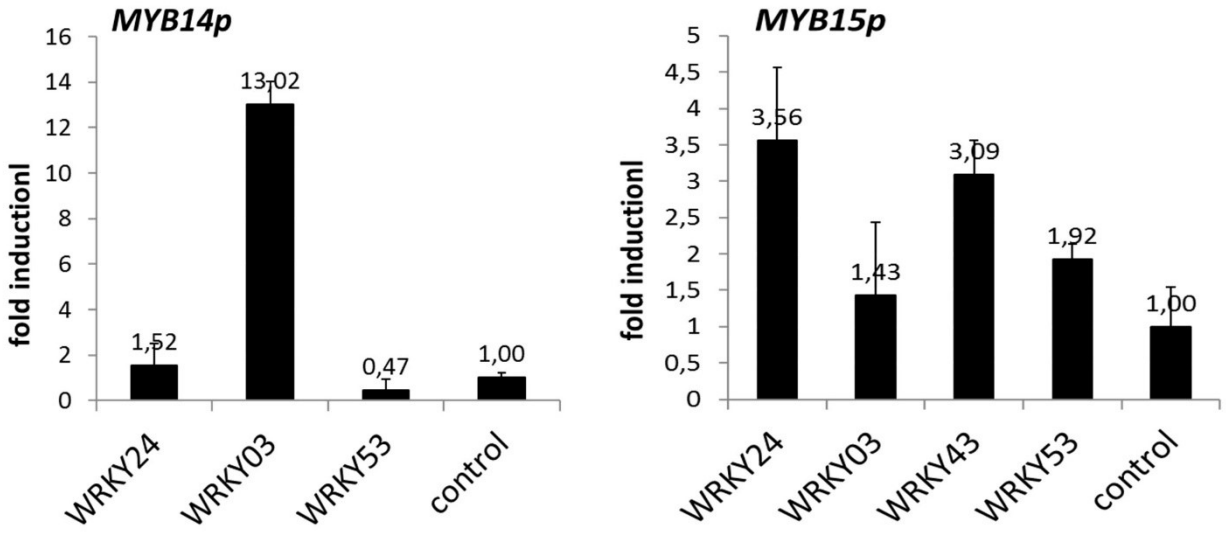

Figure 2.18 – Dual luciferase™ reporter assays showing the effect of several VvWRKY TFs in the activation of *VvMYB14* and *VvMYB15* promoters. The figure shows a graphical representation of data obtained from one representative experiment out of at least three independent ones with error bars indicating SE.

Discussion

The present study was carried out in order to increase our current knowledge about the regulation and biosynthesis of stilbenes, a class of phenylpropanoid that play important roles both at the plant level, conferring protection against many environmental stresses and at the pharmacological one, being related to many beneficial effects on human health.

In order to identify possible regulators of stilbene biosynthesis in grapevine gene co-expression networks (GCN) were infferred from two large transcriptome datasets produced by means of NimbleGen microarray technology. The first GCN was obtained from the *V. vinifera* cv. Corvina expression atlas and encompassing 54 samples corresponding to various tissues and organs at different developmental stages (Fasoli et al., 2012), whereas the second one was constituted by a 170 sample dataset corresponding to Corvina berries collected at 3 developmental stages (véraison, mid-ripening and harvest) in 3 consecutive years and in 11 different locations in Valpolicella (Dal Santo et al., 2013). GCN analysis allowed identifying three transcription factors other than *VvMYB14*/*15* already characterized by Höll et al. (2013) belonging to WRKY TFs, which are co-expressed with *VvSTSs* and may potentially be involved in the transcriptional regulation of the stilbene synthase pathway in grapevine. Interestingly MYB15, one of the two R2R3-MYB TFs already characterized by Holl et al. (2013) was not detected in the GCN obtained from berry samples suggesting this TFs may not be involved in the specific processes leading to stilbene accumulation in berries. Together with R2R3-MYB and WRKY TFs, it's worth to mention some other TFs which showed high level of correlation with VvSTS genes. Amongst them are a gene encoding for a bHLH protein (VIT_01s90016g02070) and two genes belonging to the NAC TF multigenic family. These genes were represented in both GCNs analysed and deserve further investigations. Although Höll et al. (2013) observed that both MYB14 and MYB15 do not appear to interact with the MYC1 bHLH in the regulation of two *VvSTS* promoters, since they carry an amino acid mutation within the bHLH-interacting site, the strong correlation observed between *VvMYB14*/*15*, *VvSTSs*, and *VIT_01s90016g02070 (bHLH)* raise the question whether this particular TF could effectively play a role in the regulation of *VvSTSs* by interacting with VvMYB14 and VvMYB15. Amongst the WRKY TFs family we identified three genes which were significantly correlated with *VvSTS* genes. Two of these, designated as *VvWRKY03* and

VvWRKY53, showed a nice correlation with VvSTS within the Corvina atlas dataset whereas a third one, designated as *VvWRKY43*, within the berry dataset (Wang et al., 2014).

Phylogenetic analysis of the whole grapevine WRKY multigenic family (non-climacteric) including the three WRKY candidate proteins along with the ones from peach (*Prunus persica*) (climacteric) and *Arabidopsis thaliana* for a total number of 188 accessions revealed that *VvWRKY03* and *VvWRKY43* are grouped together within subgroup II-c, while *VvWRKY53* belongs to subgroup II-b. This results are in agreement with the results of phylogenetic analysis based on an alignment of whole WRKY multigenic family of both grapevine and Arabidopsis (Wang et al., 2014). Moreover, alignment based on both the WRKY deduced full-length proteins and core domains with other WRKY TFs functionally characterized from other species showed that both *VvWRKY03* and *VvWRKY43* cluster with a group of regulators including *CjWRKY01* (Accession number AB267401) and *VvWRKY01* (Accession number: AY585679) along with *AtWRKY23* (Accession number AY052647) from Arabidopsis, while *VvWRKY53* closely associated with *SlWRKY73* (Accession number NM_001247873) and *MtWRKY100630* (Accession number EU526034) together with *AtWRKY18* (Accession number NM_119329.4) from Arabidopsis. Looking at the function of these phylogenetically-related genes, *CjWRKY01* appears to be involved in the regulation of the antimicrobial agent benzylisoquinoline alkaloid berberine in *Coptis japonica* protoplasts (Kato et al., 2007). *VvWRKY1* was also overexpressed in tobacco and was found to confer enanched resistance to pathogen infection. In grapevine, it was demonstrated to increase the resistance against the downy mildew leading to activation of the Jasmonic acid signalling pathway (Marchive et al., 2013). *AtWRKY23* has been shown to be involved in the regulation of several branches of the phenylpropanoid pathway (Grunewald et al., 2012). *SlWRKY73* is involved in transactivation of monoterpenes synthase promoters in tomato (Spyropoulou et al., 2014). According to Naoumkina et al., 2008, *MtWRKY100630*, which belongs to group II-b, is involved in the activation of lignin biosynthetic genes and consequantly increase the levels of lignin in tobacco. *AtWRKY18* has been reported to stimulate SA-signalling (Xu et al., 2006), JA-signalling via suppression of JAZ repressors and to negatively regulate the expression of the defense genes (Brotman et al., 2013). Moreover, this gene has also been reported to be tightly associated with the bacterial volatile responses which are determinants for eliciting plant induced systemic resistance (Wenke et

al., 2012; Sharifi and Ryu, 2016). From the phylogenetic analysis and the alignment of multiple amino acid sequences we found a higher similarity between both *VvWRKY03* and *VvWRKY43* compared to *VvWRKY53*. The structure of exon-intron compositions and the 3D structures of the three candidate genes revealed that both *VvWRKY03* and *VvWRKY43* shared a comparable gene structure, containing one single intron, and their 3D structures were also similar comparing with the closest *AtWRKY23*. Differently, *VvWRKY53* possess five introns and encodes for a protein with a fairly different 3D structure. Expression analyses through quantitative RT-PCR in *V. vinifera* cv. Pinot noir leaf discs treated with abiotic stresses (wounding and UV-C) have been investigated in order to validate the observations of GCN analysis. It is not surprising that chalcone synthases (*CHS*), the key enzymes of the flavonoid pathway and the parallel pathway of stilbenes, were down-regulated. On the other hand, genes encoding for STSs were up-regulated upon both wounding and UV-C treatments. These results are in harmony with the previous studies (Kodan et al., 2002; Emiliani et al., 2009; Jeandet et al., 2010; Vannozzi et al., 2012; Jeandet et al., 2014; Xi et al., 2014; Suzuki et al., 2015).

Under abiotic stress imposed (wounding and UV-C), candidate WRKY TFs, *VvWRKY03*, *VvWRKY43* and *VvWRKY53* showed a significant co-induction with selected *VvSTS* genes and with the R2R3-MYB TFs *VvMYB14* and *VVMYB15*. *VvWRKY03* represented the most responsive WRKY TF under wounding stress, whereas *VvWRKY43* was the most responsive one under UV-C stress. These results are in line with previous studies (Wang et al., 2007 Chen et al., 2009, 2010a, 2012 and Nilsson et al., 2010) where it was observed that WRKY genes were rapidly induced by wounding treatment, chemical treatments and UV radiation, indicating their regulatory function in these signalling pathways.

Together with WRKY TFs, the role of MYB TFs (*VvMYB14* and *VvMYB15*) belong to the plant R2R3-MYB subgroup (Stracke et al., 2001) in the regulation of stilbene biosynthetic pathway has also been verified. It's evident that, these TFs are induced whenever the STSs are induced too, and they show a very high correlation in terms of expression and the obtained results are in agreement with results of Höll et al. (2013) and Wong et al. (2016) who cleared that both *VvMYB14* and *VvMYB15* play an important role in the transcriptional regulation of the stilbene biosynthetic pathway in grapevine under normal and stressed conditions. Transcript analyses showed a nearby connection between the expression of *VvMYB14* and

VvMYB15 and selected members of the STS gene family and the resulting accumulation of stilbenes and it can act as direct activators of structural genes (Schaart et al., 2013; Liu et al., 2015). Moreover, R2R3-MYB TFs were also suggested to be involved in the response of plant cells to biotic and abiotic stress (Abe et al., 2003; Denekamp and Smeekens, 2003; Nagaoka; Takano, 2003). Moreover, R2R3-MYB TFs family reported to be a common denominator in the regulation of phenylpropanoid-derived compound (Lai et al., 2013). In grapevine MYB TFs (MYB5a and MYB5b) (Deluc et al., 2006; 2008) and VvMYBPA1 Bogs et al. (2007) they reported that, those MYB TFs modulate several branches of the flavonoid pathway, regulating structural genes, such as LEUCOANTHOCYANIDIN REDUCTASE (LAR), ANTHOCYANIDIN REDUCTASE (ANR), FLAVONOID 39-HYDROXYLASE, and CHALCONE ISOMERASE leading to the production of proanthocyanidins (PAs).

The functional approach of Dual-luciferase^{TM} reporter assay system was investigated to study the activity of a cloned *VvSTS* promoter DNA fragment and obtain direct evidence of the interaction role of both VvMYB and VvWRKY TFs in the regulation of VvSTS transcription in grapevine. Our results confirmed the role of selected VvMYB and VvWRKY TFs together on the activity of STS promotors. In this system chardonnay cell suspensions transiently expressing VvSTS promoter-luciferase expression constructs showed a statistically significant increase in *VvSTS* promoter activity whenever co-transformed with both VvMYB (VvMYB14/15) and VvWRKY TFs (VvWRKY03, VvWRKY43 and VvWRKY53) indicating a real interaction between these TFs and transcription of VvSTS. Interestingly, the action of WRKY effectors alone did not show any effect on the luciferase activity. But the combined co-bombardment of both WRKY and MYB TFs appeared to act synergistically with VvMYB14 and VvMYB15, leading to an increase in the luciferase activity, reaching fold change values even higher than those observed with the MYB effectors alone. In other words, this values represents the expression of two structural genes involved in the stilbene synthase pathway, i.e. *VvSTS29* and *VvSTS41*. These results of our investigation are in harmony with the conclusion given by Höll et al., 2013 and Wong et al., 2016 who noticed that, the role of MYBs TFs in the regulation of *VvSTSs*. After that, to investigate whether, these WRKY TFs act upstream (or downstream) the MYB TFs in the regulation of VvSTS or whether; they interact together with MYBs at the protein level, as

observed in the regulation of other structural genes of the parallel flavonoid pathway. To this end, several WRKY TFs were used together with *VvMYB14*/*15* as target promotors using Dual-luciferase™ reporter assay system. The results indicated a significant increase in the VvMYB promoter activity when co-bombarded with VvWRKY suggesting this effect could act upstream (Yu et al., 2001 and Qiu et al., 2007) in the regulation of the VvSTS pathway.

Conclusions

Plant stilbenes are a small group of phenylpropanoid (pp) compounds that have been detected in limited number of unrelated plant species, including grapevine (*Vitis vinifera* L.). Plants accumulate stilbenes in their leaves, roots and fruits in response to both biotic and abiotic stresses such as wounding, UV-C exposure and pathogen infection. Nowadays, plant stilbenes have a particular interest not only because of their roles in the plant defense mechanism, but also because of their roles from pharmacological point of view. The biosynthetic pathway leading to their production is a side branch of the general PP biosynthetic pathway and can be considered as an extension of the flavonoid pathway. Stilbene synthase (*STS*) is the structural enzyme responsible for the biosynthesis of resveratrol, which is the basic unit of the biosynthesis of plant stilbenes. However, just little information is available on the transcriptional regulation of *VvSTSs*. Recently, two transcription factors (TFs) belonging to the R2R3-MYB TF family, namely *VvMYB14* and *VvMYB15*, have been demonstrated to be involved in the transcriptional regulation of the stilbene synthases (*VvSTSs*; Höll et al., 2013). Nevertheless, many questions are still open about the role of these R2R3 factors on the grape response to stress and about the possible role of TFs belonging to different families than R2R3 MYBs in the regulation of the STS pathway. In this study we tried to increase the present level of *VvSTS*s transcriptional regulation, which lead to the stilbenes synthesis in grapevine.

In view of the thought that the genes involved in similar or related processes may exhibit similar expression patterns over a range of experimental conditions, we performed a co-expression network analysis (GCN) on various grapevine gene expression datasets identifying candidate TFs belonging to the WRKY family namely *VvWRKY03*, *VvWRKY43* and *VvWRKY53* potentially involved in the regulation of the stilbene biosynthetic pathway. These genes show co-expression correlation values even higher than those observed for *VvMYB14*/*15* and are strongly induced in response to the same abiotic stress treatments which lead to *VvSTSs* induction. These WRKY candidate genes belong to group II. Two of them, namely *VvWRKY03* and *VvWRKY43* appear to have high degree of similarity than *VvWRKY53*. Multiple sequence alignment with other WRKY TFs functionally characterized from different plant species indicated that those WRKY candidate TFs grouped together with other WRKY TFs already demonstrated to be involved in specialised metabolite production

regulation including falvonol and lignin in the phenylpropanoid pathway (Naoumkina et al., 2008; Grunewald et al. 2012), jasmonic acid signalling pathway (Marchive et al., 2013) benzylisoquinoline alkaloid berberine (Kato et al., 2007), monoterpenes (Spyropoulou et al., 2014) and in response to pathogen infection (Wenke et al., 2012). Expression analyses through quantitative RT-PCR confirmed that the already characterized R2R3-MYB and the three WRKY TFs chosen for this study, together with selected *VvSTSs*, are induced upon both wounding and UV-C stresses and show similar behaviour both in terms of expression and timing. Conversely, the enzyme *VvCHSs* of competitive flavonoid pathway are down-regulated upon the same conditions. The results based on the Dual-luciferase^{TM} reporter assay system indicated that, WRKY TFs alone were not able to show any effect on the luciferase activity. VvWRKY43 and VvWRKY53, act synergistically with VvMYB14/15 showing an additive effect on the *VvSTS* promoter activity. On the contrary, VvWRKY03 did not show any direct effect on *VvSTS* promoter activity but it may have a role in the regulation of the stilbene pathway through regulation of VvMYB14 expression. In addition, the study showed that, other than *VvMYB14* and *VvMYB15*, VvWRKY TF may also be potentially involved in the regulation of the stilbene pathway.

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