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# Physiological responses of transgenic tobacco plants expressing the dehydration-responsive *RD22* gene of *Vitis vinifera* to salt stress

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**Abstract:** Salinity is one of the most common environmental stresses affecting grapevine productivity. In order to analyze the contribution of the dehydration-responsive *RD22* gene of *Vitis vinifera* L. (*VvRD22*) to salt tolerance improvement, *Agrobacterium*-mediated transformation in the tobacco model plant (*Nicotiana benthamiana*) was carried out and transgenic lines were subjected to in vitro and ex vitro salt treatments for physiological response evaluation. Under in vitro salt stress, the transgenic lines exhibited higher seed germination and growth than the wild type (WT). The ex vitro assays after salt treatment showed better growth and higher chlorophyll content in *VvRD22*-expressing plants than in the WT. Ion analysis revealed less increase in the levels of sodium ($Na^+$) in leaves and chloride ($Cl^-$) in all organs within the transgenic lines compared to the WT. Interestingly, stabilized calcium ($Ca^{2+}$) contents were registered in the leaves of transgenic lines. Moreover, the occurrence of an osmotic adjustment based on an overproduction of total soluble sugars was observed within the transgenic lines. These physiological responses suggest that the protective effect of *VvRD22* transgenic expression is enabling other physiological mechanisms to function and subsequently contributing to the ability to cope with salt stress. The *VvRD22* transgenic expression would be useful in engineering salt stress-tolerant grapevines.

**Key words:** *RD22* gene, salt tolerance improvement, transgenic tobacco, *Vitis vinifera*

## 1. Introduction

Salinity limits the production ability of agricultural soils in large areas of the world (Arzani, 2008). Exposition to high saline conditions may induce either a water deficit that results from relatively high solute concentrations in the soil or a second ion-specific stress resulting from altered potassium/sodium ($K^+/Na^+$) ratios and $Na^+$ and chloride ($Cl^-$) ion concentrations that are inimical to plants (Blumwald, 2000). These adverse effects contribute to plant growth inhibition and even to plant death. In response to those adversities, plants have developed various strategies that increase induced tolerance or adaptation to stress conditions, e.g., by altering gene expression profiles, leading to adaptive responses at the cellular or systemic levels (Xiong et al., 2001). One phytohormone mediator that results in the alteration of gene expression in plants is abscisic acid (ABA), which was shown to mediate plant responses to salinity (Finkelstein et al., 2002). At present, there is limited evidence that new methods to enhance crop yield stability on saline soils, based on remediation of salinized soils, are feasible (Tester and Davenport, 2003). Comprehensive studies for developing abiotic stress tolerance are in progress (Sekmen Esen et al., 2012), involving genes from different pathways including osmolyte synthesis, ion homeostasis, antioxidative pathways, and regulatory genes (Agarwal et al., 2012). Therefore, recent trends are shifting towards genetic transformation of multiple genes or transcription factors controlling different tolerance-related physiological mechanisms (Apse and Blumwald, 2002; Wang et al., 2003; Bartels and Sunkar, 2005; Chinnusamy et al., 2005; Mahajan and Tuteja, 2005; Zhao et al., 2006; Chen et al., 2007; Tuteja, 2007; Xu et al., 2013). A large number of crop plants are being engineered with salt stress-tolerant genes and have shown salt stress tolerance, mostly at the laboratory level (Agarwal et al., 2012).

Among the various genes induced by salt stress, a member of the RD22-like subfamily was first identified during a search for dehydration-responsive genes in *Arabidopsis thaliana* (L.) Heynh. (Yamaguchi-Shinozaki and Shinozaki, 1993). The expression of the *AtRD22* gene was found to be induced by water deficit, salinity stress, and abscisic acid application, but not by cold and heat stresses (Urao et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993; Iwasaki et al., 1995; Abe et al., 1997). The RD22 proteins belong to the BURP domain protein family

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and share a conserved BURP domain at the C terminus (Hattori et al., 1998). The stress inducible nature of the *RD22*-like gene is supported by the identification of the NaCl-inducible *BnBDC1* (Yu et al., 2004) from *Brassica napus* L. From *Bruguiera gymnorrhiza* (L.) Lam., 4 genes (*BgBDC1*, *2*, *3*, and *4*) were responsive to salt, ABA, and water stresses (Banzai et al., 2002). *PpRD22* from *Prunus persica* L. Batsch and *GhRDL* from *Gossypium hirsutum* L. were also up-regulated by stress (Callahan et al., 1993; Li et al., 2002). A protective effect was reported for *Gm*RD22 from *Glycine max* (L.) Merr. that enhances abiotic stress tolerance by increasing lignin production (Wang et al., 2012). MYC *At*MYC2 (RD22BP1) and MYB *At*MYB2 transcription factors bind to MYC and MYB recognition sites, acting as cis-elements in the *RD22* promoter and cooperatively activating the expression of *RD22* in *Arabidopsis thaliana* (L.) Heynh. (Abe et al., 2003). These 2 transcription factors play roles in the late stage of the plant's response to different stresses. In fact, Abe et al. (2003) reported that transgenic *Arabidopsis thaliana* (L.) Heynh. plants overexpressing *MYC* and *MYB* had a higher sensitivity to ABA and exhibited an osmotic stress tolerance.

Within grapevine cultivars, several studies were achieved in order to identify proteins and genes involved in salt tolerance (Cramer et al., 2007; Vincent et al., 2007; Daldoul et al., 2010; Jellouli et al., 2010). Cramer et al. (2007) revealed through microarray transcript profiling, quantitative reverse-transcription polymerase chain reaction (RT-PCR), and metabolite profiling that the *RD22* gene was up-regulated in *Vitis vinifera* L. 'Cabernet Sauvignon' thoroughly subjected to gradually applied and long-term (16 days) water-deficit stress and equivalent salinity stress. Using a combined approach of suppression subtractive hybridization and microarray, Daldoul et al. (2010) identified the *RD22* gene among 7 cDNA clones that were up-regulated by salt stress in a tolerant variety (Razegui). Furthermore, in wild grapevines (*Vitis vinifera* L. subsp. *sylvestris* (C.C.Gmel.) Hegi), Askri et al. (2012) observed an increased *RD22* gene expression after 14 days of salt treatment. By a candidate gene approach, the *VvRD22* gene, with full-length cDNA of 1.358 kb, was identified from a *Vitis vinifera* 'Cabernet Sauvignon' berry cDNA library at the veraison stage (Accession No. AY634282). The predicted open reading frame (ORF) encodes a protein of 364 amino acids with a calculated molecular mass of 39.2 kDa. This *Vv*RD22 protein contains the conserved BURP domain, similarly to other RD22 proteins of higher plants (Hanana et al., 2008). By northern analysis, these authors showed that *VvRD22* mRNA is induced by salt stress and water deficit. Indeed, salt treatment (100 mM NaCl) was able to induce early and late expression of *VvRD22* in the Razegui tolerant variety, but not in a sensitive one (Syrah), suggesting that this gene is involved in the response to salt stress. The structural parameters, conserved domains, and posttranslational modification sites identified in *Vv*RD22 would support its putative function in salt stress tolerance (Hanana et al., 2008).

Despite this characterization and the previously mentioned studies in gene expression patterns, the functionality of the *VvRD22* gene and its transgenic expression effect in salt tolerance improvement remain unknown. It is therefore essential to lead further investigations on its function and utilizations in a program targeting the improvement of grapevine genetic abiotic stress tolerance.

In the present work, we conducted genetic transformation experiments using the *VvRD22* candidate gene driven by the constitutive *CaMV35S* promoter, within *Nicotiana benthamiana* Domin. The transgenic plants were examined for the presence and expression of the transferred gene at the molecular level. Furthermore, we evaluated the physiological responses of 2 transgenic T2 lines using in vitro germination and ex vitro assays in order to explore the contribution of *VvRD22* and the level to which its transgenic expression in tobacco may enhance salt tolerance.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Tobacco seeds from wild-type (WT) *Nicotiana benthamiana* Domin were sterilized for 1 min in 70% (v/v) ethanol, incubated for 7 to 10 min in 7% (w/v) calcium hypochlorite, and finally rinsed with sterile distilled water. In vitro culture was carried out on Murashige and Skoog (MS) (1962) medium at 24–25 °C with a 16-h photoperiod and 70 μmol $m^{-2}$ $s^{-1}$ light intensity. Acclimatization was established under controlled greenhouse conditions (24 °C, 60% relative humidity, and 16-h photoperiod).

### 2.2. *Agrobacterium*-mediated gene transfer and molecular analysis

To investigate the transgenic expression effect of the *VvRD22* gene in tobacco, the full ORF was cloned in the vector pGreen under the control of the *35S* promoter and *nopaline synthase* (*nos*) terminator (Hanana et al., 2008). Tobacco genetic transformation of WT *Nicotiana benthamiana* Domin using the *Agrobacterium tumefaciens* ATHV strain was achieved as described by Horsch et al. (1985). Selection was carried out using 100 mg $L^{-1}$ kanamycin.

The detection of the integrated *VvRD22* fragment in putative transgenic *Nicotiana benthamiana* Domin was performed by PCR. Genomic DNA of tobacco was extracted in vitro from leaves following the method of Edwards et al. (1991).

PCR was conducted using 2 *VvRD22* specific primers (*RD22*-1: 5'-TGGAATTCAGTTCTGCCAAACAC-3'; *RD22*-2: 5'-TCCCTTTACCAACACCAACATATACA-3')

to amplify a *VvRD22* fragment of 300 bp. The 25-µL reaction mixture contained 40 ng of genomic DNA, 0.4 mM dNTPs, 0.24 µM of each transgene primer, 3 mM $MgCl_2$, and 1.25 U of Taq DNA polymerase (Promega). PCRs were performed under the following conditions: 2 min at 94 °C, and then 35 cycles of 45 s at 94 °C, 30 s at 56 °C, and 45 s at 72 °C, with a final extension of 2 min at 72 °C. As a control, a *Tubulin* gene fragment (Jia et al., 2008) was amplified using sense primer 5'-AGAACACTGTTGTAAGGCTCAAC-3' and antisense primer 5'-GAGCTTTACTGCCTCGAACATGG-3' under similar conditions to those described for *VvRD22*, except with annealing at 50 °C and 21 cycles.

Total RNA was extracted from leaves using the plant TRIzol reagent (Invitrogen). After RNase-free DNase treatment (Promega), RT-PCR reaction was carried out using a one-step RT-PCR system (Invitrogen). Transgenic plants were subsequently selected for 2 further generations to identify transgenic lines that are homozygous for the transgene.

**2.3. In vitro germination assay**

Tobacco seeds were allowed to incubate at 4 °C for 3 days to promote synchronous germination and growth at 25 °C. Fifty seeds from WT and transgenic plants were cultivated in 9-cm petri plates. Seeds were assumed to have germinated if the radicle tips had fully expanded the seed coat. Percentage of germinated seeds was scored as the germination rate. The sensitivity of T2 seed germination to NaCl was assayed on MS medium agar plates including 0, 100, 150, and 200 mM NaCl. Germination was scored every 3 days up to 21 days. After 5 weeks, percentages of cotyledonary leaves were scored. Young seedlings of WT and transgenic lines developed in salt-free MS or 150 mM NaCl media were transplanted in jars containing the same medium for continuous proliferation.

**2.4. Greenhouse assays**

T2 seeds were first germinated on MS containing 200 mg $L^{-1}$ kanamycin. After acclimatization under controlled conditions (24 °C /18 °C, 60%–70% relative humidity, 16-h photoperiod, 25 $W/m^2$ minimal light intensity), 6 plants per line (WT and transgenic) were grown in pots filled with sand. Prior to stress application, rooted tobacco plants were periodically irrigated with a nutrient solution of Long Ashton (Hewitt, 1966) to field capacity (100% of soil equivalent humidity) every 3 days for 6 weeks. Salt stress was then applied during the 6 following weeks using the same nutrient solution supplemented with NaCl to a final concentration of 0, 150, 300, and 400 mM. Plants were salt-treated every 3 days to field capacity.

**2.5. Growth parameters**

Plant heights were recorded after 6 weeks of salt stress treatments. To determine dry weight (DW), fresh leaves were collected and dried at 70 °C for 48 h.

Total leaf areas from control and salt-stressed (400 mM NaCl) WT and transgenic tobacco plants were measured after 6 weeks using a planimeter (LI-3000A, LI-COR).

**2.6. Chlorophyll content**

Total chlorophyll content was extracted from leaves and estimated at the end of the stress treatment (400 mM NaCl) following the standard method of Torrecillas et al. (1984). Thus, 5 small disks (1 cm in diameter) were cut from young leaves and incubated in 5 mL of acetone (80%) in the dark at 4 °C for 3 days (until complete chlorophyll extraction). The total chlorophyll content was then determined by measuring the optical density at 649 and 665 nm using a spectrophotometer (Ultrospec 200, Pharmacia) and calculated according to Strain and Svec (1966) as follows:

Total chlorophyll ($\mu g\ mL^{-1}$) = 6.45 × (A665) + 17.72 × (A649).

Concentrations were then expressed in $\mu g\ cm^{-2}$ leaf area according to Dinç et al. (2012).

**2.7. Mineral contents**

$Na^+$, $Cl^-$, $K^+$, and $Ca^{2+}$ contents were determined from dried leaves, shoots, and roots after 6 weeks of salt stress treatments with 0, 150, and 300 mM NaCl. $Na^+$ and $K^+$ contents were measured using a flame spectrophotometer (Corning Flame Photometer 410). $Ca^{2+}$ content was determined with an atomic absorption spectrophotometer (Perkin Elmer Atomic Absorption Spectrometer 3110) and $Cl^-$ content was measured using a chloridometer.

**2.8. Osmotic potential**

Leaf disks (5 mm) from control and salt-stressed (400 mM NaCl) WT and transgenic tobacco plants were excised, frozen, thawed, and mechanically disrupted following the method of Martínez-Ballesta et al. (2004). The resulting sap was analyzed for osmolarity determination. Osmolarity was assessed using an osmometer (OSMOMAT 030) and converted from $mOsmol\ kg^{-1}$ to MPa to determine the osmotic potential ($\Psi_s$) according to the Van't Hoff equation: $\Psi_s = -m \times R \times T$, where m is the osmolality, R the universal gas constant, and T is the temperature (K).

**2.9. Relative water content**

Leaves from control and salt-stressed plants were excised 6 weeks after salt treatment (400 mM NaCl) to measure relative water content (RWC) according to Turner (1981). RWC was calculated based on the following formula: RWC (%) = 100 × [(fresh weight – dry weight) / (turgid weight – dry weight)]. Leaf samples were of a similar physiological stage as those collected for chlorophyll content determination.

**2.10. Sugar content**

Leaf soluble sugar content was determined from control and stressed plants (400 mM NaCl) according to the method of Morris (1948). Absorbance values were recorded at 640 nm using a spectrophotometer (Ultrospec

200, Pharmacia) and regressed as sugar concentrations (mmol $g^{-1}$ DW) according to a standard curve previously established based on a set of glucose solutions.

### 2.11. Statistical analysis

Data are means of 3 replicates from 3 different plants from control and stressed sets. STATISTICA software was used for statistical analysis. Comparisons were done using least significant difference (LSD) tests.

## 3. Results

### 3.1. Molecular characterization of transgenic tobacco lines

After *Agrobacterium*-mediated transformation, 5 lines (L7, L8, L15, L17, and L20) out of 10 were approved by PCR analysis as transgenic after 3 subcultures (Figure 1A). Our *VvRD22* transgenic lines did not display any marked phenotypic modification compared to WT plants. RT-PCR analysis was further used to confirm *VvRD22* transgene expression. Amplification of cDNAs from the *VvRD22* transgenic plants, with *VvRD22*-specific primers, yielded the expected 300-bp band (Figure 1B). The presence of a single expected transcript indicates that transcription initiation and termination of *VvRD22* mRNA occurred in the *VvRD22* transgenic plants. The transgenic lines L15 and L20 were randomly selected for further in vitro and ex vitro physiological studies.

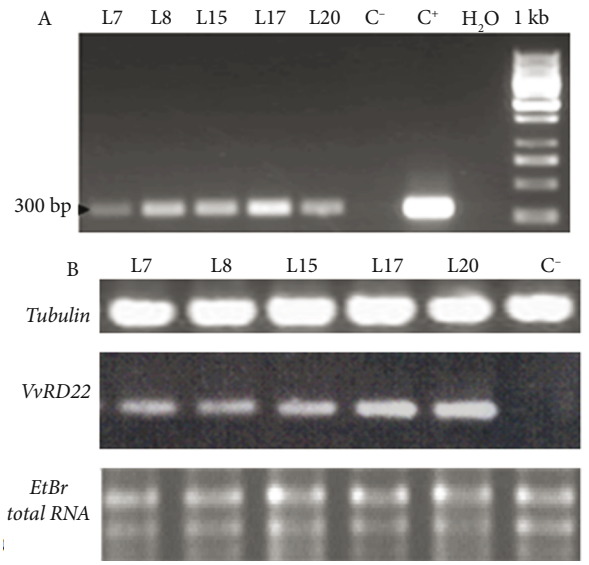


**Figure 1.** Molecular analysis of the transgenic tobacco plants. A- PCR amplification of genomic DNA of WT and transgenic *VvRD22* tobacco plants using *VvRD22* gene specific primers for 300-bp fragment amplification. $H_2O$: internal negative control; $C^-$: untransformed *Nicotiana benthamiana* Domin WT; $C^+$: pGreen-*VvRD22* vector; L7, L8, L15, L17, and L20: transgenic tobacco plants; 1 kb: 1 kb DNA ladder. B- RT-PCR analysis of transgenic *VvRD22* tobacco plants. *Tubulin* is the control plant gene.

### 3.2. In vitro germination and growth of transgenic and WT lines under NaCl treatment

Figure 2 displays the seed germination rates of WT and transgenic lines on MS medium under different salt concentrations (100, 150, and 200 mM NaCl) in comparison to the control. In the NaCl-free medium (Figure 2A), 97.5% germination rates were registered after 6 days of culture. However, differences were observed in the germination percentages between WT and L15 and L20 at the third day, when 72% and 52% germination rates were registered for the L15 and L20 lines, respectively, while 19% germination was recorded in WT. The higher germination rates recorded in the transgenic lines compared to the WT could be attributed to *VvRD22* expression. This hypothesis was confirmed after 100 mM salt application (Figure 2B). Indeed, L15 and L20 germination rates were similar to those of the control. In contrast, the WT germination ability was inhibited under salt stress. Concomitantly, L15 and L20 reached a 92% average germination. Under 150 mM salt concentration (Figure 2C), L15 and L20 lines managed to reach maximal germination percentage rates, even though a germination delay was observed. The WT germination rates did not exceed 39% after 21 days of salt application. At 200 mM NaCl concentration (Figure 2D), seed germination was strongly affected in both WT and transgenic lines. Nevertheless, after 9 days, L15 and L20 germination rates diverged from that of the WT and increased markedly until day 21 after salt application.

Regarding seedlings, transgenic lines showed more developed green rosette leaves and lateral roots than the WT when cultivated on salt-free MS medium. Under 150 mM NaCl, the percentage of seedlings with green cotyledons in transgenic lines was also significantly higher than that of the WT. When NaCl concentration increased up to 200 mM, no seedlings were observed within the WT, whereas some seedlings with green cotyledons were still observed in the transgenic lines.

To seek subsequent proliferation, both WT and transgenic seedlings developed on salt-free MS or 150 mM NaCl-supplemented media were transferred to jars containing the corresponding medium for a 1-month period under the same in vitro conditions. Under control conditions, shoot and root growth in the WT was similar to those of transgenic lines, even though many more precocious and green leaves developed in the latter plants. On the contrary, under 150 mM NaCl, significant differences were observed between the WT and lines expressing *VvRD22*. Indeed, WT plants could not develop roots and leaves, while transgenic lines displayed well-developed plantlets (Figure 3). These results may indicate an increased salt tolerance in the transgenic tobacco in vitro plants.

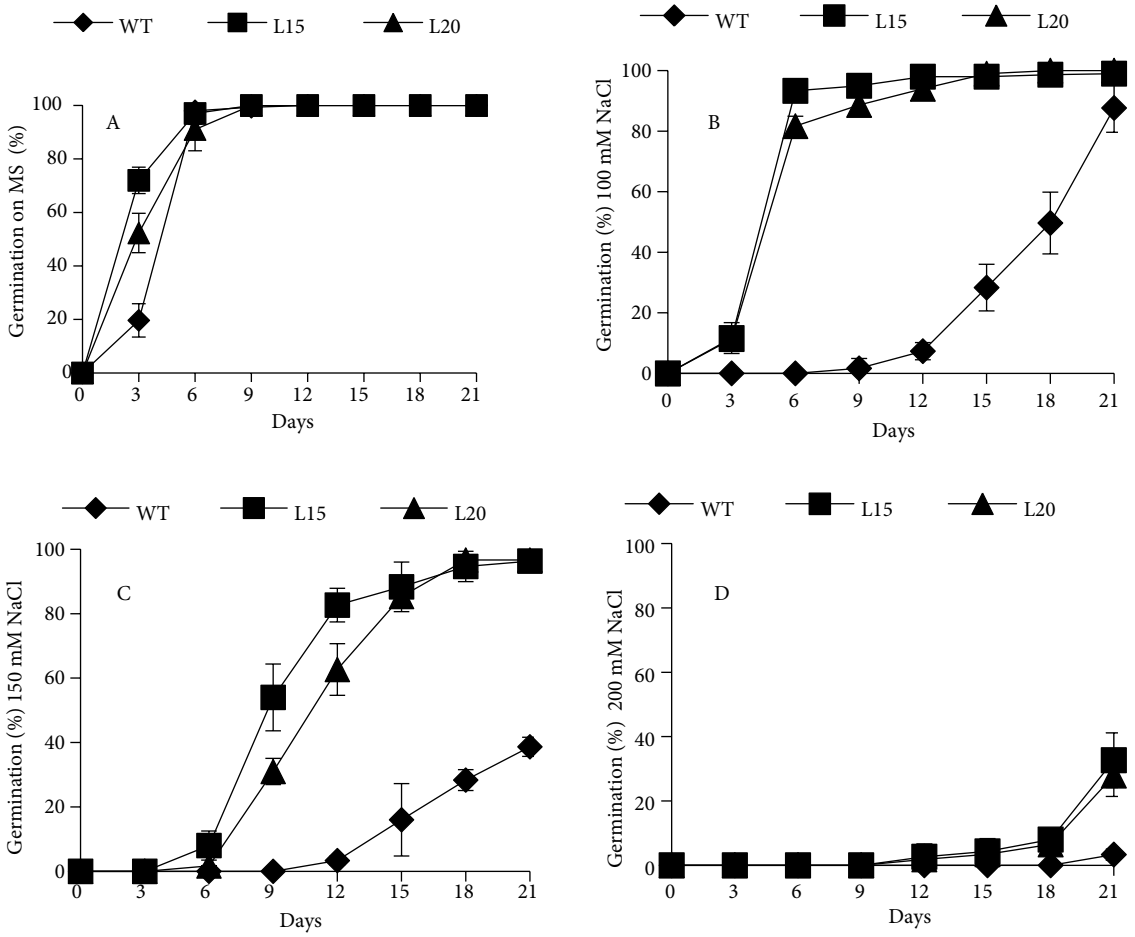


**Figure 2.** Germination of *VvRD22*-transgenic tobacco seeds under NaCl treatments for 21 days. A- 0 mM, B- 100 mM, C- 150 mM, and D- 200 mM NaCl. Data are means ± standard error (SE) of 3 replicates.

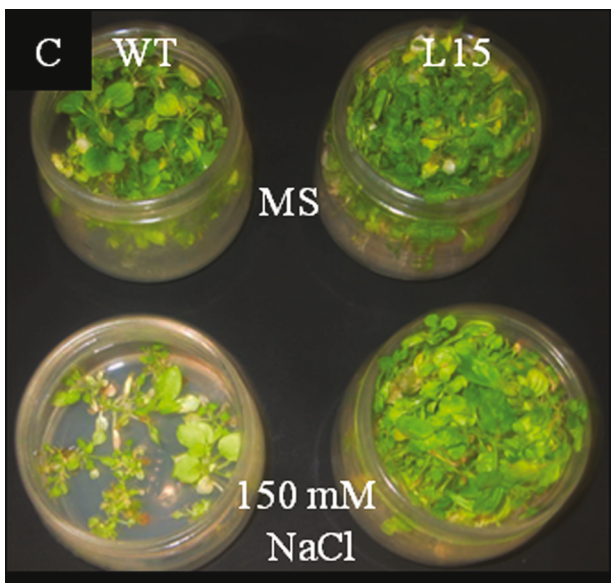


**Figure 3**. In vitro plant development from WT, L15, and L20 tobacco lines on salt-free and 150 mM NaCl-supplemented MS media after a 5-week culture period.

### 3.3. Ex vitro growth of transgenic and WT lines under NaCl treatment

When cultivated ex vitro in sand pots for 6 weeks under 0, 150, 300, and 400 mM salt concentrations, transgenic lines exhibited better growth, with significantly increased plant heights compared to the WT (Figure 4). Indeed, under 150 and 300 mM NaCl treatments, L15 and L20 did not present any significant difference from the control plants. However, the WT displayed markedly affected plant growth under 150 and 300 mM NaCl (Figures 4A–4C). When subjected to 400 mM NaCl, despite their significant shoot height decrease (Figure 4A), transgenic lines interestingly exhibited less severe leaf chlorosis and necrosis symptoms than WT tobacco plants (Figure 4D).

On the other hand, transgenic plants also exhibited better growth rates than the WT under 0, 150, 300, and 400 mM NaCl, with significantly higher leaf DW. Marked differences between transgenic and WT plants were particularly observed at 300 mM and 400 mM NaCl, at which the DW decrease was about 48% and 64%,

respectively, in the WT, while a less significant decline was observed in both transgenic lines (Figure 5A). After 6 weeks of 400 mM salt application, the total leaf area decrease was more significant in the WT (57.2%) than in transgenic plants (29% and 34% for L15 and L20, respectively; Figure 5B). These results would suggest an improved salt tolerance within the transgenic tobacco lines.

### 3.4. Chlorophyll contents in transgenic and WT lines under NaCl treatment

Chlorophyll content analysis was carried out on WT and transgenic tobacco lines cultivated under 400 mM NaCl for 6 weeks in comparison to control plants. WT tobacco leaves started to exhibit chlorophyll bleaching symptoms after 2 weeks of salt application. The total chlorophyll content of WT registered a 65% decrease after 6 weeks of salt stress. However, the transgenic tobacco leaves were similar to the control after 2 weeks of 400 mM NaCl application and started to exhibit chlorosis symptoms only after 4 weeks of salt stress. An average decline of 41% and 38% in the total chlorophyll contents was registered in L15 and L20, respectively (Figure 6).

### 3.5. Mineral contents in transgenic and WT lines under NaCl treatment

After 150 and 300 mM NaCl application, $Na^+$ accumulation increased significantly within the WT in all plant organs as compared to control. In the L15 and L20 transgenic lines, a higher $Na^+$ content than in the control was registered; however, $Na^+$ accumulation in the leaves, shoots, and roots was significantly lower under 300 mM salt than that in the WT (Figure 7). Regarding $Cl^-$, markedly higher contents were measured in WT leaves, shoots, and roots than in transgenic lines (Figure 8). As far as $K^+$ is concerned, a similar shoot accumulation level was observed within WT and transgenic lines under control conditions. Similarly, after salt application (150 and 300 mM), no significant

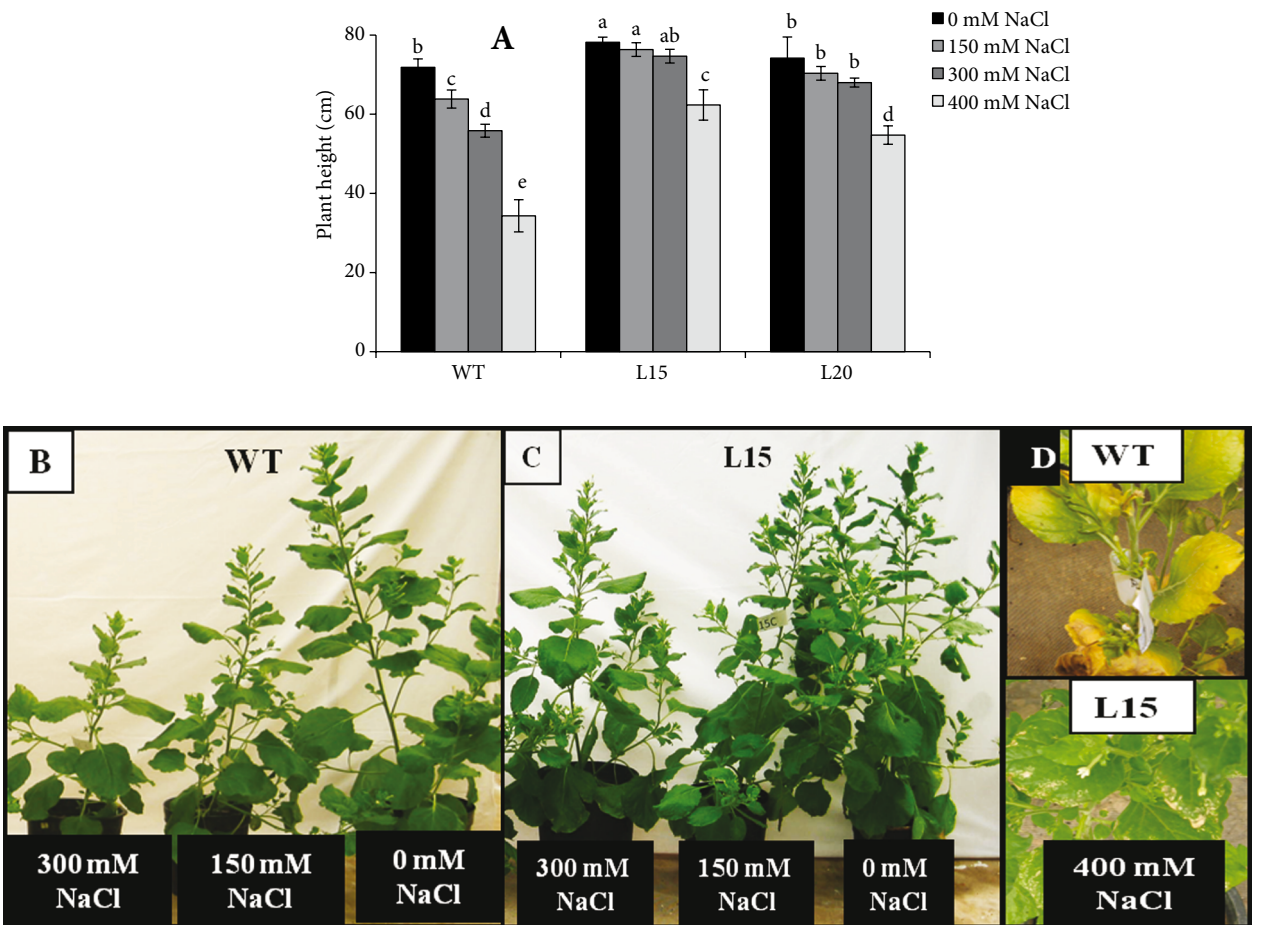


**Figure 4.** A- Plant heights of WT and *VvRD22*-transgenic lines cultivated ex vitro under 0, 150, 300, and 400 mM NaCl treatments. Data are means ± SE of 3 replicates. Different letters denote significance according to LSD test ($P \leq 0.05$). B and C- Plants from WT and transgenic lines cultivated under 0, 150, and 300 NaCl treatments. D- Leaf symptoms exhibited in transgenic lines and WT tobacco under 400 mM NaCl treatment.

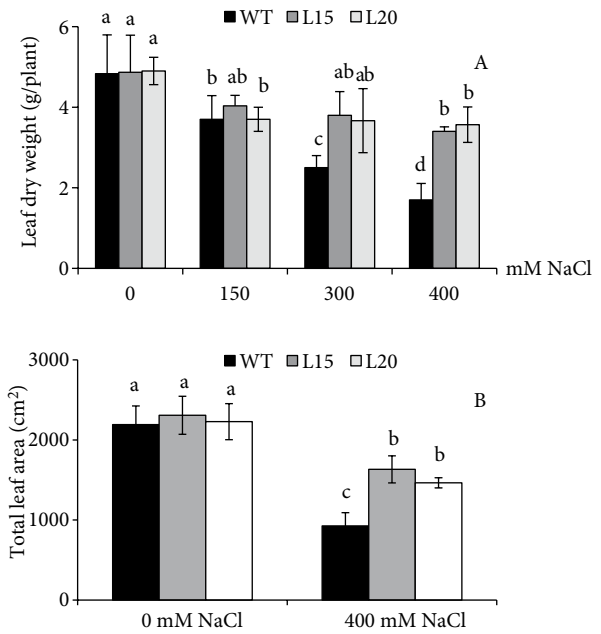


**Figure 5.** Leaf dry weight (A) and total leaf area (B) of WT and transgenic plants after 6 weeks of salt treatments. Data are means ± SE of 3 replicates. Different letters denote significant differences according to LSD test ($P \leq 0.05$).

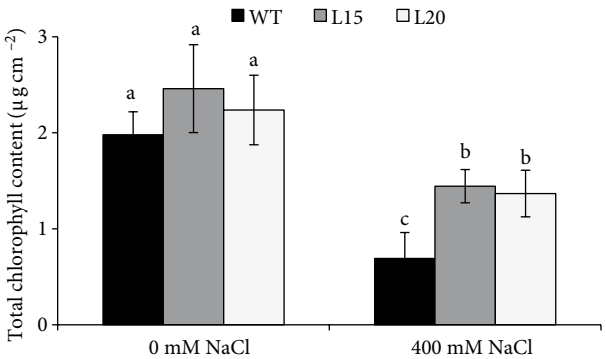


**Figure 6.** Chlorophyll content of WT and transgenic plants expressing *VvRD22* after 6 weeks of salt treatments (0 and 400 mM NaCl). Data are means ± SE of 3 replicates. Different letters indicate significant differences according to LSD test ($P \leq 0.05$).

differences were observed in $K^+$ accumulation between the WT and transgenic lines (Figure 9). Indeed, a significant decrease in $K^+$ levels was mainly observed in the aerial parts (leaves and shoots) of the WT and transgenic lines (L15 and L20), while $K^+$ content in the roots was less affected by salinity in all studied tobacco lines. Finally, analysis of $Ca^{2+}$ content clearly showed more accumulation in the leaves compared to the shoots and roots in both the WT and transgenic lines under control conditions. Under increased NaCl concentrations, $Ca^{2+}$ content decreased significantly in the leaves of WT plants. However, transgenic lines maintained significantly higher $Ca^{2+}$ contents than the WT when subjected to 150 mM and 300 mM NaCl treatments (Figure 10).

### 3.6. Osmotic potential and sugar contents in transgenic and WT lines under NaCl treatment

The osmoregulatory capacity was evaluated at the end of salt application (400 mM NaCl) through osmotic potential (Ψs), RWC, and sugar content measurement. Under control conditions, less negative Ψs values were registered in WT plants compared to transgenic lines. When subjected to 400 mM NaCl application, Ψs decreased in both WT and transgenic plants. However, the Ψs of transgenic lines was significantly more negative than that of WT plants (Figure 11A), indicating an increased solute concentration within cells. On the other hand, RWC under control conditions were similar in both WT and transgenic lines with an average value of 76%. After 6 weeks of 400 mM NaCl application, a significant RWC decline was registered within the WT (40%) compared to the control, while no significant RWC decline (11%) was recorded in the transgenic lines compared to control plants (Figure 11B). Such a stable RWC within transgenic lines would confirm the occurrence of an enhanced osmotic adjustment as indicated through Ψs measurement. Subsequently, total soluble sugar content in transgenic lines was 2.5 times higher than that registered in the WT under salt stress conditions. In contrast, WT plants did not present any significant increase in osmolyte content when subjected to 400 mM salt stress as compared to the control (Figure 12).

## 4. Discussion

This study highlights the transgenic expression effect of the *VvRD22* gene in promoting adaptation to salinity in a tobacco model plant from seed germination to adult plant stages. *VvRD22* expression effects were observed throughout in vitro germination assays and ex vitro physiological responses of transgenic tobacco lines in comparison to WT plants. Deep insights were provided into the involvement of this candidate gene in salt tolerance improvement.

Based on the in vitro assays, *VvRD22* gene expression promoted seed germination and seedling growth rates under salt stress in transgenic tobacco as compared to WT plants. These results would intend that *VvRD22* possesses a direct cellular protective aptitude under salinity constraint. Such an effect was mentioned by Ding et al. (2009) and Wang et al. (2012) in transgenic tobacco BY-2 cells ectopically expressing *GmRD22*. Additionally, an improved osmotic stress tolerance in both seeds and vegetative parts was reported to be associated with the up-regulation of the *RD22* gene in transgenic *Arabidopsis* overexpressing the *AtMYC2* and *AtMYB2* transcription factors (Abe et al., 2003). These previous findings would

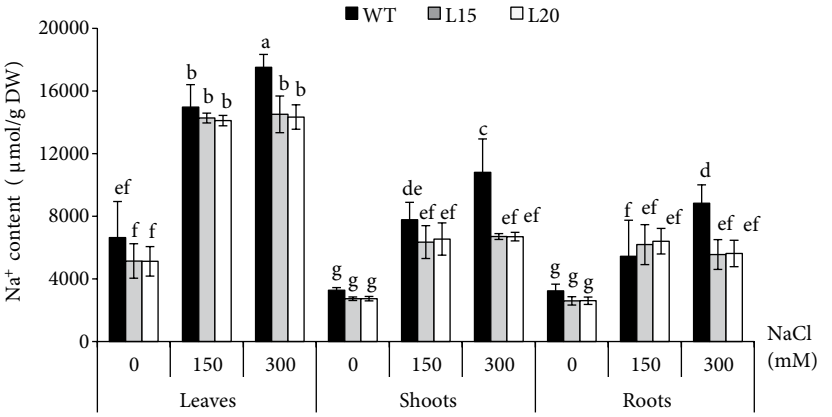


**Figure 7.** Leaf, shoot, and root sodium contents of WT and transgenic plants under 0, 150, and 300 mM NaCl concentrations after 6 weeks. Data are mean ± SE of 3 replicates. The letters indicate significant differences according to LSD test ($P \leq 0.05$).

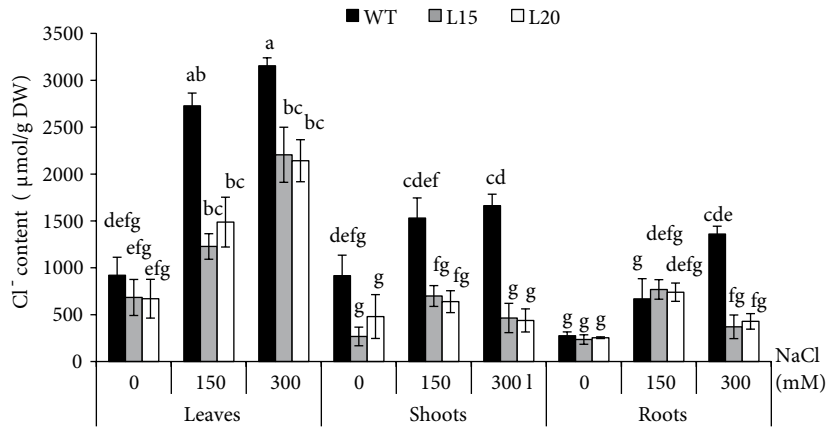


**Figure 8.** Leaf, shoot, and root chloride contents of WT and transgenic plants under 0, 150, and 300 mM NaCl treatments after 6 weeks. Data are mean ± SE of 3 replicates. Different letters indicate significant differences according to LSD test ($P \leq 0.05$).

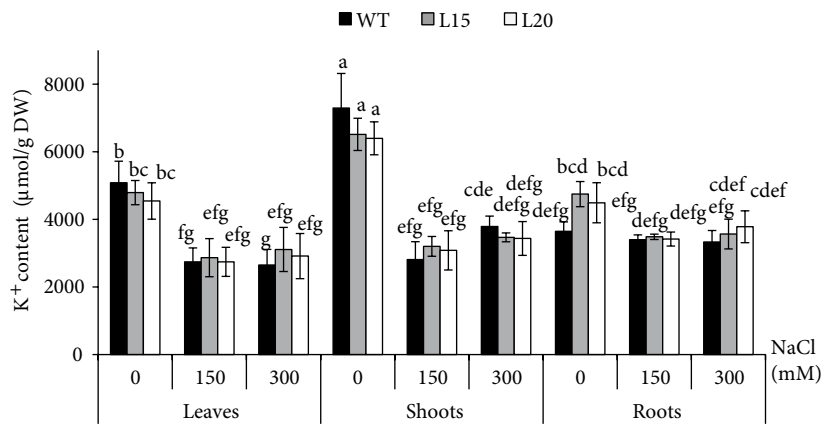


**Figure 9.** Leaf, shoot, and root potassium content of WT and transgenic plants under 0, 150, and 300 mM NaCl treatment after 6 weeks. Data are mean ± SE of 3 replicates. Different letters indicate significant differences according to LSD test ($P \leq 0.05$).

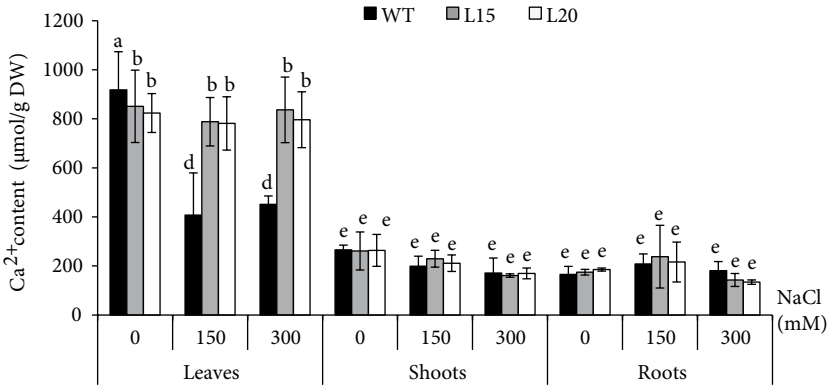


**Figure 10.** Leaf, shoot, and root calcium contents of WT and transgenic plants under 0, 150, and 300 mM NaCl treatment after 6 weeks. Data are mean ± SE of 3 replicates. Different letters indicate significant differences according to LSD test ($P \leq 0.05$).

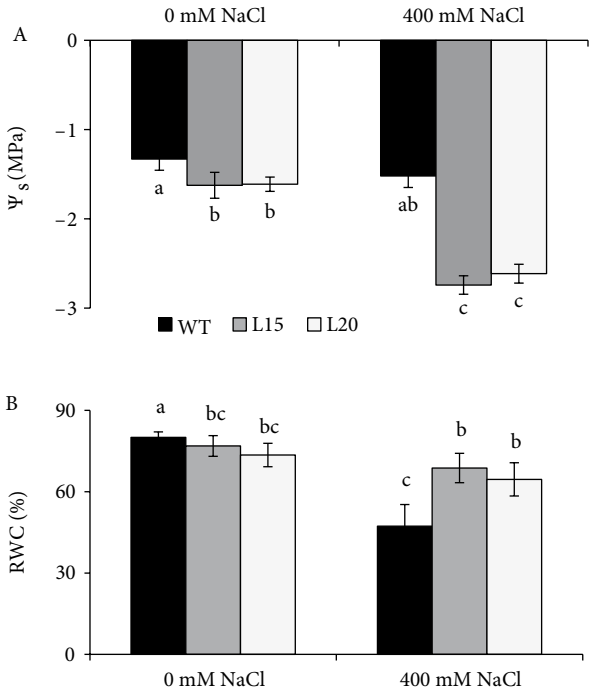


**Figure 11.** Osmotic potential $\Psi_s$ (A) and relative water content RWC (B) of WT and transgenic plants cultivated under 0 and 400 mM NaCl for 6 weeks. Data are means ± SE of 3 replicates. Different letters indicate significant difference according to LSD test ($P \leq 0.05$).

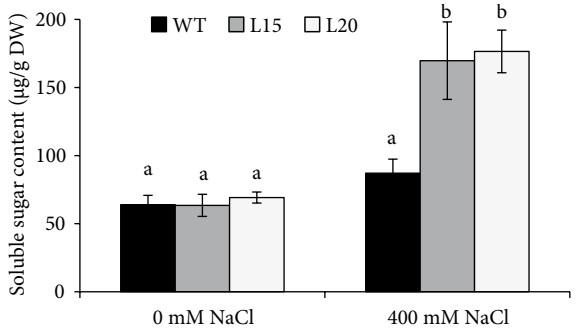


**Figure 12**. Total soluble sugar contents within WT and transgenic tobacco lines subjected to 0 and 400 mM NaCl treatment. Data are means ± SE of 3 replicates. Different letters denote significant differences according to LSD test ($P \leq 0.05$).

support our results on the implication of *VvRD22* in promoting seed germination and seedling growth.

In vivo, salt stress inhibits plant growth, even though transgenic plants with improved tolerance usually presented better growth than WT plants (Shen et al., 2003). Based on the ex vitro assay, both WT and transgenic lines exhibited similar phenotypes under controlled conditions. However, under salt stress, transgenic plant growth (plant height and leaf DW) was more significant than that of the WT. Additionally, a slight leaf area decrease was registered in transgenic lines compared to the WT. Such parameters were deemed reliable to assess the tolerance capacity of plants to abiotic stresses (Bansal and Nagarajan, 1987; Roxas et al., 2000). Plant growth inhibition under salt stress is primarily due to the osmotic effect, whereas toxicity produced by excessive salt accumulation in the plant cells becomes evident at the later stages of growth (Munns, 2002). It thus appears that *VvRD22*-expressing plants are able to tolerate the initial osmotic stress as well as the toxic effect of salt in the late growth stage. This would illustrate the beneficial effects of *VvRD22* expression in promoting adaptation to salinity through its involvement in the shoot growth. In this context, Seo et al. (2009) revealed that in *MYB96*-overexpressing *Arabidopsis* treated by ABA, a higher *RD22* gene expression was seen in the shoot than in the root, which was associated with drought tolerance enhancement. Moreover, transgenic cotton overexpressing the *AtRD22-like 1* gene *GhRDL1*

exhibited an increased fiber length and seed mass (Xu et al., 2012). Other related studies inferred a vegetative protective effect of *GmRD22* in transgenic *Arabidopsis* and rice under NaCl treatment (Wang et al., 2012). In our case, the protective effect induced at the germination and seedling stages of transgenic tobacco encouraged us to ask whether this *VvRD22* expression effect might enable other physiological mechanisms and contribute subsequently to an improved salt tolerance, based on chlorophyll, mineral contents, and osmoregulatory capacity evaluation. Incidentally, such parameters were not checked in previous studies (Yamaguchi-Shinozaki and Shinozaki, 1993; Yu et al., 2004; Wang et al., 2012; Xu et al., 2012). Thus, in contrast to the WT, transgenic tobacco leaves did not display any significant difference in the chlorophyll content under a high NaCl concentration as compared to the control. This might indicate that chlorophyll biosynthesis was not affected by the inhibitory effects of accumulated ions. In this context, the chlorophyll a/b binding protein (Chla/bBP protein) was proven to have a role in high salinity stress tolerance (Joshi et al., 2009). Similarly, an up-regulation of *Chla/bBP* and *RD22* genes was detected in *35S:AtMYC2*/*AtMYB2* transgenic *Arabidopsis* by microarrays analysis. Presumably, these genes would work cooperatively to improve osmotic stress tolerance (Abe et al., 2003). We suggest consequently that the maintained chlorophyll content in transgenic tobacco plants is likely to be a consequence of *VvRD22* expression, which would provide a photosynthetic protection ensuring optimal growth. On the other hand, ion analysis revealed that $Na^+$ and $Cl^-$ were increased under salt treatment but accumulated at lower levels in transgenic lines than in WT plants. Even though $Na^+$ and $Cl^-$ toxicities within cells cause a reduced development (Ali et al., 2004), the specific damaging effects of such ions were markedly reduced in our transgenic plants. As far as the $K^+$ ion is concerned, our results revealed decreased contents in both WT and transgenic lines under salt stress conditions in comparison to the control. Since $K^+$ is one of the principal mineral solutes contributing to osmotic adjustment in many crop species (Damon et al., 2011), it seems that our transgenic lines adopted other strategies for their osmotic adjustment, which do not involve $K^+$ ion accumulation. However, when analyzing leaf $Ca^{2+}$ contents, transgenic lines kept a stable content under salt stress compared to WT plants. This result may justify the enhanced salt tolerance of transgenic lines, as accumulated $Ca^{2+}$ acts in part as a secondary messenger to ABA (Hirschi, 2004) for transducing adaptive stress responses (Hasegawa et al., 2000). Furthermore, the modulation of intracellular calcium levels was reported to be partly regulated by calcium-binding proteins such as calmodulin, which is activated by increased calcium concentrations. Incidentally, this protein was suggested to be involved in the NaCl-stress signal transduction pathway (Cunningham and Fink, 1994). In a similar context, the up-regulation of both calcium-binding protein *RD20* and *RD22* genes was detected in the *35S:AtMYC2*/*AtMYB2* transgenic *Arabidopsis* (Abe et al., 2003). Consequently, we can assume that *VvRD22* expression seems to be associated with that of a calcium binding protein leading to the stabilization of calcium content in the transgenic tobacco under salt stress. Our results suggest that *VvRD22* transgenic expression could reduce sodium and chloride and maintain $Ca^{2+}$ contents in plants under salt stress. This would contribute to the protection of enzymatic processes from salt stress damages.

To investigate the osmoregulatory capacity in transgenic tobacco lines, the RWC and osmotic potential, which are important indexes for determining leaf water status (McCaig and Romagosa, 1991; Yıldıztugay et al., 2013), were measured. Under salt stress, transgenic plants had a significantly decreased osmotic potential, a stable RWC, and an enhanced production of total soluble sugars compared to the WT. Such soluble osmotic molecules were reported to be accumulated in several plants in response to environmental stresses (Gupta and Kaur, 2005) as osmoprotectants. Sugars are also considered as important signaling molecules (Hanson and Smeekensn, 2009) and may play important roles in stress-adaptive mechanisms, such as sucrose induction (Ramel et al., 2009). Therefore, the exhibition of a sugar accumulation-based osmotic adjustment in L15 and L20 lines lets us postulate that *VvRD22* transgenic expression involves sugar regulation. In this context, the relation between the *Sus*-encoding (*sucrose synthase 1*, *Sus1*) gene expression and the perception of a leaf osmotic potential decrease previously reported by Dejardin et al. (1999) would support our suggestion. Taken together, our results and those reported by Abe et al. (2003) on the co-up-regulation of osmotic stress inducible genes encoding *rd22*, *Sus1*, calcium binding protein gene *RD20*, and *Chla/bBP* in *35S:AtMYC2/AtMYB2 Arabidopsis* plants let suggest the involvement of *VvRD22* gene expression in the cellular protection against salt stress' deleterious effects.

In conclusion, our findings strongly suggest that the transgenic *VvRD22* expression confers an improved salt tolerance that involves an osmotic adjustment strategy and a protective effect enabling other physiological mechanisms to counteract salinity effects at the cellular and the whole-plant levels. Evidently, *VvRD22* transgenic expression would be useful in engineering stress-tolerant grapevines and understanding other specifically involved physiological mechanisms.

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