The grapevine *VvWRKY2* gene enhances salt and osmotic stress tolerance in transgenic *Nicotiana tabacum*

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Abstract

Our study aims to assess the implication of WRKY transcription factor in the molecular mechanisms of grapevine adaptation to salt and water stresses. In this respect, a full-length *VvWRKY2* cDNA, isolated from a *Vitis vinifera* grape berry cDNA library, was constitutively over-expressed in *Nicotiana tabacum* seedlings. Our results showed that transgenic tobacco plants exhibited higher seed germination rates and better growth, under both salt and osmotic stress treatments, when compared to wild type plants. Furthermore, our analyses demonstrated that, under stress conditions, transgenic plants accumulated more osmolytes, such as soluble sugars and free proline, while no changes were observed regarding electrolyte leakage, H₂O₂, and malondialdehyde contents. The improvement of osmotic adjustment may be an important mechanism underlying the role of *VvWRKY2* in promoting tolerance and adaptation to abiotic stresses. Principal component analysis of our results highlighted a clear partition of plant response to stress. On the other hand, we observed a significant adaptation behaviour response for transgenic lines under stress. Taken together, all our findings suggest that over-expression of *VvWRKY2* gene has a compelling role in abiotic stress tolerance and, therefore, would provide a useful strategy to promote abiotic stress tolerance in grape *via* molecular-assisted breeding and/or new biotechnology tools.

Keywords *Vitis vinifera* · WRKY · Over-expression · Salt tolerance · Osmotic regulation

Abbreviations

CI	Confidence interval
FW	Fresh weight
H ₂ O ₂	Hydrogen peroxide
PCA	Principal component analysis
ROS	Reactive oxygen species
TBA	Thiobarbituric acid

TF	Transcription factor
WT	Wild type

Introduction

Among the abiotic stresses, salinity and drought are two of the most widespread soil degradation factors on the earth. The majority of crops are highly susceptible to these constraints; therefore, there is an urgent need to deal with this problem. Grapevine (*Vitis vinifera* L.) is considered to be one of the major fruit crops in the world based on the cultivated surface area and economic value with total fruit production in 2013 as 771 Million quintals (FAO 2016: <http://faostat.fao.org/beta/en/#data/QC> 24/10/2016). Despite the multigenic nature of abiotic stress tolerance, the classical breeding approach for abiotic tolerance in grape is relatively slow (Yamaguchi and Blumwald 2005). For this reason, the transgenic approach seems to be more attractive and appropriate, than the classical breeding method, to improve grapevine species. In fact, the expression of many genes is regulated under these stress constraints, enabling

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plants to adapt to these conditions (Dorothea and Ramanjulu 2005). There are some studies indicating abiotic stress tolerance improvement by grapevine genes over-expression using *Nicotiana tabacum* plant model, namely, those related to *VvSUC27* (Cai et al. 2017), *Vv-α-gal/SIP* (Daldoul et al. 2018), *VvDhn* (Jardak et al. 2017), *VvRD22* (Jamoussi et al. 2014), *VvbZIP23* (Tak and Mhatre 2013a) and *VvSDIR1* (Tak and Mhatre 2013b) as candidate genes. The transcriptional regulation of these genes is under the control of various transcription factors (TFs) (Seki et al. 2002; Zhu 2002); such as the WRKY TFs. The latter belong to a multigenic family characterized by the presence of one or two WRKY conserved domains of about 60 amino acids containing the WRKYGQK sequence and followed by a zinc-binding motif (Eulgem et al. 2000).

The WRKY genes are well-known to be part of various developmental and physiological mechanisms, including disease resistance (Wu et al. 2016), senescence (Rinerson et al. 2015) growth and developmental processes (Guillaumie et al. 2010; Chen et al. 2016). In addition, several genetic and molecular studies demonstrated that WRKY genes are involved in plant response to abiotic stresses (Nuruzzaman et al. 2014; Tripathi et al. 2014; Xi et al. 2016). In fact, dehydration, salt, and cold stresses were reported to enhance the expression of numerous WRKY genes in rice, *Arabidopsis thaliana* and other crop plants (Seki et al. 2002; Song et al. 2009; Wu et al. 2016; Phukan et al. 2016).

After the release of the whole genomic sequences of highly homozygous and heterozygous grapevine (Jaillon et al. 2007; Troggio et al. 2007; Velasco et al. 2007), a genome-wide identification of WRKY family members and the characterization of their response to cold stress were performed by Wang et al. (2014). Moreover, the evolution study and the expression analysis of this family upon a broad range of abiotic and biotic stresses revealed its key role in the grape adaptation to environmental constraints (Guo et al. 2014). Many genes from this family have been already identified and characterized in different grapevine species such as *VvWRKY1* (Marchive et al. 2007) and *VvWRKY2* (Mzid et al. 2007) from *Vitis vinifera*, in addition to *VpWRKY1*, *VpWRKY2* (Li et al. 2010) and *VpWRKY3* (Zhu et al. 2012) from *Vitis pseudoreticulata*.

Particularly, *VvWRKY2* is constitutively, yet variably, expressed in the different organs of healthy grapevine plants (supplemental file 1). In leaves, *VvWRKY2* is induced by wounding and after infection with *Plasmopara viticola*. Its over-expression in tobacco plants induced an enhanced tolerance to necrotrophic fungal pathogens (Mzid et al. 2007). However, the role of this gene in improving the tolerance to abiotic stress such as salinity and drought remains unknown.

The current work aimed to investigate the biological role of *VvWRKY2* gene in salt and osmotic stress tolerance based on physiological studies of three independent transgenic

lines of tobacco, which can be used in genetic programme to improve the tolerance of grapevine to abiotic stress.

Materials and methods

Plant material and growth conditions

Full length of *VvWRKY2* coding sequence (AY596466) was amplified by PCR from cDNA library with the primers W2-5# (5'-AAGTAATATTGATCAATGGCTGAA-3') and W2-3 # (5'-GATATTCTACATCGCCTTGCC-3') using Pfu polymerase (Promega). This cDNA was first cloned into the pGEM-T vector for sequencing validation and then transferred into the pGREEN vector (<http://www.pgreen.ac.uk>) under the 35S promoter of the cauliflower mosaic virus using restriction enzymes. The construct was introduced into *Agrobacterium* GV3010 strain for tobacco (*Nicotiana tabacum* cv. Xanthi) genetic transformation. Regenerated plants were transferred to soil pots and grown in greenhouse. The genotyping of these plants was carried out by PCR with a specific set of primers.

Wild type (WT) tobacco seeds and transgenic tobacco plants obtained from homozygous T2 generations over-expressing *VvWRKY2* gene were used in the present study. After performing a semi-quantitative RT-PCR, three transgenic lines (W2-5, W2-11, and W2-12) with different expression levels were analyzed (Mzid et al. 2007). The untransformed WT *N. tabacum* cv. Xanthi line was used as a control. Tobacco plants were grown in vitro at 25 °C on half strength MS medium (Murashige and Skoog 1962) under a 16 h/8 h photoperiod and a temperature range of 25/20 °C. In all experiments, plant tissues were harvested, frozen in liquid N₂ and stored at − 80 °C before analysis.

Performance of transgenic tobacco plants under stress treatment

Seeds of WT and transgenic plants were treated with 70% ethanol for 5 min and then sterilized with 15% bleach. After washing five times with sterile distilled water, the seeds were placed on solidified half strength MS medium. They were stratified at 4 °C for 3 days and then germinated under long-day conditions (15 h light/9 h dark) at 25 °C.

For NaCl or mannitol treatment, 25 seeds from WT and each transgenic line were placed in 120 mm square plastic Petri dishes that were sealed with Parafilm to prevent evaporation. The sensitivity of T2 seed germination to NaCl or mannitol (to mimic a water deficit) was assayed on half-strength MS agar plates including NaCl (0, 100, 200 and 300 mM) or mannitol (0, 100, 200 and 400 mM). Germination kinetic was followed every 3 days up to 21 days. The

percentage of germinated seeds was scored as the germination rate.

For physiological analysis, seedlings were transplanted in a magenta box containing fresh medium supplemented with the same concentration of NaCl or mannitol to monitor plantlet growth.

Analysis of relative electrolyte leakage

Membrane damage was assessed by measuring relative electrolyte leakage (REL) from leaf discs. Electrical conductivity was measured (value A), after which the tubes were autoclaved to release all electrolytes for the second determination of the total content of electrolytes in each sample. Ion leakage from the leaf discs was measured as the conductivity of the solution with a pH meter conductimeter (model Jenway). After cooling to room temperature, the conductivity of each sample solution was measured again (value B). For each measurement, REL was expressed as percentage leakage (%), i.e. (value A/value B) $\times$ 100.

Measurement of malondialdehyde (MDA) contents

The level of lipid peroxidation in leaf tissues was determined in terms of the peroxidation by MDA product in samples. The MDA content was determined by the reaction of the thiobarbituric acid (TBA) as described by Hagege et al. (1990). Briefly, 100 mg of plant material were ground in 4 ml of 20% trichloroacetic acid solution (TCA). A volume of 0.1 ml TBA (5 mg/ml) was added to the homogenized samples and the homogenates were incubated at 95 °C for 30 min, centrifuged at 15,000 $\times$ g for 10 min and 0.55 ml of the supernatant fraction was mixed with 0.55 ml of TBA solution (25% thiobarbituric acid). The mixture was heated at 95 °C for 30 min, chilled on ice, and then centrifuged at 10,000 $\times$ g for 5 min. The absorbance of the supernatant at 532 nm was read and the value of MDA was estimated using the molar extinction coefficient of 155 mM⁻¹ cm⁻¹.

Hydrogen peroxide assay

H₂O₂ concentration in leaf samples was determined following the protocol of Velikova et al. (2000). Fresh material (0.1 g) was homogenized with 5 ml of 0.1% TCA and centrifuged at 12,000 rpm for 15 min. To 0.5 ml of the supernatant, 0.5 ml of phosphate buffer (10 mM pH 7), and 1 ml of 1M potassium iodide were added. The absorbance of the solution was taken at 390 nm using a UV visible spectrophotometer. The content of H₂O₂ was determined using a calibration curve obtained by plotting the rate of intensity increase as a function of H₂O₂ concentration.

Sugar content analysis

The total soluble sugar contents (sucrose, glucose, fructose and their methylated derivative polysaccharides) were determined using the method of Dubois et al. (1956). Plant materials (100 mg) were extracted using 10 ml of 80% ethanol (V/V) and preheated to 80 °C. The mixture was vigorously vortexed and then placed in a water bath at 70 °C for 30 min. Few drops of ethanol were added occasionally to replace evaporative losses. The alcoholic extract (0.05 ml) was mixed with 5 ml anthrone (100 mg anthrone, 50 ml of concentrated sulphuric acid) and 2.45 ml of ethanol 80% (V/V)). Samples were placed in a boiling water bath for 10 min. The light absorption of the samples was estimated at 640 nm using a PD-303 model spectrophotometer. The concentrations of soluble sugars in samples were calculated by referring to a standard curve of glucose. Soluble sugar contents were expressed as mg glucose g⁻¹ FW of leaves. Finally, the results of the optical densities were reported on the calibration curve of soluble sugars (expressed as glucose).

Determination of proline contents

Proline contents in leaves were determined following the method of Bates (1973) with slight modifications. Fresh plant materials (100 mg) were frozen in liquid N₂ and treated with 3 ml of sulphosalicylic acid (3%) for 1 h at 100 °C. About 0.2 ml of the filtered phase was added to 2 ml of solution mixture (composed of 30 ml of glacial acetic acid, 20 ml distilled water and 0.5 g of ninhydrin reagent). The mixture was boiled for one hour. The reaction was stopped by placing tubes in cold water. Samples were vigorously mixed with 4 ml of toluene. The absorbance of toluene phase was estimated at 520 nm against toluene blank. Proline concentration was estimated by referring to a standard curve of proline. Free proline content was expressed as µg g⁻¹ FW of plant sample.

Phylogenetic tree construction

For the phylogenetic analyses, amino acid sequences (for VvWRKY2 protein and some selected AtWRKY proteins) were aligned by CLUSTALW program (<http://www.genome.jp/tools/clustalw/>) (Higgins et al. 1994) and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 6 (<http://www.megasoftware.net>) (Tamura et al. 2013). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were carried out using the neighbour-joining method (Saitou and Nei 1987), the minimum evolution method (Rzhetsky and Nei 1992), the UPGMA method (Sneath and Sokal 1973), the maximum-likelihood method (Jones et al. 1992) and the maximum parsimony method

(Eck and Dayhoff 1966), along with a statistical bootstrapping procedure involving 10,000 replicates. Numbers at the branches with confidence values are based on Felsenstein's bootstrap method (Felsenstein 1985). Evolutionary distances used to infer the phylogenetic tree were computed using the Poisson correction method (Zuckerandl and Pauling 1965).

Data treatment and statistical analysis

Results presented in all histograms, tables and figures are the means of three independent replicates $\pm$ Confidence Interval (CI). Data were subjected to a one-way ANOVA test using XLSTAT software v. 2014 (Addinsoft; <http://www.xlstat.com>) and means were compared according to Duncan's multiple-range test at 5% level of significance.

The principal component analysis (PCA) was done using XLSTAT software v. 2014, considering variables centred around their means and normalized with a standard deviation of 1.

Results

Phylogenetic analysis of the VvWRKY2

To investigate the relationships of the VvWRKY2 protein with other WRKYs from *A. thaliana*, a phylogenetic analysis was performed based on their amino acid sequences using the neighbour-joining method. The results demonstrated that VvWRKY2 was closely related to group I of WRKY family members, including AtWRKY3 and AtWRKY4. In addition, a clear orthology relationship associated VvWRKY2 to AtWRKY3 and AtWRKY4 sequences with, respectively, 58.44 and 54.21% of similarity. These results further confirmed the belonging of VvWRKY2 protein to the group I of WRKY family (Fig. 1).

Plants over-expressing VvWRKY2 gene showed improved germination under saline and drought constraints

To investigate the function of VvWRKY2 in the germination process, the seeds of transgenic tobacco lines over-expressing VvWRKY2 were cultivated under saline conditions. The seedling behaviours of three different transgenic lines were compared under different saline concentrations (0, 100, 200 and 300 mM NaCl). Germination rates of WT seeds and transgenic lines under control conditions were similar and did not show any significant difference (Fig. 2a). However, under NaCl concentrations higher than 100 mM, the germination percentages of WT were lower than those of transgenic lines. In particular, transgenic line 5 behaved better than the others, showing a

significantly higher germination percentage than control plants under salinity.

Under 300 mM of NaCl, the germination capacity of WT seeds decreased to less than 5%, whereas for transgenic lines it reached 30%, indicating clearly the involvement of VvWRKY2 in salinity tolerance at the germination stage (Fig. 2a, 3a1, a2).

For further evaluation of the salt tolerance of transgenic plants, 2-week-old seedlings grown in soil were exposed to 300 mM NaCl solution for 2 weeks. The results showed that, under high salinity, more transgenic plants survived than the control ones and some of them grew vigorously (Fig. 3b, c). Although, in Fig. 3 it seems that transgenic plants grew faster than WT under control condition; this observation needs more significant analyses to be well established.

To assess the implication of VvWRKY2 in osmotic constraint adaptation, tobacco seeds of WT and transgenic lines were germinated under osmotic stress using different concentrations of mannitol (0, 100, 200 and 400 mM). As shown in Fig. 2b, there was no visible difference between WT and transgenic lines on control medium without mannitol. However, starting from 200 mM mannitol, the WT germination rate began to fall up to 50%, whereas the transgenic lines maintained the same level of germination as for 0 mM mannitol. Under higher mannitol concentrations, the transgenic lines displayed better germination rates, particularly under 400 mM where they reached approximately 35% of germination, showing thus the implication of VvWRKY2 in osmotic stress adaptation.

The increased germination in the three independent transgenic (W2-5, W2-11 and W2-12) lines excluded any insertional effects and confirmed the role of VvWRKY2 in the tolerance phenotype. Furthermore, these results indicate that the over-expression of the VvWRKY2 gene improved the germination of seeds under both salt and osmotic constraints. The transgenic lines W2-5, displaying the highest germination rate under both salt and osmotic stresses, had the highest level of accumulation of VvWRKY2 transcripts (Mzid et al. 2007). VvWRKY2 expression patterns analysis (Supplemental File 1a) in 54 tissues/organs samples at various developmental stages of grapevine rootstock (cv Corvina) showed diverse transcription levels, mainly in mid-ripening berries and young leaves. Moreover, expression studies of VvWRKY2 under abiotic stressful conditions (Supplemental File 1b and c) revealed that VvWRKY2 is slightly induced by salt treatment and water deficit. Our results point out the importance of VvWRKY2 transcription levels in plant adaptation.

Oxidative stress evaluation in transgenic VvWRKY2 and WT lines under saline and osmotic stresses

To study whether the over-expression of VvWRKY2 gene increases the tolerance of transgenic plants through better

Fig. 1 The Phylogenetic tree constructed by the MEGA program version 6 (Tamura et al. 2013). Different methods were used; they gave similar results. The presented tree was the optimal tree obtained using the neighbour-joining method (Saitou and Nei 1987) and corresponds to a bootstrap consensus tree inferred from 10,000 replicates. The numbers at the branches are confidence values based on Felsenstein's bootstrap method (Felsenstein 1985). The tree is drawn to scale, with branch lengths corresponding to the number of amino

acid substitutions per site. All positions containing gaps and missing data were eliminated, leaving a total of 58 positions in the dataset. The evolutionary distances used to infer the phylogenetic tree were computed using the Poisson correction method (Zuckerandl and Pauling 1965). Proteins for which a functional role at the plant level has been ascribed are indicated in bold. All positions containing gaps and missing data were eliminated. The analysis involved 66 amino acid sequences

Fig. 2 Seed germination of WT (control) and *VvWRKY2* transgenic tobacco under salt (a), and water stress conditions (b). Each value is a mean of four separate biological replicates. Error bars are $\pm$ CI. For each parameter, bars labelled with different letters are significantly different according to Duncan's test at $P \leq 0.05$

oxidative stress management, REL, MDA and H_2O_2 contents were analyzed.

The improved stress (osmotic and salt) tolerance of *VvWRKY2* over-expressing plants was not correlated with changes in REL, H_2O_2 or with MDA contents. Indeed, under normal and stress growing conditions, the ion leakage, H_2O_2 and MDA contents of both WT and *VvWRKY2* transgenic lines were similar (Tables 1, 2). These results indicate that the tolerance of transgenic plants was not related to oxidative stress

Over-expression of the *VvWRKY2* increases proline and sugar accumulation under saline and osmotic stresses

To elucidate and understand the physiological changes of *VvWRKY2* transgenic lines, contents of free proline and

soluble sugars were measured upon application of salt and osmotic stresses.

Proline accumulation in the leaves was determined in the WT and the *VvWRKY2* over-expressing plants. Under control condition, proline levels were similar in transgenic and wild type plants (Fig. 4a, b). However, under osmotic and salt conditions, when compared with the WT plants, the transgenic lines showed remarkably higher levels of both prolines (Fig. 4a, b) and soluble sugars (Fig. 5a, b), highlighting their role in osmotic stress adaptation through osmo-regulation mechanism.

Thus, the results of physiological characterization suggest that over-expression of *VvWRKY2* increased proline and sugar contents, with no change in MDA, H_2O_2 and ion leakage accumulation under salt and dehydration conditions.

Principal component analysis (PCA)

The trait-by-trait analyses were completed by Principal Component Analysis (PCA) which took into account traits characterizing transgenic plants that were cultivated under stress conditions.

Taking into account the PCA performed for NaCl treatments, by considering six parameters and two plants lines (WT and transgenic tobacco plant over-expressing *VvWRKY2*), the first two components (F1 and F2) explained 92.63% of the total variation. The first component (axis 1) explained 78.2% of the variation, followed by 14.43% for the second component (axis 2) (Fig. 6).

Likewise, we found that NaCl was correlated with the studied parameters. In fact, we noticed that all studied parameters showed high correlation coefficients with saline treatments ($R^2 = -0.87$ for germination rate, 0.94 for leaf relative electrolyte leakage, 0.81 for sugar content, 0.76 for proline content, 0.88 for H_2O_2 content and 0.92 for MDA content) (Table 3). The analysis showed also statistically significant positive correlations between transgenic tobacco plants grown under salt treatments and the contents of sugar and proline ($R^2 = 0.39$ and 0.35, respectively) (Table 3).

Congruous results were showed based on PCA carried out for mannitol treatments. In fact, the first two components explained 92.54% of the total variation. The first component (axis 1) explained 79.72% of the variation, followed by 12.82% for the second component (axis 2) (Fig. 7).

Similarly to results shown by NaCl, mannitol was correlated with all studied parameters ($R^2 = -0.85$ for germination rate, 0.96 for leaf relative electrolyte leakage, 0.68 for sugar content, 0.84 for proline content, 0.87 for H_2O_2 content and 0.94 for MDA content) (Table 4). Transgenic plants grown under mannitol treatments showed positive correlations (significant at 0.05 level) with germination rate ($R^2 = 0.35$), sugar content ($R^2 = 0.47$) and proline content ($R^2 = 0.33$) (Table 4).

Fig. 3 Effect of salt stress (300 mM NaCl) on VvWRKY2 transgenic tobacco plants grown under in vitro (**a1, a2**) and greenhouse conditions (**b1, b2, c1, c2**)

Fig. 3 (continued)

Table 1 Relative electrolyte leakage, free H₂O₂ and MDA contents in seedlings of WT and VvWRKY2 tobacco lines (W2-5, W2-11 and W2-12) under control and salt stress

NaCl (mM)	Genotype	Relative electrolyte leakage (%)	H ₂ O ₂ (nmol/g FW)	MDA (nmol/g FW)
0	WT	28.00 ± 5.67 g	260.24 ± 59.32 f	3.52 ± 1.02 cd
	W2-5	33.69 ± 3.30 g	310.52 ± 10.15 def	3.12 ± 0.59 d
	W2-11	32.43 ± 2.90 g	303.57 ± 8.55 ef	4.41 ± 1.26 cd
	W2-12	36.41 ± 2.40 fg	280.24 ± 7.85 f	3.87 ± 1.08 d
100	WT	44.74 ± 1.52 def	375.00 ± 11.32 bc	7.94 ± 0.90 cd
	W2-5	40.20 ± 1.92 efg	351.86 ± 19.38 cde	5.91 ± 1.79 cd
	W2-11	48.61 ± 1.36 de	367.14 ± 12.44 cd	11.27 ± 7.30 bc
	W2-12	46.6 ± 2.15 de	378.57 ± 53.97 bc	7.92 ± 2.94 cd
200	WT	58.33 ± 10.99 c	432.62 ± 17.75 b	17.00 ± 0.74 b
	W2-5	51.90 ± 0.88 cd	370.48 ± 9.74 bcd	15.31 ± 1.18 b
	W2-11	57.3 ± 6.21 c	430.38 ± 8.91 b	17.37 ± 10.49 b
	W2-12	59.4 ± 1.50 c	500.14 ± 6.47 a	15.17 ± 1.26 b
300	WT	68.98 ± 8.42 b	517.86 ± 4.04 a	27.56 ± 5.49 a
	W2-5	73.96 ± 10.64 ab	403.57 ± 31.31 bc	24.73 ± 3.04 a
	W2-11	75.33 ± 8.49 ab	515.00 ± 2.14 a	23.52 ± 2.71 a
	W2-12	79.47 ± 4.00 a	516.19 ± 20.47 a	24.15 ± 3.57 a

Values represent the mean ± CI of three experiments. For each parameter, values labelled with different letters are significantly different according to Duncan's test at $P \leq 0.05$

PCA analyses confirm clearly the positive effects of VvWRKY2 gene over-expression on the tolerance of tobacco plants to salt and osmotic stresses, particularly throughout the adjustment of sugar and proline contents.

Discussion

Numerous studies demonstrated that several members of WRKY TFs family are involved in the responses of many plants to multi-environmental stresses (Zhao et al. 2015;

Table 2 Relative electrolyte leakage, free H₂O₂ and MDA contents in WT and VvWRKY2 tobacco (W2-5, W2-11 and W2-12) seedlings under control and osmotic stress

Mannitol (mM)	Genotype	Relative electrolyte leakage (%)	H ₂ O ₂ (nmol/g FW)	MDA (nmol/g FW)
0	WT	28.24 ± 0.85 h	326.19 ± 7.85 f	5.82 ± 2.25 de
	W2-5	34.75 ± 2.24 g	331.43 ± 15.61 f	3.22 ± 1.25 e
	W2-11	31.11 ± 1.44 h	350.48 ± 6.48 ef	4.60 ± 2.05 e
	W2-12	36.14 ± 1.17 g	322.14 ± 5.30 f	7.32 ± 2.19 de
100	WT	44.79 ± 0.56 f	375.00 ± 11.32 e	11.56 ± 1.66 cd
	W2-5	43.82 ± 3.59 f	455.24 ± 32.39 cd	15.39 ± 1.37 bc
	W2-11	47.57 ± 2.60 ef	428.10 ± 8.30 d	16.05 ± 2.27 bc
	W2-12	48.94 ± 2.46 e	316.86 ± 10.93 f	12.94 ± 2.99 bc
200	WT	59.43 ± 1.40 d	432.62 ± 17.75 d	17.52 ± 7.32 b
	W2-5	62.73 ± 1.22 cd	537.14 ± 19.67 b	16.43 ± 6.02 bc
	W2-11	60.30 ± 3.45 d	468.81 ± 2.84 c	17.72 ± 3.12 b
	W2-12	64.59 ± 3.23 c	427.57 ± 6.15 d	17.33 ± 2.56 b
400	WT	71.82 ± 1.85 b	517.86 ± 4.04 b	28.38 ± 2.28 a
	W2-5	72.85 ± 1.16 b	581.43 ± 60.77 a	28.06 ± 1.88 a
	W2-11	72.88 ± 4.47 b	542.14 ± 5.66 b	27.46 ± 2.44 a
	W2-12	77.03 ± 2.57 a	514.29 ± 42.77 b	29.41 ± 4.24 a

Values represent a mean ± CI of three experiments. For each parameter, values labelled with different letters are significantly different according to Duncan's test at $P \leq 0.05$

Song et al. 2016; Jiang et al. 2017). However, their functions in the response of grapevine to stress remain unclear. To go further, our previous study on VvWRKY2 biological roles (Mzid et al. 2007), we explored the putative involvement of this gene in the response to salinity and dehydration stresses.

Amino acid comparisons and phylogenetic analyses showed a clear orthologous relationship of VvWRKY2 with AtWRKY3 and AtWRKY4, all of which belong to the group I of WRKY family. The AtWRKY3 was induced by SA and pathogens (Lai et al. 2008; Rushton et al. 2010), while the AtWRKY4 was regulated by JA, SA, pathogens, sucrose, senescence, cold and salinity (Hammargren et al. 2008).

Ramamoorthy et al. (2008) demonstrated that WRKY proteins play a crucial role in abiotic stress tolerance alongside their roles in biotic stress tolerance. Guo et al. (2014) indicated that the expression of VvWRKY2 was induced by salt and by drought after 48 and 144 h, respectively. This result was in line with the finding of Wang et al. (2012) and Liu et al. (2013) who revealed the induction of BcWRKY46 (*Brassica campestris* ssp. *chinensis*) and DgWRKY3 (*Dendranthema grandiflorum*) by the salt and drought treatments. Consequently, it is clear that VvWRKY2 is a functional WRKY TF involved in the response to salt and water stresses. Being induced by NaCl treatments, we performed a functional analysis on VvWRKY2 to confirm its implication in salinity tolerance and response. In the current study, we demonstrated that the over-expression of VvWRKY2 in tobacco improved the tolerance of transgenic plants to salt and water-deficit stresses at germination and seedling stages. In fact, the germination under

salt and drought stress conditions was higher in transgenic lines than for WT plants. Likewise, many previous studies demonstrated that the constitutive expression of some grapevine WRKY genes, including VvWRKY11, VpWRKY2 and VpWRKY3, could enhance tolerance to abiotic stress in transgenic lines (Li et al. 2010; Liu et al. 2011). These results are similar to those previously obtained for tobacco plants over-expressing the wheat TaWRKY10 (Wang et al. 2013) and the cotton GhWRKY41 genes (Chu et al. 2015), which showed an increased germination rate under salt and water stress conditions. More recently, Zhou et al. (2015) revealed that over-expression of GhWRKY34 in the model plant *A. thaliana* led to a significantly higher germination rate in transgenic lines, as compared with WT in presence of NaCl. Other authors demonstrated that the over-expression of (*Reaumuria trigyna*) RtWRKY1 gene in *Arabidopsis* was responsible for the salt tolerance improvement by regulating plant growth, osmotic balance, Na⁺/K⁺ homeostasis and the antioxidant system (Du et al. 2017). Nevertheless, when over-expression a cotton GhWRKY25 gene in *Arabidopsis*, a low tolerance to drought was obtained, while better salt tolerance was observed (Liu et al. 2016).

Several studies reported that seed germination is regulated by WRKY TFs through different mechanisms. For example, AtWRKY2-mediated seed germination and post-germination stages are inhibited by ABA, and OsWRKY78, which might proceed as a regulator in stem elongation and seed development (Jiang and Yu 2009; Zhang et al. 2011). Besides, Lagacé and Matton (2004) revealed the implication of WRKY proteins in

Fig. 4 Changes of free proline contents in WT and *VvWRKY2* tobacco plants (W2-5, W2-11 and W2-12) under salt (a) and water stress conditions (b). Values are means of three replicates. For each parameter, bars labelled with different letters are significantly different according to Duncan's test at $P \leq 0.05$

Fig. 5 Changes of sugar contents in WT and *VvWRKY2* tobacco plants (W2-5, W2-11 and W2-12) under salt (a), and water stress conditions (b). Values are means of three replicates. For each parameter, bars labelled with different letters are significantly different according to Duncan's test at $P \leq 0.05$

the embryogenesis of the wild potato species (*Solanum chacoense*). In fact, they demonstrated the expression of *ScWRKY1* in ovules bearing late torpedo-staged embryos. Additionally, and more recently, Rainieri et al. (2016) showed that the sunflower transcription factor *HaWRKY10* could improve the germination of *A. thaliana* through the stimulation of reserve mobilization. Other studies highlighted that the WRKY TF over-expression can inhibit the germination process under stress conditions. For instance, Yan et al. (2014) reported that *GhWRKY17* played a negative role in regulating ABA-mediated seed germination and seedling growth in *Nicotiana benthamiana* transgenic plants. This was already pointed out by Jia et al. (2015) for the *GhWRKY68*.

After 4 weeks of drought or salt stress conditions, transgenic lines accumulated high levels of proline and soluble sugars (Figs. 4, 5). This result is in accordance with others

demonstrating that the accumulation of compatible solutes such as proline and a variety of sugars is effective in improving tolerance of plants to salt and osmotic stresses (Huang et al. 2009; Petronia et al. 2011), playing highly protective roles under stress constraints (Hayat et al. 2012; Murakeozy et al. 2003). Thus, our results indicated that the transgenic lines display drought and salt resistances through a better mechanism of osmo-regulation. Similarly, constitutive expression of different WRKY genes, including *TaWRKY20* and *TaWRKY44*, resulted in a greater accumulation of proline and sugars in transgenic lines (Wang et al. 2013; Wang et al. 2015).

Electrolyte leakage represents a rapid, sensitive and quantitative method to evaluate the effect of stress on plant cells (Verslues et al. 2006). Moreover, the MDA was used to analyse lipid peroxidation (Loreto and Velikova 2001). We thus, evaluated the accumulation of H_2O_2 in the leaf after

Fig. 6 Principal component analysis. The significance of parameters used to study the response of transgenic and WT tobacco plants under salt stress conditions. These parameters, as well as the different growth conditions, are projected onto the F1–F2 principal factorial plane that explains 92.63% of the variation. Plants were exposed to saline conditions with increasing NaCl concentrations (0, 100, 200 and 300 mM). Black circles represent different analysis parameters. Black diamonds represent the different growth conditions, and white circles represent the different study lines (wild type and the different transgenic lines)

Table 3 Pearson's correlation matrix analyzing the effect of salt stress on the germination rate, relative electrolyte leakage, sugar content, proline content, H_2O_2 content, MDA content in WT and VvWRK2 tobacco plants lines

Variables	Germination rate	Relative electrolyte leakage	Sugar content	Proline content	H_2O_2 content	MDA content	NaCl	WT	Transgenic lines
Germination rate	1	− 0.8437	− 0.5292	− 0.4976	− 0.8201	− 0.8537	− 0.8704	− 0.2528	0.2528
Leaf relative electrolyte leakage		1	0.7638	0.7276	0.8625	0.8815	0.9441	− 0.0771	0.0771
Sugar content			1	0.9498	0.5970	0.7271	0.8112	− 0.3850	0.3850
Proline content				1	0.5032	0.6737	0.7636	− 0.3452	0.3452
H_2O_2 content					1	0.8037	0.8810	0.0126	− 0.0126
MDA content						1	0.9245	0.0470	− 0.0470

Variables were centred around their means and normalized with a standard deviation of 1

Bold values represent significant correlation levels at 0.05

stress conditions. We found that no difference was observed within these parameters for both control (WT) and transgenic (VvWRKY2) plants under salt stress conditions.

Maintenance of membrane functionality due to unchanged oxidative stress indicators in grapevine under salt stress may be attributed to the adequate response of the antioxidant system to salt stress-induced oxidative damage, as also observed in some other salt- or drought-treated plants

such as *Pisum sativum* (Noreen and Ashraf 2009), maize (Jiang and Zhang 2002), sorghum (Zhang and Kirkham 1996) and *Plantago maritima* (Sekmen et al. 2007).

The over-expression of VvWRKY2 gene did not change the response of the oxidative indicators observed in wild plants under salt stress. These results are in contrast with those of Liu et al. (2013) and Liang et al. (2017) showing reduced accumulation of MDA and H_2O_2 in transgenic

Fig. 7 Principal component analysis. The significance of parameters used to study the response of transgenic and WT tobacco plants under mannitol conditions. These parameters, as well as the different growth conditions, are projected onto the F1–F2 principal factorial plane that explains 92.54% of the variation. Plants were exposed to saline conditions with increasing mannitol concentrations (0, 100, 200 and 400 mM). Black circles represent different analysis parameters. White diamonds represent the different growth conditions, and white circles represent the different study lines (wild type and the different transgenic lines)

Table 4 Pearson's correlation matrix analyzing the effect of mannitol on the germination rate, relative electrolyte leakage, sugar content, proline content, H₂O₂ content, MDA content in WT and VvWRKY2 tobacco plants lines

Variables	Germination rate	Relative electrolyte leakage	Sugar content	Proline content	H ₂ O ₂ content	MDA content	Mannitol	WT	Transgenic lines
Germination rate	1	− 0.8290	− 0.3543	− 0.5158	− 0.6883	− 0.7816	− 0.8460	− 0.3450	0.3450
Leaf relative electrolyte leakage		1	0.7554	0.8279	0.8496	0.9103	0.9569	− 0.0911	0.0911
Sugar content			1	0.8738	0.7115	0.6945	0.6813	− 0.4688	0.4688
Proline content				1	0.8477	0.7941	0.8401	− 0.3256	0.3256
H ₂ O ₂ content					1	0.8352	0.8737	− 0.1323	0.1323
MDA content						1	0.9412	− 0.0263	0.0263

Variables were centred around their means and normalized with a standard deviation of 1. Bold values represent significant correlations at 0.05 level

chrysanthemum over-expressing *DgWRKY* genes under salt stress, when compared with control plants (WT). In another study, the *GhWRKY68*-over-expressing plants have a reduced resistance to drought and salt. They exhibited reduced tolerance to oxidative stress after drought and salt

stress treatments. It was correlated with the accumulation of ROS, reduced enzyme activities, elevated MDA content and altered ROS-related gene expression (Jia et al. 2015). All these results clearly showed that the relationship between *WRKY* genes and salt-induced oxidative stress is variable

among plant species. In our study, the improvement of the tolerance to drought and salt stress in transgenic *WRKY* plants cannot be associated with an improved ROS detoxification process.

In conclusion, our results indicated that over-expression of *VvWRKY2* in transgenic tobacco enhanced salt and drought stress tolerance when compared with wild types. On one hand, these functions are achieved by accumulating proline and soluble sugar contents, but on the other hand, the plant ROS amount and MDA content remain unchanged. Hence, based on overall results, it could be presumed that *VvWRKY2* over-expression in grapevine would improve its salt and drought stress tolerance. Moreover, the mechanism of osmotic stress tolerance must be further investigated. Therefore, the role of *VvWRKY2* in grapevine and its target genes in various signalling pathways must be studied to better understand and reveal the exact mechanisms of plant responses to these constraints.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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