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Dissertação
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“Inspiration exists, but it has to find us working”

PABLO PICASSO

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RESUMO

O processo de enxertia consiste na união de duas plantas com genótipos diferentes, o enxerto e o porta-enxerto. Na vinha é utilizado, principalmente, no combate à praga filoxera (*Daktulosphaira vitifoliae)*, de origem norte-americana e introduzida na Europa no século XIX. Apesar da enxertia ser um processo muito antigo, a incompatibilidade entre enxertos, caracterizada pela quebra da planta pela zona da enxertia, é ainda um grave problema, que pode passar despercebido durante anos.

Histologicamente, a diferença entre enxertos compatíveis e incompatíveis está bem descrita, sendo principalmente caracterizada pelo grau de desenvolvimento e reunião vascular entre o enxerto e o porta-enxerto, mas este é ainda um processo particularmente desconhecido do ponto de vista molecular e a expressão genética que caracteriza a (in)compatibilidade permanece, em larga escala, desconhecida. No sentido de contribuir para o conhecimento nesta área, considerámos de particular interesse a análise da expressão de factores de transcrição (TFs) envolvidos no processo de compatibilidade da enxertia, uma vez que estes possuem a capacidade de controlar a expressão de numerosos genes, regulando vários processos biológicos, incluindo stress biótico, stress abiótico e desenvolvimento. Com esse objectivo, foram utilizados hetero-enxertos, auto-enxertos e variedades não enxertadas (os dois últimos para controlo) de duas variedades de Touriga Nacional, TN112 (A) e TN21 (B), com diferentes taxas de compatibilidade, e o porta-enxerto Richter-110, previamente testados para despiste de infecção viral. As enxertias foram realizadas utilizando a técnica *omega* e a amostragem da zona de enxertia foi feita em três fases temporais: fase da calogénese (I - 21 dias), fase de enraizamento (II - 3 meses) e no fim do primeiro ciclo vegetativo (III - 12 meses). Todas as amostras foram lavadas, cortadas, etiquetadas e armazenadas a -80ºC.

Para extracção de RNA, foi recolhido o tecido da zona de enxertia de três plantas diferentes, por combinação, num total de 15 amostras, imediatamente congeladas em nitrogénio líquido. O RNA total de cada amostra foi extraído com clorofórmio:ácido isoamílico (24:1) seguindo um protocolo adaptado de Chang *et al.* (1993) e Le Provost *et al.* (2007). A concentração total de RNA foi avaliada por espectroscopia UV-Vis (absorvância a 260 nm), usando o espectrofotómetro Nanodrop ND-2000C. A pureza e integridade do RNA foram determinadas pela relação da absorvância a 260/280 nm e visualização após corrida em gel de eletroforese. O DNA genómico contaminante foi removido do RNA com o kit DNA-free

Turbo de Ambion® (de acordo com as instruções do fabricante) duas vezes.

Amostras de RNA de três repetições biológicas por combinação foram enviados para análise do transcriptoma pelo método de sequenciação MACE (Massive Analysis of cDNA Ends), desenvolvido pela GenXpro GmbH. Foram construídas quatro bibliotecas (AI/AII, BI/BII, AI/BI e AII/BII) com 23999 genes cada, e analisadas no programa Microsoft Excel®, sendo que os níveis de expressão considerados representam a média normalizada dos níveis de expressão em cada grupo de réplicas biológicas. Um total de 466 (bibliotecas A/B) e 3037 (bibliotecas I/II) genes foram seleccionados com base no FDR (FDR <0,5) e na razão da expressão entre grupos (Log_2FoldChange). Para as bibliotecas AI/AII e BI/BII foi considerado um Log_2FoldChange arbitrário > |1.9|, enquanto que para as bibliotecas AI/BI e AII/BII foi considerado um Log_2FoldChange > |1|. Para cada gene foram obtidas as sequências peptídicas em formato FASTA, utilizando o recurso informático Biomart em http://plants.ensembl.org/biomart/, e carregadas no programa Blast2GO em https://www.blast2go.com, onde foi realizada uma pesquisa BLAST utilizando o algoritmo BLASTp (e-value<$1e^{-6}$). Foram identificados 19 (bibliotecas A/B) e 66 (bibliotecas I/II) genes com actividade de factor de transcrição e regulação da expressão genética. A anotação de cada gene foi confirmada em VTCdb — Vitis co-expression database, e Planttfdb, em http://vtcdb.adelaide.edu.au e http://planttfdb.cbi.pku.edu.cn/, respectivamente.

As taxas de sobrevivência foram obtidas no final do ciclo (quando as plantas entraram em fase de dormência) e permitiram identificar a combinação TN21/R110 como o hetero-enxerto mais compatível. No entanto, contrário ao esperado, não foi o auto-enxerto TN21/R110 a apresentar a maior taxa de sobrevivência, mas sim a combinação TN112/R110. Com base na bibliografia existente sugere-se a hipótese de que um evento de transferência horizontal genética (HGT) poderia produzir estes resultados, ao superar a barreira da incompatibilidade genética e sincronizar as respostas de defesa e regeneração entre as células das duas plantas, pelo que seria interessante estudar a ocorrência de HGT em enxertias de *Vitis vinifera*.

Da análise dos vinte genes diferencialmente expressos entre as fases de calogénese e enraizamento pôde-se verificar que, embora não diferencialmente expressos entre enxertias, doze começaram por ser ligeiramente mais expressos na combinação compatível TN21/R110, em particular genes relacionados com as hormonas auxina e giberelina, bem

como uma proteína kinase. No geral, é provável que os genes encontrados estejam relacionados com a saída do período de dormência.

Comparando hetero-enxertias, observou-se a expressão diferencial de genes pertencentes a cinco famílias: AP2/ERF, WRKY, Zinc Finger do tipo C_2H_2, NAC e TALE. Cinco genes encontravam-se diferencialmente expressos na fase de calogénese e treze na fase de enraizamento. Não foram encontrados, diferencialmente expressos, genes envolvidos na regulação das hormonas auxina ou citocinina.

Na calogénese, foi observada principalmente a expressão de dois genes pertencentes à família AP2/ERF (RAP2.4 e ERF109). De acordo com a bibliografia, estes resultados sugerem uma maior resistência a stress oxidativo no enxerto mais compatível. No enxerto menos compatível, o stress oxidativo poderá ser responsável pela fraca ou inexistente vascularização da zona de enxertia, condição que caracteriza a incompatibilidade. Se a produção inicial de etileno e ROS ou se a capacidade para recuperar a homeostase, ou ambos, são responsáveis na probabilidade de sucesso da enxertia, requer uma investigação mais aprofundada.

Na fase de enraizamento, nota-se uma predominância na expressão de TFs relacionados com stress biótico, nomeadamente pertencentes à famíla WRKY. Os resultados são compatíveis com estudos anteriors e sugerem que a combinação menos compatível TN112/R110 poderá ter experienciado um prolongamento do período de defesa, visto a expressão destes genes ser maior nesta combinação. Tal prolongamento pode colocar em risco a regeneração e vascularização. Além disso, este tipo de resposta imunitária pode ser provocada por incompatibilidade genética entre as duas plantas do enxerto, podendo em última análise resultar em apoteose celular. Dada a elevada expressão dos TFs WRKY18 e WRKY70, propomos que estes sejam considerados para marcadores moleculares de incompatibilidade da enxertia.

Palavras-chave: Expressão genética, *Vitis vinifera*, enxertia, factores de transcrição, resposta a stress.

ABSTRACT

Grafting is an ancient technique widely used in viticulture mainly as a control tool in grape phylloxera infected soils. Still, graft incompatibility is a problem, specially because it can go undetected for years. Although the histological and biochemical traits of (in)compatibility have been well established, the molecular mechanisms underlying the process are still poorly understood. Because late rejection seems to be predetermined already at the initial steps of union formation, the transcriptome of 21 days (callogenesis) and 3 months (rooting) old graft tissue from two rootstock/scion combinations of Touriga Nacional and Richter-110, with different rates of graft success, was analysed. Interestingly, graft success was higher for the heterograft of one of the Touriga Nacional clones compared to its autograft, and we discuss a possible relation to horizontal gene transfer events. For the most incompatible heterograft, gene expression analysis showed that it was subject to a higher oxidative stress response at callogenesis and a stronger immune-type response at rooting, compared to the most compatible heterograft. As severe oxidative stress and a prolonged immune-type response can arrest the progression of cell division and differentiation, their role in the incomplete and/or disorganized vasculature typically observed in incompatible grafts was discussed. Moreover, results suggest that initial ethylene production at callogenesis is higher in incompatible grafts and its predictive value for graft (in)compatibility is also discussed. Additionally, two strong candidates for incompatibility molecular markers at rooting were identified, namely WRKY18 and WRKY70.

Keywords: Gene expression, *Vitis vinifera*, grafting, transcription factors, stress response.

CONTENTS

List of figures and tables

1. INTRODUCTION

Grafting is an ancient technique used in agriculture to potentially enhance resistance to deleterious biotic and abiotic conditions, increase production and improve quality, offering economic benefits to agricultural production (Lee *et al.*, 2010). Still, some issues in grafting need to be solved, and one is how to overcome graft incompatibility, and thus widen its application in agriculture (Wang, 2011). In viticulture, grafting is used mainly as a control tool in grape phylloxera - *Daktulosphaira vitifoliae* (Fitch) (Homoptera: Phylloxeridae) - infected soils, a pest introduced in Europe by the end of the 19th century (Granett *et al.* 2001). Grapes were among the first fruit species to be domesticated and today are the world's most economically important fruit crop (Keller, 2010). Because the scions and rootstocks belong to different species, graft incompatibility is a problem, specially because it can go undetected for years before the graft breaks down.

There is a poor understanding of the early stages of the grafting process and the molecular mechanisms involved in the communication between two different genotypes at the graft interface (Cookson *et al.*, 2014). Despite, it is certain that cellular recognition and the establishment of cellular communication through plasmodesmata formation is a crucial step in graft compatibility (Jeffreey *&* Yeoman, 1983; Kollmann *&* Glockmann, 1985; Waigmann *et al.*, 1998 , Pina *et al*., 2012; Atmodjo *et al.*, 2013). This cellular communication allows the exchange of water, nutrients, nucleic acids and plant-encoded proteins, including RNAs and transcription factors between different cells of the scion and stock, thus establishing an effective pathway for local and long-distance signalling (Tiedemann, 1989; Lucas *et al.*, 1995, 2001, 2004; Stegemann *&* Bock, 2009) along with the establishment of a functional vascular system (Wang *&* Kollmann, 1996; Dolgun *et al.*, 2008; Aloni *et al.*, 2008).

Histologically and biochemically, incompatibility at the graft interface between different genotypes has been related to poor plasmodesmata formation, disorganized and/or incomplete vascularization, often accompanied by the accumulation of phenolic compounds and reactive oxygen species (ROS) (Errea *et al.*, 1994; Errea, 1998; Ermel *et al.*, 1999; Aloni *et al.,* 2008; Pina *&* Errea, 2008; Flaishman *et al,* 2008; Basheer-Salimia *&* Hamdan 2009; Nocito *et al.*, 2010; Zarrouk *et al.*, 2010; Pina *et al*., 2012). Late rejection seems to be predetermined already at the initial steps (15 days - 3 months) of union formation (Errea *et al.,* 1994; Flaishman *et al*., 2008; Pina *et al*., 2012; Mahunu *&* Adjei, 2012; Yin *et al.*, 2012; Cookson *et al.*, 2014; Brian *&* Duron, 1971 *in* Ermel *et al.*, 1998).

Only a few studies focused on the occurring changes at the molecular level. Graft interface-specific genes were identified in autografts of hickory (*Carya cathayensis*) (Zheng, *et al.* 2010), *Arabidopsis (*Yin *et al.,* 2012*),* and *Vitis vinifera (*Cookson *et al.,* 2013). In hickory, gene expression changes analysed with cDNA-AFLP, 3-14 days after grafting, revealed the up-regulation of genes related to metabolism, cell cycle and IAA transport-related proteins (including auxin responsive transcription factors - ARFs). It was argued that increased concentration of endogenous auxin was probably the key response to graft union development, as it canalizes the pathway to direct the reconnection of vascular tissue between the scion and stock (Zheng *et al*, 2010). In *Arabidopsis*, micrografts were collected 22–26 h after grafting and prepared for whole-genome microarray analysis. Although *callus* formation was not observed, unlike previous studies and quite different from what happens in woody plant species grafting, the graft union was initiated by a wound-induced program with the activation of ethylene and jasmonic acid biosynthesis pathways, followed by the initiation of cell-to-cell communication associated to sucrose- and plasmodesmata-related proteins, and concluding with re-establishment of the vascular communication network which relied on hormone signalling, particularly local accumulation of auxin (indole-3-acetic acid – IAA) (Yin *et al.,* 2012). Using whole-genome microarrays during a time course of 3-28 days, graft union development in grapevine revealed the up-regulation of many genes involved in cell-wall modification like senescence-associated genes, LRR-receptor kinases, wound responses involving jasmonate signalling, flavonoids and peroxidases metabolism, and hormonal signalling (cytokinin, ethylene and auxin) including ethylene-responsive transcription factors (ERFs), a lateral organ domain transcription factor (LBD4) and an auxin influx carrier (AUX1) and also pathogenesis-related (PR) proteins (Cookson *et al.*, 2013).

Only recently the gene expression changes induced by grafting different species was examined in grapevine, one analising the impact of grafting in the shoot apical meristem gene expression (Cookson *&* Ollat, 2013) and the second focused on graft-interface specific gene expression (Cookson *et al*., 2014). Cookson and Ollat (2013), using whole-genome microarray, studied the gene expression changes in the shoot apical meristem four months after grafting. This revealed the induction of extensive transcriptional reprogramming related to the process that included the up-regulation of PR- and oxidative-related proteins, like flavonoids. They further observed that the choice of rootstock genotype had little effect on the gene expression in the shoot apex, suggesting that auto- and hetero-grafting was the major factor regulating gene expression. Gene expression was studied by Cookson *et al*. (2014) at the graft interface in hetero- and autografts of grapevine during a time course (3, 7, 14, and

28 d) after grafting using whole-genome microarrays. They found that grafting with nonself rootstock triggers the differential expression of genes involved in plant defence and/or stress responses, particularly genes involved in oxidative stress (e.g. peroxidases) associated with the induction of an oxidative burst at the graft interface that potentially induces programmed cell death (PCD), pathogenesis-related (PR) proteins that can be related to hormone signalling and LRR-receptor kinases. Particularly, comparing hetero- and auto-grafts, during the first two weeks after grafting, jasmonate and abscisic acid (ABA) signalling genes were differentially expressed. Ethylene signalling genes were strongly up-regulated at day 14, and both up- and down-regulated at day 28. Auxin signalling genes showed to be up-regulated at day 14, but no differential expression was noted at other time points.

2. OBJECTIVE

Overall, several whole-genome studies show that wound-response and hormonal signalling are important players in the rootstock-scion union and communication (Sorce *at al*., 2002; Aloni *et al*., 2010), but the specific occurring changes at the graft interface remain largely uncharacterised. The aim of this study is to investigate the molecular mechanisms behind graft in(compatibility) by specifically analysing the transcription factors (TFs) involved. TFs are of particular interest given their ability to control the expression of numerous genes, and thus their ability to regulate biological pathways and developmental processes. TFs form intricate networks (Riechmann *&* Ratcliffe, 2000; Babu *et al.*, 2004) that influence gene transcription, either positively or negatively (Latchman, 1998, 2001), mediate hormonal crosstalk (Liu *et al.*, 1999; Depuydt *&* Hardtke, 2011) and regulate development (e.g. Aloni *et al*, 2006; Love *et. al.*, 2009; Waduwara-Jayabahu *et al.*, 2012; Etchells *et al.,* 2012).

We used two rootstock-scion combinations with different rates of grafting success, grafted in the onset of spring and collected at the end of callogenesis and after rooting. Transcriptome analysis of MACE sequencing *data* of differential expressed transcription factors between heterografts was analysed. Results revealed transcriptomic differences between compatible and incompatible grafts concerning mainly oxidative stress and immune-type responses.

3. MATERIAL AND METHODS

3.1 PLANT MATERIAL AND GRAFTING PROCEDURE

Two clones of Touriga Nacional, TN112 (A) and TN21 (B) with different rates of grafting success and the rootstock Richter-110 (R110) were used for this experiment. Traditionally, TN112 hetrografts are expected to have increased survival rates over TN21 heterografts. Autografts of both scions and rootstock were performed as control groups. The material used was collected at CAN (Colecção Ampelográfica Nacional, INIAV, Dois Portos) or provided by Viveiros Plansel. All plant material used for grafting was previously tested for virus infection and only free virus material was used for the experiments.

Initially, 300 grafts were used for each combination and 45 were collected at each time-point for analysis. Hetero- and autografting (for control purpose) was conducted in April of 2012 and 2014, repectively. Also, in April 2012, non-grafts of each variety were planted for control purpose. Mechanical omega grafting was performed on scion/rootstock pairs of approximately the same diameter and the graft zone was dipped into melted wax containing a combination of phytohormones. Grafts were heat-treated (21 days at 30ºC) in order to induce budding and graft callusing. After *callus* development the grafted combinations were transferred to the field (fig.1). Sampling was done at three time-points: end of callogenesis, complete rooting and end of cycle, 21 days, 3 and 12 months after grafting, respectively. In this work, the first two time-points will be considered. All samples were washed, cut, labelled and stored at -80ºC. Survival rates were obtained at the end of the cycle (when plants went to dormancy). All grafting procedures were executed at the Viveiros Plansel facilities.

3.2 RNA EXTRACTION

For each combination, *callus* tissue was collected from three different plants, with a total of 15 samples, and immediately snap frozen in liquid nitrogen. Because the *callus* is a stressed tissue, total RNA from frozen samples of all combinations was extracted using a protocol adapted from Chang *et al.* (1993) and Le Provost *et al.* (2007). Briefly:

- 100mg of plant tissue was grinded in liquid nitrogen using a mortar and pestle
- Pre-heated (65ºC) extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L Spermidine) with 2% β-mercaptoethanol was added, followed by 15 min incubation at 65ºC

- Three extractions with Chloroform:isoamyl alcohol (CIA 24:1) were performed and phases separated by centrifugation (14650 x g at at room temperature, 15 minutes)
- Equal volume of 100% ethanol and 0,1% of 3.0 M NaOAc (pH 5.2) was added to the supernatant and incubated for precipitation (-20ºC, minimum 1 hour)
- After centrifugation (14650 x g, 30 minutes, at room temperature) the supernatant was discarded and the pellet resuspended in 500 µL of SSTE buffer (1 M NaCl, 0.5% SDS, 10mM Tris - HCl (pH 8.0), 1mM EDTA (pH 8.0))
- 450 µL of CIA was added and mixed by inverting the tubes, followed by centrifugation (9,500g, 30 minutes, at room temperature) for phase separation
- The pellet was washed with 70% and 100% ethanol (-20ºC). RNA was dried and resuspended in 20 µL of Milli-Q Rnase-free water.

Total RNA concentration was assessed by spectrophotometry (absorbance at 260 nm), using a UV-Vis Spectrophotometer Nanodrop ND-2000C. RNA purity and integrity were determined by the absorbance ratio at 260/280 nm and visualization after electrophoresis (Biassoni and Raso, 2014). Genomic DNA contamination was removed from the RNA with the Turbo DNA-free kit from Ambion® (according to the manufacturer's instructions) twice.

RNA samples were kept on ice during all procedures.

3.3 SEQUENCING AND TRANSCRIPTOME ANALYSIS

RNA samples from three biological replicates *per* combination were sent for transcriptome analysis by a deep sequencing method, the Massive Analysis of cDNA Ends (MACE), a high-throughput next generation sequencing-based 3′ end sequencing protocol developed by GenXpro GmbH (Zawada *et al.*, 2014). Briefly:

- Total RNA is reverse-transcribed using an anchored and biotinylated oligo(dT) primer that preferentially hybridizes to the proximal end of poly(A) tails
- Reverse-transcribed cDNA is fragmented by sonication and subsequently bound to a streptavidin matrix. Unbound fragments are discarded and biotinylated fragments are released from the matrix for ligation of the sequencing adaptor
- The adaptor-ligated fragments are amplified *via* PCR and finally sequenced on the Illumina HiSeq2000 platform

Because each cDNA molecule is represented by one cDNA fragment (tag) of 94 bps, originating from a region 100–500 bps from the 3′(poly-A) end of the transcript, high throughput sequencing of tags provides high resolution gene expression values and reveals differential expression of low-abundant transcripts, beyond the scope of microarrays and Rnaseq (Muller *et al.*, 2014). Also the increased tag length, comparing to e.g. SuperSage (Matsumura *et al*., 2012), considerably improves the efficiency of tag-to-gene annotation.

Four MACE libraries with 23999 genes each were constructed (AI/AII, BI/BII, AI/BI and AII/BII) and analysed with Microsoft Excel®. Expression levels were summarized as the normalized mean of the expression levels in each group of biological replicates.

Differential expressed genes were selected based on FDR (FDR<0.5) (Reiner *et al.*, 2003) and on the ratio of the normalized expression means between groups (FoldChange), shown as the logarithm to the basis 2 of the ratio of FoldChange. An arbitrary Log2FoldChange >| 1.9| was considered for AI/AII and BI/BII libraries, while a Log2FoldChange >|1| was considered for the AI/BI and AII/BII libraries. Additionally, 16 genes from the AI/AII library with Log2FoldChange>1.5 were manually selected.

For each gene, peptide sequences in FASTA format were obtained with Biomart (Kasprzyk, 2011) from http://plants.ensembl.org/biomart/ and uploaded to Blast2GO (Conesa *et al.*, 2005; Conesa & Gotz, 2008) at https://www.blast2go.com where a BLAST search was performed using the BLASTp algorithm (e-value<$1e^{-6}$). The tool provides the Gene Ontology (GO) terms to assign sequence IDs into the GO categories (Molecular Function, Biological Process and Cellular Component). All genes with sequence-specific DNA binding transcription factor activity and some additional transcription regulators were selected (Tables S1, S2, S3 and S4). Annotation was confirmed at VTCdb — Vitis co-expression database (Wong *et al*., 2013) and Planttfdb (Jin *et al.*, 2014) at http://vtcdb.adelaide.edu.au and http://planttfdb.cbi.pku.edu.cn/, respectively. NCBI database BLAST only provided PREDICTED proteins and for that was not considered.

4. RESULTS

4.1 SUCCESS RATE

In order to better understand the molecular response mechanisms that take place during the graft (in)compatibility reactions, we analysed the gene expression of two clones of Touriga Nacional with different rates of compatibility, grafted onto the rootstock Richter-110 and collected at callogenesis and rooting, 21 days and 3 months after grafting respectively. Autografts and non-grafts of both scions and rootstock were used as control groups. At the end of the season, a survey on the number of surviving plants was performed (fig. 1) in order to assess the most compatible heterograft (fig. 2).

Fig. 1 Success rates at the end of the season.

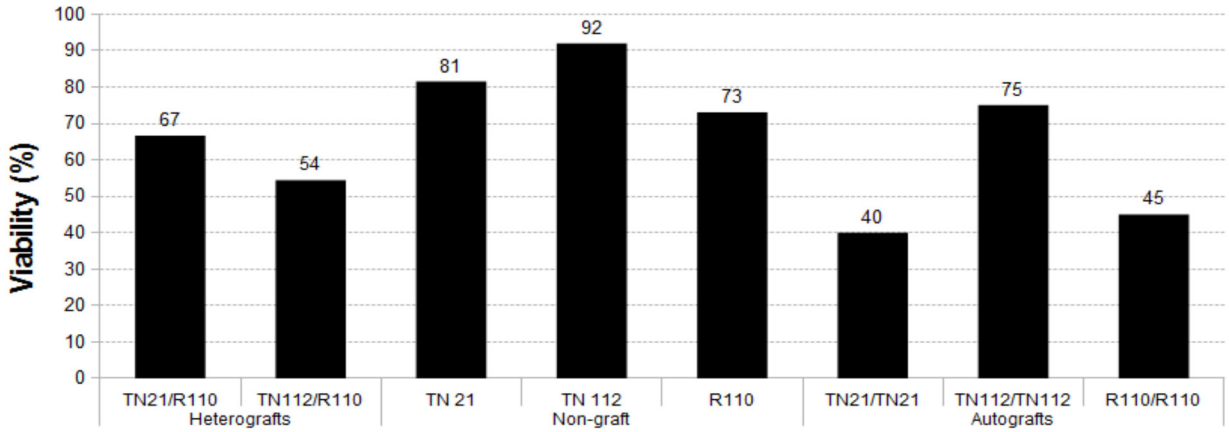
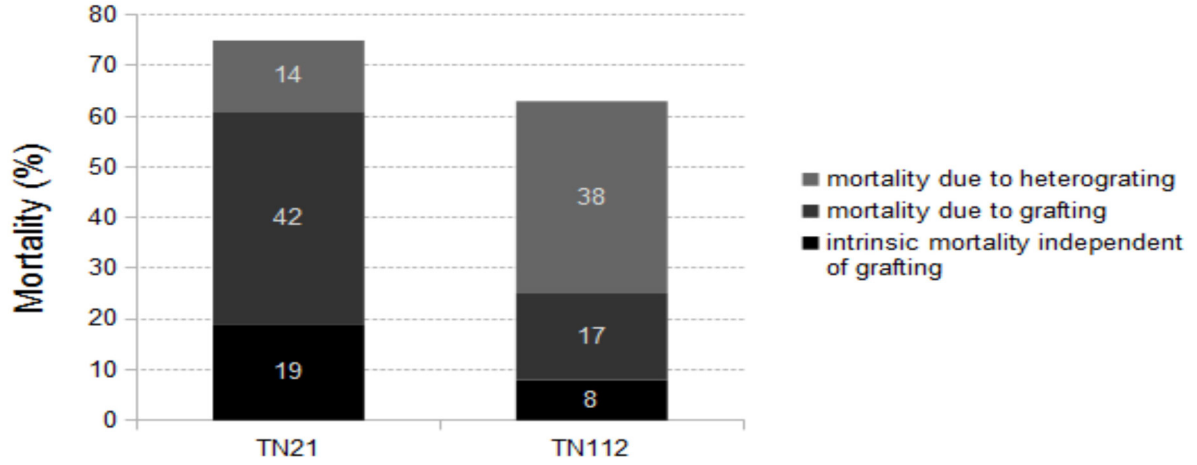


Fig. 2 Assessment of mortality rates.



We considered the intrinsic mortality independent of grafting as the mortality rate of non-grafts. The mortality rate due to grafting was calculated as the difference between non-grafts and autografts mortality. The mortality rate due to heterografting was calculated as the difference between non-grafts and heterografts mortality.

The TN112 clone outperformed the TN21 clone in both non-grafts and autografts, but the TN21/R110 combination outperformed the TN112/R110 by 24%. Although unexpected, results show that the TN21/R110 combination is the most compatible.

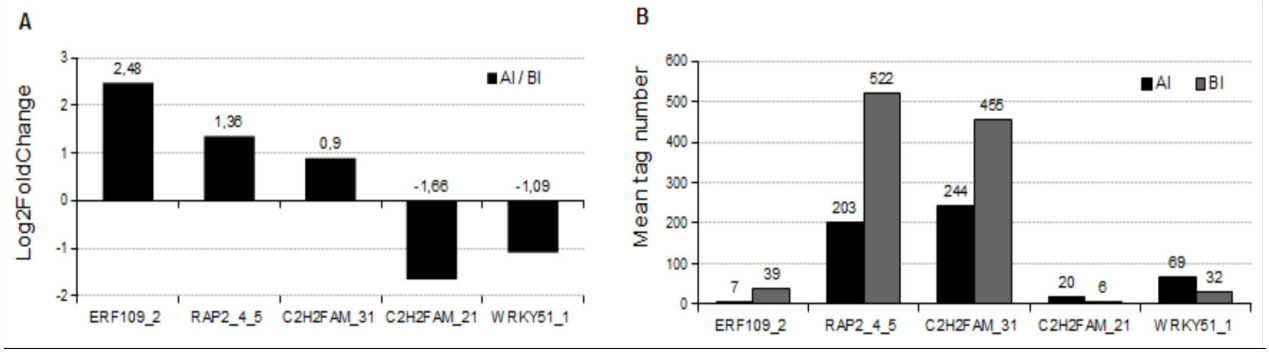
4.2 TRANCRIPTOME ANALYSIS

Four MACE libraries (AI/BI, AII/BII, AI/AII, BI/BII) were generated from *callus* tissue of two clones of *Vitis vinifera* cv Touriga Nacional (A and B) grafted onto the Richter-110 rootstock, collected at two different time points, callogenesis (I) and rooting (II). In order to analyze differential expressed genes between heterografts, two MACE libraries were analysed (AI/BI, AII/BII). The total number of genes (FDR>0.5) and the number of differential expressed genes (FDR<0.5, Log_2FoldChange≥|1|) identified is expressed in table 1.

Table 1 Number of differentially identified genes between heterografts.

Library	Total	Log2FC≥|1|	TF activity
AI vs BI	254	148	5 (3,4%)
AII vs BII	465	318	14 (4,4%)
Total	719	466	19 (4,1%)

Fig. 3 Differential TFs expression between heterografts at callogenesis (phase I).

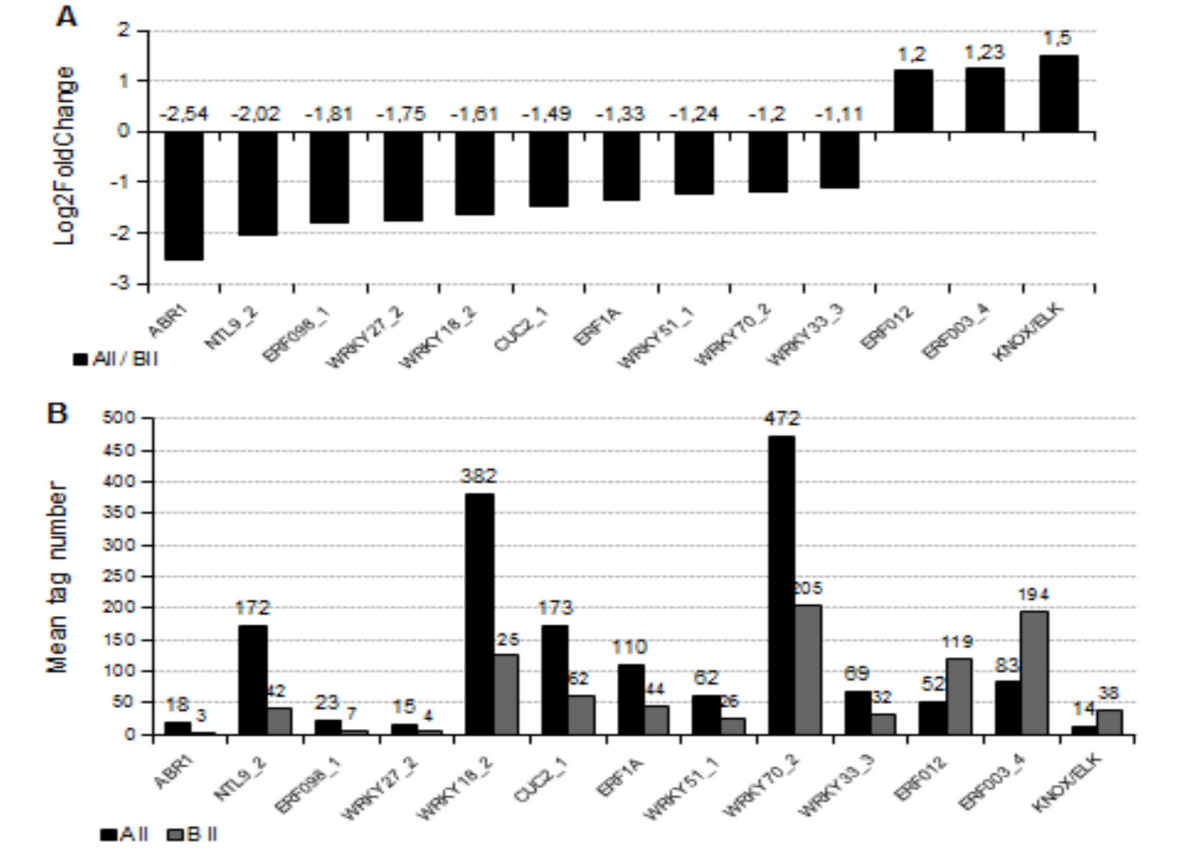


A. Bars represent the Log_2FoldChange of differential expressed genes between TN112/R110(A) and TN21/R110(B). **B.** Expression levels are presented as the normalized mean of the expression levels in the group replicates.

At callogenesis, five genes were found to be differential expressed between heterografts (fig. 3A and table S1). Two ethylene-responsive transcription factors from the AP2/ERF family, ERF109 (VIT_03s0063g00460) and RAP2.4 (VIT_00s0662g00030), as also a C2H2-type Zinc Finger protein, C2H2FAM_31 (VIT_06s0004g06240), were up-regulated in TN21/R110, whilst a member of the WRKY family of transcription factors, WRKY51 (VIT_04s0069g00970) and another member of the ZF-C2H2 family of transcription factors, C2H2FAM_21

(VIT_13s0019g02120) were up-regulated in TN112/R110. Although C2H2FAM_31 and RAP2.4_5 were not the most differential expressed gene between heterografts, they were the highest expressed.

Fig. 4 Differential TFs gene expression between heterografts at rooting (phase II).



A. Bars represent the Log_2FoldChange of differential expressed genes between TN112/R110 (A) and TN21/R110 (B). **B.** Expression levels are presented as the normalized mean of the expression levels in the group replicates.

At rooting, thirteen genes were found to be differential expressed between heterografts (fig. 4, table S2). Five ethylene-responsive transcription factors from the AP2/ERF family, five members of the WRKY family, two members of the NAC family and a KNOX/ELK homeobox member of the TALE family. Two AP2/ERF members and the KNOX/ELK homeobox TF were up-regulated in TN21/R110, whilst all the others were up-regulated in TN112/R110. WRKY51_1 was the only gene found to be differential expressed at both callogenesis and rooting, with very similar expression levels in both. Amongst the most differential expressed genes were the Ethylene-responsive transcription factor ABA repressor 1 (ABR1) (VIT_07s0031g01980) from the AP2/ERF family, and NTL9/VvNAC17 (VIT_15s0048g02300) from the NAC family, both with Log_2FoldChange>-2. Amongst the most expressed genes were two WRKY family members, WRKY18 (VIT_04s0008g05760) and WRKY70

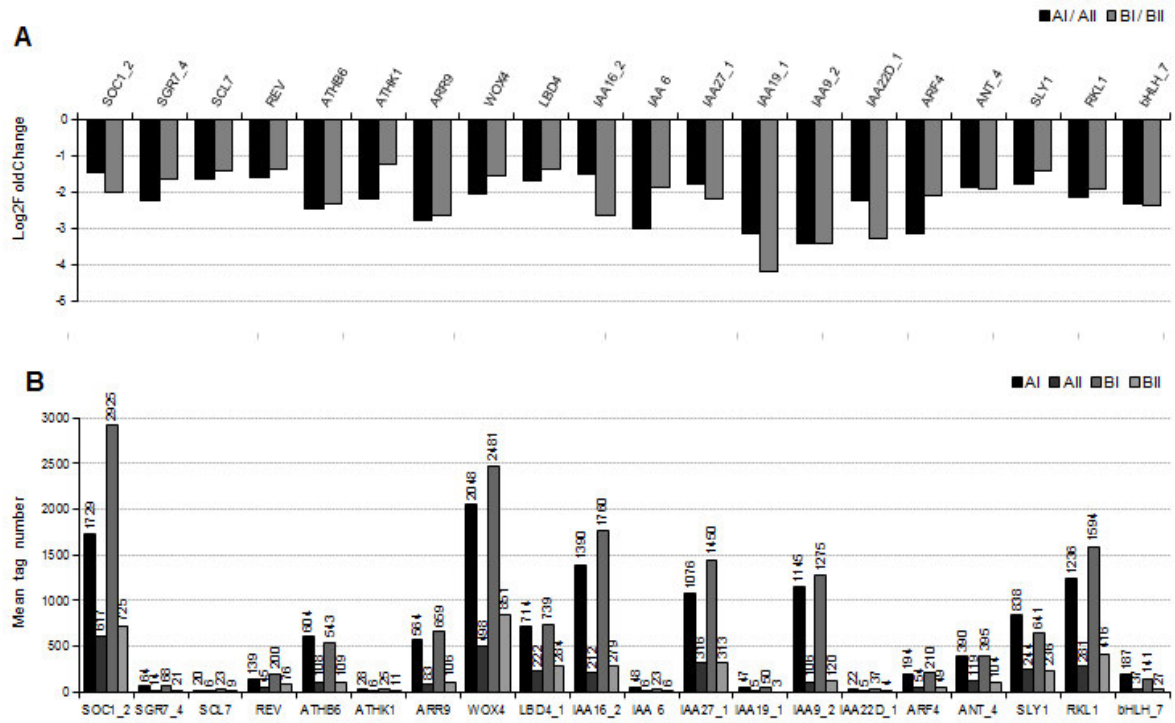
(VIT_08s0058g01390), two NAC family members, VvNAC55 (VIT_15s0048g02270) and VvNAC17 (VIT_15s0048g02300), and three AP2/ERF family members, ERF003 (VIT_09s0002g09120), ERF012 (VIT_18s0001g03240) and ERF1A (VIT_16s0013g00890).

We further searched the transcriptome for evidences of auxin and cytokinin signalling and/or vascular-related transcriptional regulators. For that, we analysed two MACE libraries of differential expressed genes between time-points. The total number of genes (FDR>0.5) and the number of differential expressed genes (FDR<0.5, Log_2FoldChange≥|1| and Log_2FoldChange≥|1.9|) identified is expressed in table 2. Because we wanted to analyse genes with a strong differential expression, we considered a Log2FoldChange≥|1.9| for gene identification and further analysis.

Table 2. Number of differentially identified genes between time-points.

Library	Total	Log2FC≥|1.9|	TF activity
AI vs AII	8787	1432	35 (2,4%)
BI vs BII	7063	1605	31 (1,9%)
Total	15850	3037	66 (2,2%)

Fig. 5 Genes differential expressed between time-points.



A. Bars represent the Log_2FoldChange of differential expressed genes between callogenesis and rooting. **B.** Bars represent the normalized mean expression levels in the group replicates. A: TN112/R110; B: TN21/R110; I: callogenesis; II: rooting.

Between time-points, we have selected a set of 20 genes for discussion, based on its vascular development influence. Some genes that only appeared in one of the two libraries were manually selected from the library from which they were not initially identified (tables S3 and S4). Although we can observe that, at callogenesis, most of the genes start to be slightly more expressed in the TN21/R110 heterograft, particularly SOC1_2 (VIT_17s0000g01230), WOX4 (VIT_18s0001g10160), IAA16_2 (VIT_07s0141g00290), IAA27_1 (VIT_11s0016g03540), and RKL1 (VIT_08s0007g04150), the differences between heterografts have no statistical meaning. In the same manner, although the genes SLY1 (VIT_07s0129g01000) and ATHB6 (VIT_18s0001g06430) are slightly up-regulated in the TN112/R110 heterograft, the differences between heterografts have no statistical meaning. Both SGR7_4 (VIT_07s0129g00030), SCL7 (VIT_04s0023g01660), ATHK1 (VIT_04s0023g03680) and the auxin-related IAA6 (VIT_04s0008g00220), IAA19_1 (VIT_09s0002g05150) and IAA22D_1 (VIT_07s0141g00270), although differential expressed between time-points, showed very low levels of expression, as did, to a lesser extent, ARF4 (VIT_06s0004g03130), REV (VIT_06s0004g02800), AtHB6 (VIT_18s0001g06430), ARR9 (VIT_18s0001g02540), ANT_4 (VIT_18s0001g08610) and SLY1 (VIT_07s0129g01000).

4.3 TF FAMILIES DIFFERENTIAL EXPRESSED BETWEEN HETEROGRAFTS

4.3.1 The AP2/ERF superfamily of transcription factors

The AP2/ERF superfamily is one of the largest groups of transcription factors in plants and regulate a number of biological processes including development, reproduction, adaptation to biotic and abiotic stresses and hormonal responses (Gutterson & Reuber, 2004; Xu *et al.*, 2011; Mizoi *et al.*, 2012; Chang *et al.*, 2013; Licausi *et al.*, 2013; Zhao *et al.*, 2014). In Vitis, 149 distinct AP2/ERF putative transcription factors were identified and divided in three families according to type and number of domains. Proteins with a single AP2/ERF domain were assigned to the ERF family, proteins with tandem repeated double AP2/ERF motif were assigned to the AP2 family and proteins with a single AP2/ERF DNA binding domain together with a B3 type domain were assigned to the RAV family. The ERF super-family was further divided in eleven groups, according to their similarity to Arabidopsis ERF sequences. The AP2 family was further subdivided into the AP2 and ANT groups according to the amino acid sequence of the double AP2 domain and the nuclear localization sequence (Licausi *et al.,* 2010; 2013). More recently, 38 genes from the family of dehydration responsive element-binding (DREB) transcription factors were identified in the grapevine genome and further classified into six subgroups (A1–A6) based on their AP2 domain similarities (Zhao *et al.*,

2014). The abiotic stress-associated DREB subfamily of the AP2/ERF family of transcription factors (DREBs) regulate downstream genes that contain a core DRE sequence (A/GCCGAC) (Yamaguchi-Shinozaki *&* Shinozaki, 1994; Feng *et al*., 2005; Nakano *et al.*, 2006; Agarwal *et al.*, 2006), while ERF proteins typically bind to an AGCCGCC sequence, called GCC box (Ohme-Takagi *&* Shinshi, 1995), although some ERF transcription factors regulate the expression of genes containing both a core DRE sequence or a GCC box in their promoters (Lin *et al.*, 2008; Zhang *et al*., 2011; Cheng *et al*., 2013; Lee *et al.*, 2014). The *RAP2* (related to APETALA2) genes encode two classes of proteins, AP2-like and EREBP-like, that are defined by the number of AP2 domains in each polypeptide as well as by two sequence motifs referred to as the YRG and RAYD elements that are located within each AP2 domain (Okamuro *et al*., 1997). The AP2-like branch of the *RAP2* gene family is comprised of three genes *AP2, ANT*, and *RAP2.7*, each of which encodes a protein containing two AP2 domains and a conserved WEAR/WESH amino acid sequence motif located in the YRG element of both AP2 domain repeats, while genes belonging to the EREBP-like branch encode proteins with only one AP2 domain and a conserved 7-amino acid sequence motif referred to as the WAAEIRD box in place of the WEAR/WESH motif located in the YRG element and include RAP2.1–RAP2.6, RAP2.8 –RAP2.12, and TINY (Okamuro *et al.,* 2007).

Vitis RAP.4 (VIT_00s0662g00030) and ERF012 (VIT_18s0001g03240) are members of the ERF/DREB family of transcription factors. Vitis RAP2.4, also known as VvDREB37, belongs to DREB sub-group A6 and its best Arabidopsis homolog is RAP2.4 (AT1G22190/AT1G78080) (Licausi *et al.*, 2010; Zhao *et al.*, 2014). Several members of the RAP2 class of genes were shown to be involved in abiotic stress responses. In Arabidopsis, mutants over-expressing RAP2 genes showed enhanced tolerance to salt, hypoxia and drought stress (Hinz *et al.*, 2010; Krishnaswamy *et al.*, 2011). Vitis ERF012 was identified by Licausi *et al*. (2010) as VvERF008, a group-II ERF member, while Zhao *et al*. (2014) assigned it to the sub-group A5 of the DREB family as VvDREB26. Unlike its Arabidopsis homolog DREB26 (AT1G21910), VvDREB26 contains an ERF-associated amphiphilic repression (EAR) motif (KPDLNxxP) (Krishnaswamy *et al.*, 2011; Zhao *et al*., 2014). EAR motif-containing repressor proteins play a key role in modulating plant defences and stress responses by inhibiting the expression of other stress-related genes, thus preventing over-responses to stress treatment, and to prevent a stress response when there is not a need for one (Kazan, 2006; Dong and Liu, 2010).

Both Vitis ERF098 and ERF1A genes belong to group-IX, subfamily B-3 of the ERF superfamily (Licausi *et al.*, 2010; Zhao *et al.*, 2014). Arabidopsis ERF98/TDR1 (AT3G23230) and ERF13 (AT2G44840) are Vitis ERF098 and ERF1A homologs, respectively. ERF98/TDR1 and other sub-family members contain an EDLL motif which represents an activator domain (Tiwari *et al.*, 2012). Arabidopsis TDR1 and ERF13 expression is involved in JA-mediated induced systemic response to several pathogens (McGrath *et al.*, 2005; Brotman *et al.*, 2012; Schweizer *et al.,* 2013) and oxidative stress response (González-Pérez *et al*., 2012; Chen *et al*., 2013).

Vitis ERF003 gene belongs to group-V of the ERF superfamlily (Licausi *et al.*, 2010). Group-V genes are characterized by two conserved motifs corresponding to motifs CMV-1 and CMV-2, although CMV-1 may be incomplete (Nakano *et al.,* 2006, 2014). Group-V genes include the Arabidopsis WAX INDUCER 1 (WIN1)/SHINE1, (SHN1), SHN2, and SHN3, which possess two complete CMV motifs, and the Arabidopsis ETHYLENE AND SALT INDUCIBLE (ESE1-3) and tomato (Solanum lycopersicum) ETHYLENE-RESPONSIVE FACTOR 52 (SlERF52) genes which contain a complete CMV-2 and an incomplete CMV-1 motifs (Nakano *et al*., 2006, 2014). Vitis ERF003 only contains the CMV-2 motif (F. Licausi, personal communication) which was demonstrated in SlERF52 to act as an activation domain (Nakano *et al.*, 2014). These genes regulate cutin biosynthesis, flower development and are involved in abiotic-stress responses (Lin *et al.*, 2008; Shi *et al.*, 2011; Zhang *et al*., 2011; Cheng *et al*., 2013; Lee *et al.*, 2014; Aharoni *et al.*, 2014).

Vitis ERF109 and Ethylene-responsive transcription factor ABA repressor 1 (ABR1) genes belong to group-X, sub-family B-3/B-4 of the ERF super-family. Other members of this group include ERF114 and the EREBP-like genes RAP2.6 and RAP2.6L (Nacano *et al*., 2006; Okamuro *et al.*, 2007; Licausi *et al.*, 2010). Arabidopsis ERF109/RRTF1 was shown to be involved in redox homeostasis regulation during biotic and abiotic stress (Khandelwal *et al.*, 2008; Mehterov *et al*., 2012; Kerchev *et al.*, 2013) and in vascular development (Etchells *et al.*, 2012), while Arabidopsis ABR1 gene acts as a repressor of ABA-regulated gene expression (Pandey *et al*., 2005).

4.3.2 The WRKY family of transcription factors

WRKY genes are characterized by an almost invariant WRKY amino acid sequence at the N-terminus, and a zinc-finger structure at the C-terminus. In Vitis, 59 WRKY distinct WRKY

putative transcription factors were identified and divided in four groups according to the number of WRKY domains and the structure of their zinc fingers. In this study, five WRKYs were identified, WRKY18, WRKY27, WRKY33, WRKY51 and WRKY70. Group I WRKY33 has two complete WRKY domains and a C2H2-type zinc finger motif. Group II WRKY18, WRKY27 and WRKY51 have a WRKY domain and a C2H2-type zinc finger motif and belong to subgroups IIa, IIe and IIc, respectively. Group III WRKY70 contains a WRKY domain and a C2HC-type zinc finger motif (Rushton *et al*., 2010; Cheng *et al.*, 2012; Wang *et al.*, 2014). WRKY proteins are particularly involved in pathogen defence-responses (Eulgem, 2006; Wang *et al*., 2006; Eulgem *&* Somssich, 2007; Pandey *&* Somssich, 2009; Rushton *et al*., 2010; Moore *et al.*, 2011), oxidative-stress (Vranová *et al.,* 2002; Gadjev *et al.*, 2006; Chen *et al.,* 2012) and in autoimmune-like responses (Bomblies *et al*., 2007).

4.3.3 The C_2H_2-type Zinc Finger family of transcription factors

In this study, two C2H2-type zinc finger proteins were identified, C2H2FAM_21 and C2H2FAM_31. Zinc finger proteins are among the most abundant and functionally versatile proteins in eukaryotic genomes (Laity *et al.*, 2001) known to bind to DNA, RNA, protein and lipid substrates (Matthews *&* Sunde, 2002; Brown, 2005; Hall, 2005; Gamsjaeger *et al.,* 2007; Brayer *&* Segal, 2008). C2H2-type zinc finger proteins, also called the classical or TFIIIA-type finger (Miller *et al.*, 1985), are characterized by two cysteines and two histidines that coordinate a zinc atom to form a compact structure containing a beta-hairpin and an alfa-helix that binds to DNA (Takatsuji *et al.*, 1999; Marco *et al*., 2003). Based on the number and the pattern of the fingers, most of the proteins can be classified into one of three groups: triple-C2H2, multiple-adjacent-C2H2, and separated-paired-C2H2 finger proteins. In contrast to proteins with triple-C2H2 fingers, proteins with multiple-adjacent-C2H2 fingers can bind multiple, different ligands. Proteins with a number of separated-paired fingers bind to the target by means of only a single pair (Iuchi, 2001). Vitis C2H2FAM21 best Arabidopsis homolog is zinc finger protein 10 (ZFP10) (AT2G37740), a member of the ZFP subfamily of C2H2-zinc finger factors, whose proteins have one zinc finger (Ciftci-Yilmaz *&* Mittler, 2008), while Vitis C2H2FAM_31 best Arabidopsis homolog is the C2H2 zinc finger AT2G29660. C2H2 zinc finger transcription factors are involved in development, biotic and abiotic stresses (Xiong *&* Zhu, 2002; Rizhsky *et al*., 2004; Sakamoto *et al*., 2000, 2004; Payne *et al*., 2004; Davletova *et al*., 2005; Miller *et al*., 2008; Mehterov *et al*., 2012; Zhou *et al.*, 2013; Balazadeh *et al.*, 2012; Zhang *et al*., 2014; Shi *&* Chan, 2014).

4.3.4 The NAC family of transcription factors

In this study, two NAC genes, CUC2/VvNAC55 (VIT_15s0048g02270) and NTL9/VvNAC17 (VIT_15s0048g02300) were identified. NAC (NAM, ATAF1/2 and CUC2) transcription factors comprise a conserved NAM domain in the N-terminus and are one of the key regulatory gene families involved in plant development and stress, including senescence (Olsen *et al*., 2005; Guo & Gan, 2006; Nuruzzaman *et al*., 2013; Jensen & Skriver, 2014). The Vitis genome comprises 74 NAC genes, divided in eight phylogenetic clades (Wang *et al*., 2013). Group V VvNAC55 and its best Arabidopsis homolog ANAC116 (AT4G35580) encode a calmodulin (CaM) binding NAC protein (CBNAC), which is positively induced by ET and JA (Nakano *et al.*, 2006). Group III VvNAC17 (VIT_15s0048g02300) best Arabidopsis homolog ANAC072/ATRD26 (AT4G27410) is known to be a transcriptional activator of defence, senescence and detoxification genes induced by abiotic stress and responsive to JA, ABA and ROS (Fujita *et al.*, 2004).

4.3.5 The TALE family of transcription factors

The plant three-amino-acid-loop-extension (TALE) class of homeoproteins comprises the KNOTTED-like homeodomain (KNOX) and BEL1-like homeodomain (BELL) homeobox proteins that function as heterodimers. The homeodomain (HD) is a conserved 60-amino acid motif which folds into a characteristic three-helix structure that is able to interact specifically with DNA and is present in transcription factors found in all the eukaryotic organisms. One KNOX/ELK gene, VIT_10s0116g00190, was found differential expressed between heterografts. Its best Arabidopsis match is the KNOX/ELK gene AT1G62360, also known as SHOOTLESS (SHL). These genes are particularly involved in meristem maintenance and initiation (Satoh *et al*., 1999, 2003; Hamant *et al*., 2002; Hay *et al.,* 2002; Scofield & Murray, 2006; Hay & Tsiantis, 2010; Hamant & Pautot, 2010).

5. DISCUSSION

5.1 OXIDATIVE STRESS AT CALLOGENESIS

The Vitis ethylene-responsive transcription factor ERF109 was also previously identified as a grapevine graft interface-specific gene, up-regulated from 3 d to 28 d after grafting (Cookson *et al.*, 2013). Its Arabidopsis homolog ERF109/RRTF1 was shown to be involved in

redox homeostasis regulation during biotic and abiotic stress (Khandelwal *et al.*, 2008; Mehterov *et al*., 2012; Kerchev *et al.*, 2013) and in vascular development (Etchells *et al.*, 2012).

In a study with photosynthetic induced oxidative stress, RRTF1 knockout plants showed greater sensitivity to this stress through an inability to reach homeostasis (Khandelwal *et al*., 2008). Foyer *et al.* (2014) suggested that RRTF1 stimulates the production of protective pigments such as anthocyanin through an association with *PAP1*, which is a transcription factor that is involved in the regulation of anthocyanin biosynthesis. In a study with oxidative stress resistant (atr7) and sensitive (loh2) Arabidopsis mutants, RRTF1 was up-regulated upon oxidative stress and more abundantly expressed in the mutant resistant atr7 compared with the sensitive mutant loh2 (Mehterov *et al*., 2012). Overall these studies suggest that ER109/RRTF1 over-expression confers enhanced resistance to oxidative stress. Although its low expression, it is up-regulated in the most compatible heterograft what might suggest a higher ability for this combination to cope with oxidative stress.

Shaikhali *et al.* (2008) suggested that a member of the RAP2.4 clade, RAP2.4a, stimulates the expression of antioxidant enzymes under moderate, but not severe, oxidative stress conditions,. We might hypothesize that the expression of RAP2.4a could also be impaired by severe oxidative stress conditions, although, to our knowledge, there are no studies to support this hypothesis. If verifiable, it could explain the lower levels of RAP2.4 expression in the least compatible hetero-graft TN112/R110, while suggesting that TN112/R110 was experiencing higher oxidative stress than TN21/R110. Because cells usually arrest the progression of cell division, when confronted with an acute osmotic/oxidative stress (Xiong & Zhu, 2002), this results could explain the incomplete vascular differentiation usually reported in incompatible grafts. Moreover, a study reported that RAP2.4, also known as *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*), over-expression upon wounding is sufficient to induce vascular cell differentiation in a synergistic pathway with a B-type ARR-mediated cytokinin (Iwase *et al.*, 2011). At callogenesis, we found a cytokinin response regulator B-type ARR9 slightly up-regulated in the most compatible hetero-graft (fig.6), although the difference is not statistical relevant. Additionally, RAP2.4 regulates the expression of at least six aquaporin (AQP) genes (Rae *et al.*, 2011) and its over-expression not only confers an enhanced tolerance to drought stress (Lin *et al*., 2008) but also reduces vessel embolism (Lovisolo *et al*., 2007). AQPs are membrane proteins that facilitate the movement of water or other small solutes (Hachez *et al.*, 2006), respond to ROS gradients, and are widely

implicated in plant growth and development besides its involvement in response to biotic and abiotic stress (Lin *et al.*, 2008; Maurel *et al.*, 2008, 2009; Péret *et al*., 2012; Shi *et al.;* 2014). In fact, Péret *et al.* (2012) proposed a model where auxin promotes root development by regulating the spatial and temporal distribution of AQPs expression. These studies suggest that, while participating in cells detoxification, RAP2.4 might be also promoting vascular development, in a stress-response/development-inducing cross-talk manner.

Furthermore, the low expression levels of ERF109/RRTF1 might be explained by the repressive effect of WOX4 expression on these genes. It is known that ERF109, along with PXY and WOX4, promotes cells orientation and division rate in vascular development, in a model where RRTF1 expression is repressed by WOX4 but induced by jasmonic acid (JA) (Fisher *&* Turner, 2007, Wang *et al.*, 2008), a phytohormone that has recently emerged as a key modulator of cell division in the cambium (Sehr *et al*., 2010). JA is also a key modulator of immune response and comprises two signalling branches, the MYC branch, usually associated with the wound response and defence against insect herbivores, and the ERF branch, usually associated with defence against necrotrophic pathogens (Pieterse *et al.*, 2012). The expression of WRKY51, however, suggests repression of the JA-mediated defence response and induction of the salicylic acid (SA)-mediated responses (Gao *et al*., 2011). On the other hand, we found in our study that the expression of VIT_11s0052g00100 (BHLHDOM_7), a MYC-type basic-helix-loop-helix (bHLH) transcription factor, was higher in TN112/R110 and, although the difference between hetero-grafts had no statistic significance, it could suggest a stronger MYC-dependent JA-induced response in TN112/R110. Moreover, Arabidopsis ERF98/TDR1 (AT3G23230) and ERF13 (AT2G44840), which are Vitis ERF098 and ERF1A homologs, respectively, are up-regulated in TN112/R110 (fig. 4) and involved in JA-mediated induced systemic response to several pathogens (McGrath *et al.*, 2005; Brotman *et al.*, 2012; Schweizer *et al.,* 2013) and oxidative stress response (González-Pérez *et al*., 2012; Chen *et al*., 2013).

Studies with Arabidopsis mutants showed that plants deficient in ZAT12, a C2H2-type zinc finger, were unable to enhance the expression of an antioxidant enzyme (Apx1), as well other transcription factors expressed under oxidative stress, while its over-expression resulted in the enhanced expression of oxidative- and light stress-response transcripts (Rizhsky *et al.,* 2004; Davletova *et al.*, 2005). The up-regulation of C2H2FAM_31 (VIT_06s0004g06240) in the most compatible hetero-graft may therefore suggest a higher ability for this genotype combination to cope with oxidative stress, that could theoretically

overcome the low expression of RRTF1, although there is no data to support this hypothesis. Another C2H2-type zinc finger protein, C2H2FAM_21 (VIT_13s0019g02120), also known as zinc finger protein 10 (ZFP10), was up-regulated in the least compatible heterograft (fig.3). In rice plants, over-expression of ZFP36 was found to elevate the activities of antioxidant enzymes and to enhance the tolerance to water and oxidative stress, while an RNA interference (RNAi) mutant of ZFP36 had lower activities of antioxidant enzymes and was more sensitive to water stress and oxidative stress (Zhang *et al.*, 2014). In Arabidopsis, over-expression of ZFP3 and the closely related ZFP1, ZFP4, ZFP6 and ZFP7 zinc finger factors confers ABA insensitivity to seed germination while the *zfp3 zfp4* double mutant displays enhanced ABA susceptibility, suggesting that members of the ZFP subfamily of zinc finger factors regulate light and ABA responses during germination and early seedling development (Joseph *et al.*, 2014). This previous study suggests that ZFPs respond to stress in an ABA-dependent pathway, as do RAP2.6, RAP2.6L and ABR1 (Pandey *et al.*, 2005; Krishnaswamy *et al.*, 2011), members of the same group as ERF109/RRTF, unlike RAP2.4 that confers oxidative stress tolerance in an ABA-independent pathway (Lin *et al.*, 2008). Overall, this could suggest that the oxidative stress response at the graft-interface is not particularly ABA-dependent, what could explain the low levels of RRTF1 and ZFP10 expression at callogenesis, and ABR1 at rooting. In fact, osmotic, drought and salt stress signalling can occur in an ABA-dependent (Sharp & LeNoble, 2002; Davies *et al.*, 2005; Rosado *et al*., 2006; Tramontini *et al.*, 2013) or -independent way (Zhu, 2002; Shinozaki & Yamaguchi-Shinozaki, 2007; Wang *et al.,* 2010). Additionally, we found that the ABA-induced gene AtHB6 (VIT_18s0001g06430), a class I homeodomain leucine-zipper (HD-Zip I) transcription factor (Söderman *et al.*, 1999; Elhiti & Stasolla, 2009) was not differential expressed between heterografts (fig. 5), neither at callogenesis, nor at rooting, what might explain the similar level of expression for ERF109/RRTF1, C2H2FAM31/ZFP10 and ABR1.

5.2 IMMUNE-TYPE RESPONSE AT ROOTING

Extensive studies have established that plant WRKY transcription factors play important roles in the two branches of the plant innate immune system, which are triggered by pathogen-associated molecular patterns (PAMP-triggered immunity or PTI) and pathogen virulent effectors (effector-triggered immunity or ETI). PAMPs are recognized by pattern-recognition receptors (PRRs) that trigger PTI, with increased intracellular Ca^{2+} concentration, oxidative burst, MAP kinase (MAPK) activation, protein phosphorylation, receptor endocytosis and protein–protein interactions. Many PRRs are membrane associated receptor-like kinases

(RLKs), leucine-rich repeat RLKs (LRR-RLKs) or receptor-like proteins (RLPs) and previous studies reported LRR-RLKs to be up-regulated in grapevine heterografts (Cookson *et al.*, 2013, 2014). We found a leucine-rich repeat transmembrane protein kinase RKL1 (VIT_08s0007g04150) up-regulated in TN21/R110 at both callogenesis and rooting, although the difference was not statistically relevant (fig. 5). Previous studies suggest that the up-regulation of PAMP-responsive RLK genes might lead to an enhanced sensitivity to further PAMP stimuli, thus resulting in a faster response to subsequent pathogen challenge (Navarro *et al.,* 2004; Sanabria *&* Dubery, 2006; Newman *et al.*, 2007; Sanabria *et al*., 2012). If so, our results could suggest that TN21/R110 could be more prepared to a PAMP stimuli, resulting in a faster and more efficient defence response. A rapid defence induction upon pathogen detection minimizes the costs associated with the production and secretion of proteins with specific defensive properties, such as PR proteins, thus contributing to a more stable trade-off between defence and growth/regeneration hormones (reviewed by Huot *et al.*, 2014). PR proteins were previously shown to be involved in grapevine hetero- and auto-grafts (Cookson & Ollat, 2013; Cookson *et al*., 2013, 2014). The expression of PR proteins is part of the SA-dependent systemic acquired resistance (SAR) pathway, unlike the JA-dependent induced systemic resistance (ISR) pathway that functions independently of SA and PR gene activation, (Ward *et al.,* 1991; Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Mou *et al*., 2003; Eulgem *et al.,* 2006; Eulgem & Somssich, 2007). Typically, SAR as been associated with ETI, but recent findings suggest that both PTI and ETI are involved in SAR (reviewed by Thomma *et al.*, 2011). Additionally, both SAR and ISR require the key regulatory protein NPR1 (Wees *et al.,* 2000; Spoel *et al.*, 2003) and synergism between SA- and JA-mediated signalling has also been observed in the plant immune response (Eulgem & Somssich, 2007; Robert-Seilaniantz *et al.*, 2007; Pandey & Somssich, 2009).

In fact, both WRKY70, WRKY51, WRKY27 and WRKY18 appear to be joint-regulators connecting the SA-mediated disease resistance pathway and JA-mediated disease/wound response pathway (Li *et al*., 2004; Dong *et al.*, 2004; Mukhtar *et al.*, 2008; Wang *et al.*, 2008; Gao *et al*., 2011; Wenke *et al*., 2012; Schweizer *et al.*, 2013). Moreover, Lippoch *et al*. (2007) showed that, in Arabidopsis, WRKY33 expression was induced by both biotrophic and necrotrophic pathogens, although not by a biotrophic non-host, in a SA-, JA- and NPR1-independent manner. In fact, an association between WRKY33 and the mitogen-associated protein kinase4 MPK4, which is a negative regulator of salicylic acid (SA) and SAR, was previously established (Peterson *et al.*, 2000; Grant & Lamb, 2006). Recently, it was shown that MPK4 and WRKY33 exists in nuclear complexes, which depends on the MPK4 substrate

MKS1. Infection with the biotrophic pathogen *Pseudomonas syringae* led to MPK4 activation and MKS1 phosphorylation releasing WRKY33 for targeting the promoter of *PHYTOALEXIN DEFICIENT3* (*PAD3*) encoding an enzyme required for the synthesis of antimicrobial camalexin enzyme (Qiu *et al*., 2008). It has also been demonstrated that WRKY33 is involved in resistance to necrotrophic pathogens through repression of PR proteins (Zheng *et al.*, 2006) and interaction with the VQ-motif of the nuclear-encoded SIGMA FACTOR BINDING PROTEIN1 (SIB1) and SIB2 that stimulate the DNA-binding activity of WRKY33 (Lai *et al*., 2011). VQ proteins, like SIB1, SIB2 and MKS1, are responsive to pathogen infection and salicylic acid treatment and most likely act as co-factors of group I WRKY33 and group IIc WRKY51 transcription factors. By contrast, the WRKY domains from group IIa WRKY18 and group III WRKY70, as well as group lle WRKY22, which belongs to the same group as WRKY27, failed to interact with VQ proteins (Cheng *et al.*, 2012).

The expression of these WRKYs could suggest a cellular response to both biotrophic and necrotophic pathogens, and this synergism may provide an additive effect on the level of induced protection against pathogens (Wees *et al.,* 2000). Nonetheless, they appear to be particularly involved in SAR establishment, which is supported by a previous study that reports the induction of ROS and PR-proteins at the graft interface of grapevine heterografts (Cookson *et al*, 2014). The authors also suggested that the results could support an immune-type response caused by genetic incompatibility between heterografts. Interestingly, WRKY proteins are amongst the overrepresented genes associated with autoimmune-like responses that cause *hybrid necrosis,* a genetic incompatibility (Bomblies *et al*., 2007). Overall, it seems that an immune or autoimmune-type response is stronger in the least compatible graft.

Intriguingly, the expression of WRKY18 and WRKY70 was notably higher than the expression of the other WRKY transcripts (fig. 5). Very interestingly, WRKY18 and WRKY70, along with WRKY11 and WRKY15, were found to be negative regulators of PTI signalling, blocking the PAMP-triggered ROS burst and growth inhibition preventing an exaggerated activation of a subset of PTI defence-related genes thus restricting the output of PAMP-triggered basal defence below a detrimental threshold (Shen *et al.*, 2007; Lozano-Duran *et al.,* 2013). In the study by Shen *et al*. (2007), only *wrky18/40* double mutants, but not *wrky18* or *wrky40* single mutants, displayed exaggerated activation of a subset of PTI defence-related genes although the interaction of WRKY18 with other proteins rather than the traditional WRKY18/40/60 interaction (Xu *et al*., 2006) as been established (Wang *et al.*,

2008; Dinkins *et al.*, 2012).

Shen *et al*. (2007) also suggested that, besides the repression of PAMP-triggered basal defence, WRKY18/40 could, at the same time, function as a trigger of the primed immune system for disease-resistance (R) protein dependent defence, a component of ETI, driving host cells into suicide (Jones *&* Dangl, 2006). ETI seems to operate by alleviating the negative feedback regulation of PTI, leading to stronger defences. Often ETI is quantitatively stronger than PTI and is accompanied by a hypersensitive response form of programmed cell death. It is very important to regulate the strength and duration of innate immune responses to prevent over-reaction of the immune system (e.g. sepsis), autoimmune diseases and prolonged growth inhibition that could compromise proper vascular regeneration (reviewed by Jones *&* Dangl, 2006; Schwessinger *&* Zipfel, 2008; Huot *et al.*, 2014). Senescence is a form of PDC, linked both to defence and development, hypersensitive response, tissues sculpting and cell recycling. It is controlled by hormones, ROS and Ca^{2+}. The phytohormone Cytokinin blocks senescence, while ethylene, ROS, Ca^{2+} and salicylic acid enhances it. However, there are some differences between a defence hypersensitive response and organ senescence. Contrary to the second, death in the hypersensitive response does not remove the dead cells but does release toxins directed against an invader pathogen, leaving wide gaps in the plant tissue that are eventually crushed by expanding tissues (Yen *&* Yang, 1998; Xu *&* Hanson, 2000; Jones, 2001). This scenario resembles previous studies reporting bark discontinuity, wide gaps at the cortex of scion and stock and tissue degeneration of incompatible grafts (Ermel *et al.*, 1999; Mahunu & Adjei, 2012; Zarrouk *et al*., 2010) that could be therefore the result of a hypersensitive response. On the other hand, Nocito et al. (2010) suggested that a senescence-like status could be the result of undifferentiated cells in incompatible grafts. It seems that senescence is important in compatible grafts for the removal of the graft-interface necrotic layer caused by grafting, as senescence-associated genes have been reported at the graft-interface of 28 d grapevine auto-grafts, which corresponds to callogenesis (Cookson *et al.*, 2013). Overall, results suggest that a form of PDC could be occurring at rooting in incompatible grafts, long pass the time-point of graft-interface healing (Mahunu & Adjei, 2012) resulting in detrimental effects of constitutive activation of defence on plant growth (reviewed by Jensen & Skriver, 2014).

Four other genes were up-regulated at rooting in incompatible grafts, two NAC genes VvNAC55 and VvNAC17, and three AP2/ERF genes, ABR1, ERF1A and ERF098.

Group V VvNAC55 and its best Arabidopsis homolog ANAC116 (AT4G35580) encode a calmodulin (CaM) binding NAC protein (CBNAC), which is positively induced by ET and JA (Nakano *et al.*, 2006). CBNAC is a Ca2+-dependent CaM binding transcriptional repressor, and its repressive activity is enhanced by binding to Ca2+/CaM (Kim *et al*., 2007; Wang *et al*., 2013). In pathogen defence, CaM binds to MAPK phosphatase (MKP1), a negative regulator of mitogen-associated protein kinases (MAPKs) in a Ca2+-dependent manner (Lee *et al.*, 2008). Since MKP1 is a negative regulator of MAPKs, and MPK4 is a negative regulator of salicylic acid (SA) induced SAR (Peterson *et al*., 2000), VvNAC55 expression could suggests SAR repression. Additionally, an *Arabidopsis* signal responsive (*AtSR*) protein, which belong to a class of Ca^{2+}/calmodulin (CaM)-binding transcription factors, was found to repress the expression of EDS1, a regulator of salicylic acid level, in a Ca^{2+}/calmodulin-AtSR1 binding dependent manner, thus repressing SA-mediated plant immunity (Du *et al.*, 2009). Moreover, CBNAC genes are involved in suppression of basal resistance through negative regulation of PR1 expression and disease resistance to prevent detrimental effects of constitutive activation of defence on plant growth (reviewed by Jensen *&* Skriver, 2014).

Group III VvNAC17 (VIT_15s0048g02300) best Arabidopsis homolog ANAC072/ATRD26 (AT4G27410) was found to be a PAMP-responsive gene upon pathogen attack, regulated by ABA and hydrogen peroxide, but not ethylene, SA, JA or IAA (Huang *et al*., 2012). Since AtHB6 was not differential expressed between hetero-grafts, it suggests that the level of ABA production was similar in both TN21/R110 and TN112/R110. Moreover, the expression of ABR1 at rooting, although significantly different between hetero-grafts, was very low (fig. 4). ABR1 is induced by ABA, osmotic stress, sugar stress and drought, probably involved in regulation of ABA-mediated stress responses (Pandey *et al.*, 2005). Results might suggest that ABA was not being highly produced at this time-point, but weather AtHB6 and ABR1 expression could explain an ABA stress-induction expression of VvNAC17 is not possible without knowledge on ABA steady-state transcript levels and on the expression of additional ABA-related genes.

On the other hand, ERF003 (VIT_09s0002g09120) and ERF012 (VIT_18s0001g03240) were up-regulated in the compatible TN21/R110 combination. Vitis ERF012/DREB16 possess a ERF-associated amphiphilic repression (EAR) motif (Zhao *et al.,* 2014). Since EAR motif-containing repressor proteins inhibit the expression of stress-related genes, thus preventing a stress response when there is not a need for one (Kazan, 2006; Dong *&* Liu, 2010), results

might suggest that TN21/R110 was in a further stress-repressive state than TN112/R110.

5.3 REPRODUCTIVE PHASE

Two other genes up-regulated in TN21/R110 at rooting were ERF003 and a KNOX/ELK gene. Arabidopsis SHN and tomato SIERF52 genes are Vitis ERF003 homologs. These are involved in cutin biosynthesis and flower development (Shi *et al.*, 2011; Nakano *et al.*, 2014; Aharoni *et al.*, 2014). KNOX/ELK genes are involved in meristem maintenance and initiation (Satoh *et al*., 1999, 2003; Hamant *et al*., 2002; Hay *et al.,* 2002; Scofield *&* Murray, 2006; Hay *&* Tsiantis, 2010; Hamant *&* Pautot, 2010). Overall, the expression of these genes might relate to the reproductive phase (Araki, 2001) or lateral shoot formation from the graft site.

5.4 HORIZONTAL GENE TRANSFER BETWEEN GRAFT PARTNERS

A plant not subjected to the grafting process is expected to outperform the plants that are, and that was in fact observed in the field trials (fig. 1). All non-grafts had a greater success rate that the respective autografted plants. Given that grafting with a different genotype increases the chance of incompatibility (Cookson *&* Ollat, 2013) it was expected that autografts success would outperform the heterografts. This was indeed observed for TN112, but, interestingly, not for TN21. The TN21/R110 heterograft not only outperformed the TN112/R110 heterograft, but also both TN21 and R110 autografts. One explanation could rely on the fact that heterografts and autografts were performed one-year apart. Differences in environmental conditions from one year to the next could account for the poor autograft results, but this was only observed for one combination and not for the other, and thus conclusions can not be drawn from environmental differences. This leads us to the process of grafting. Interestingly, the TN21/R110 heterograft success rate suggests that some advantage might had come from grafting these two different genotypes. Upon grafting, signalling molecules can migrate across cells (Lucas *et al.*, 1995, 2001, 2004; Stegemann *&* Bock 2009), and because cells have genotype-specific sensitivities (Lerner, 1999) and different response thresholds (Bradford *&* Trewavas, 1994), this process can lead to hormonal unbalance, which has been suggested as a cause for graft incompatibility (reviewed in Aloni *et al.*, 2010). But narrowing the variation among cells could synchronize the response across all regulator levels (Bradford *&* Trewavas, 1994), which could be theoretical achieved with horizontal gene transfer (HGT) (Richardson *&* Palmer, 2007; Stegemann *&* Bock, 2009; Thyssen *et al*., 2012; Stegemann *et al.*, 2012) between graft

partners (see review by Gao *et al*., 2014). HGT events seem to be common at the graft-interface of graft partners and can drive genomic and phenotypic changes that increase fitness substantially (reviewed by Bock, 2010) perhaps even overcoming the genetic incompatibility that seems to be a cause for unsuccessful graft (Pina *&* Errea, 2005). Moreover, why the TN21/R110 combination was advantageous, while the TN112/R110 combination was detrimental, compared to autograft success, is another question. We might suggest that TN21 possesses some mechanim over TN112 that enables or enhances HGT events. Plant viral movement proteins (VMPs), for instance, are known to enable intercellular and long-distance traffic of single and multipartite genomes (see review by Zambryski *&* Crawford, 2000), so we may hypothesize that some VMPs could be expressed in TN21/R110 but not in TN112/R110, which could be further analysed in the transcriptome. Also, an HGT event could theoretically increase the number of different pattern-recognition receptors (PRRs), thus resulting in a faster response to subsequent pathogen challenge (Navarro *et al.,* 2004; Sanabria *&* Dubery, 2006; Newman *et al*., 2007; Sanabria *et al*., 2012), minimizing the costs associated with the production and secretion of proteins with specific defensive properties, thus contributing to a more stable trade-off between defence and growth/regeneration hormones (reviewed by Huot *et al*., 2014).

6. CONCLUSIONS AND FUTURE DIRECTIONS

Both the wound-induced responses (see reviews by Léon *et al*., 2011; Cheong *et al*., 2002; Schilmiller & Howe, 2005) as the hydraulic bottleneck caused by grafting (reviewed in Serra *et al*., 2014) alter the normal homeostasis of cells and can lead to oxidative stress with enhanced production of reactive oxygen species (ROS) (see reviews by Blokhina *et al*, 2003; Miller *et al*., 2010). Previous studies show that grafting induces the up-regulation of genes related to anti-oxidant enzymes (Fernández-García *et al*., 2004; Cookson *et al.,* 2013), and report higher accumulation of ROS and low anti-oxidant enzyme activities in incompatible grafts (Errea, 1998; Ermel *et al*., 1999; Aloni *et al.,* 2008; Pina & Errea, 2008; Basheer-Salimia & Hamdan, 2009; Nocito *et al*., 2010; Zarrouk *et al*., 2010; Pina *et al*., 2012; Cookson *et al*., 2014). Further, it has been suggested that ROS-induced oxidative stresses may have the upper-hand in incompatible graft combinations (Nocito *et al*., 2010). Interestingly, although not surprising, we found that all the five genes up-regulated at callogenesis (RAP2.4, ERF109, C2H2FAM_21/ZFP10, C2H2FAM_31 and WRKY51) are involved in oxidative stress response/homeostasis. Overall, our results suggest that the oxidative stress was more severe in the incompatible graft. Because cells usually arrest the

progression of cell division, when confronted with an acute osmotic/oxidative stress (Xiong *&* Zhu, 2002), this results could explain the incomplete vascular differentiation usually reported in incompatible grafts, since the re-establishment of the vascular communication between grafts is the final crucial step in graft recovery (reviewed by Pina *&* Errea, 2005; Dolgun *et al.*, 2008; Yin *et al.* 2012) and the time it takes to occur is crucial for graft compatibility (Errea *et al.,* 1994; Flaishman *et al*., 2008; Pina *et al*., 2012; Mahunu *&* Adjei, 2012; Yin *et al.*, 2012; Cookson *et al.*, 2014; Brian *&* Duron, 1971 *in* Ermel *et al.*, 1998).

Moreover, In a recent review on hormonal signalling in scion-rootsctock interactions, Aloni *et al.* (2010) points to the fact that the crosstalk between ethylene and other phytohormones required for vascular regeneration and proper maintenance of the rootstock–scion communication, depends largely on the concentration of these hormones and the subsequent balance required for these processes, and propose that a model for ethylene dependent rootstock–scion relationship was worth investigating, similar to the model proposed by Ruzicka *et al.* (2007) for root growth. Our results suggest that initial ethylene production could account for the difference between compatible and incompatible heterografts, since the expression of transcription factors involved in other phytohormones response was not statistically relevant. Therefore, the predictive value of the initial concentration of ethylene production upon grafting seems worth investigating. If validated, such a simple non intrusive test could be standardized to prematurely assess the probability of graft (in)compatibility before field establishment of the crops. In the same way, because a loop cross-talk between ethylene and H_2O_2 has a role in auxin signalling in response to wounding (Blancaflor & Masson, 2003; Apel & Hirt, 2004; Wang *et al.*, 2010) and control the transition from cell proliferation to differentiation (Tsukagoshi *et al.*, 2010), H_2O_2 production levels could be assessed the same way as ethylene, as a complementary field test for graft (in)compatibility.

At rooting, an immune or autoimmune-type response was significantly higher in the least compatible heterograft, as previously suggested (Cookson *&* Ollat, 2013; Cookson *et al*., 2013, 2014). These results suggest an extended period of defence-related responses in TN112/R110 compared to TN21/R110. Since a rapid defence induction upon pathogen detection contributes to a more stable trade-off between defence and growth/regeneration hormones (reviewed by Huot *et al.*, 2014), the extended period of defence-related responses in TN112/R110 may therefore be a cause for inhibited vascular regeneration.

Cookson *et al.* (2014) and several previous authors (reviewed by Pina *&* Errea, 2005) also suggested that an immune-type response caused by genetic incompatibility between heterografts could explain graft incompatibility. Interestingly, WRKY proteins are amongst the overrepresented genes associated with autoimmune-like responses that cause *hybrid necrosis,* a genetic incompatibility (Bomblies *et al*., 2007). Overall, our results suggest that an immune or autoimmune-type response is stronger in the least compatible graft at rooting, which could lead to a hypersensitive response form of programmed cell death, autoimmune disease or, at least, prolonged growth inhibition that can compromise proper vascular regeneration (reviewed by Jones *&* Dangl, 2006; Schwessinger *&* Zipfel, 2008; Mahunu *&* Adjei, 2012; Huot *et al.*, 2014). Because of the high expression levels of WRKY18 and WRKY70, we suggest that these transcription factors are strong candidates for molecular markers, particularly at rooting, given that these results are validated by qPCR.

Still, the reason(s) why the TN21/R110 heterograft is more compatible than the TN112/R110 remains elusive. We suggest that a form of HGT event could be responsible for the TN21/R110 success. HGT events seem to be common at the graft-interface of graft partners (reviewed by Bock, 2010) and could potentially narrow the variation among cells and synchronize the response across all regulator levels (Bradford & Trewavas, 1994). Moreover, HGT events could increase the PAMP-responsive RLK genes, leading to an enhanced sensitivity to PAMP stimuli, resulting in a faster response to subsequent pathogen challenge (Navarro *et al.*, 2004; Sanabria *&* Dubery, 2006; Newman *et al*., 2007; Sanabria *et al.*, 2012). Moreover, the notion that HGT events occur naturally between graft partners could interpose the worldwide resistance to genetically modified organisms and the poor acceptance of hybrids within *V. Vinifera,* owing to the industry's reliance on traditional and easily marketed classic wine grape cultivars (Vivier & Pretorius, 2000; Bisson *et al.,* 2002). Based on our results, it should be interesting to assess if HGT events occur at the graft interface and, if so, should they account for graft compatibility.

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SUPPLEMENTARY MATERIAL

Table S1. MACE Results and Annotation of identified TFs in **AI vs BI** Library.

Sequence	Annotation	Symbol	Best arabidopsis match	FDR	baseMean	baseMean AI	baseMean BI	Fold Change	log2Fold Change	pvalue
VIT_03s0063g00460	Ethylene-responsive transcription factor ERF109	ERF109_2	AT4G34410	1,10E-02	23,13	7,03	39,23	5,58	2,48	2,51E-05
VIT_04s0069g00970	WRKY DNA-binding protein 51	WRKY51_1	AT5G64810	3,83E-01	50,38	68,53	32,23	0,47	-1,09	3,42E-03
VIT_00s0662g00030	Ethylene-responsive transcription factor related to APETALA2 4	RAP2_4_5	AT1G22190/AT1G78080	4,16E-06	362,32	202,91	521,73	2,57	1,36	2,65E-09
VIT_13s0019g02120	Zinc finger (C2H2 type)/zinc-finger protein 10	C2H2FAM_21	AT2G37740	4,87E-01	13,25	20,11	6,38	0,32	-1,66	5,57E-03
VIT_06s0004g06240	Zinc finger (C2H2 type) family	C2H2FAM_31	AT2G29660	3,56E-02	349,16	243,69	454,64	1,87	0,9	1,05E-04

Table S2. MACE Results and Annotation of identified TFs in **AII vs BII** Library.

Sequence	Annotation	Symbol	Best arabidopsis match	FDR	baseMean	baseMean AII	baseMean BII	Fold Change	Log2Fold Change	pvalue
VIT_04s0008g05760	WRKY DNA-binding protein 18	WRKY18_2	AT4G31800	3,14E-03	253,39	381,93	124,85	0,33	-1,61	6,55E-06
VIT_18s0001g03240	DREB sub A-5 of ERF/AP2 transcription factor	ERF012	AT1G21910	2,90E-01	85,55	51,94	119,16	2,29	1,2	3,93E-03
VIT_16s0013g00890	Ethylene-responsive element binding factor	ERF1A	AT2G44840	9,21E-03	77,01	110,13	43,89	0,4	-1,33	2,59E-05
VIT_10s0116g00190	KNOX/ELK homeobox transcription factor	KNOX/ELK	AT1G62360	1,65E-01	25,95	13,53	38,37	2,84	1,5	1,49E-03
VIT_09s0002g09120	Ethylene-responsive transcription factor ERF003	ERF003_4	AT5G25190	3,62E-01	138,46	82,59	194,33	2,35	1,23	5,88E-03
VIT_08s0058g01390	WRKY DNA-binding protein 70	WRKY70_2	AT3G56400	2,39E-02	338,23	471,64	204,83	0,43	-1,2	9,77E-05
VIT_08s0058g00690	WRKY DNA-binding protein 33	WRKY33_3	AT2G38470	2,78E-01	50,4	68,94	31,87	0,46	-1,11	3,72E-03
VIT_07s0031g01980	Ethylene-responsive transcription factor aba repressor 1 abr1/erf111	ABR1	AT5G64750	7,17E-02	10,67	18,2	3,13	0,17	-2,54	4,38E-04
VIT_07s0005g03220	Ethylene-responsive transcription factor ERF098	ERF098_1	AT3G23230	4,57E-01	14,67	22,81	6,52	0,29	-1,81	8,82E-03
VIT_04s0069g00970	WRKY DNA-binding protein 51	WRKY51_1	AT5G64810	1,73E-01	43,91	61,63	26,18	0,42	-1,24	1,62E-03
VIT_02s0025g00420	WRKY DNA-binding protein 27	WRKY27_2	AT5G52830	4,98E-01	9,79	15,1	4,48	0,3	-1,75	1,05E-02
VIT_15s0048g02300	NAC transcription factor-like 9	NTL9_2	AT4G27410	2,43E-01	107,25	172,08	42,43	0,25	-2,02	3,08E-03
VIT_15s0048g02270	No apical meristem cup-shaped cotyledon2	CUC2_1	AT4G35580	9,63E-03	117,45	173,07	61,82	0,36	-1,49	2,87E-05

Table S3. MACE Results and Annotation of identified TFs in **AI vs AII** Library.

Sequence	Annotation	Symbol	Best arabidopsis match	FDR	baseMean	baseMeanAI	baseMeanAII	Fold Change	Log2Fold Change	pvalue
VIT_00s0125g00290	Lateral organ boundaries domain protein 4	LBD4_2	AT1G31320	6,76E-08	16,3	30,43	2,18	0,07	-3,81	1,81E-09
VIT_00s0956g00020	Leafy cotyledon1-like protein	L1L_1	AT5G47670	347,1E-10	28,66	50,34	6,98	0,14	-2,85	8,98E-10
VIT_00s2015g00020	F-box family protein	F-box	ND	1,51E-09	162,72	248,22	77,23	0,31	-1,68	2,99E-11
VIT_01s0010g00930	BZIP transcription factor bZIP58	BZIP58	AT1G13600	2,61E-09	58,29	109,99	6,59	0,06	-4,06	5,49E-11
VIT_01s0010g02070	Ethylene-responsive protein	ERUNK_3	AT1G27660	1,44E-03	13,24	24,36	2,13	0,09	-3,52	1,19E-04
VIT_01s0010g03550	Nuclear transcription factor Y subunit B related	NFYBREL_7	AT5G47670	5,42E-03	23,58	40,77	6,4	0,16	-2,67	5,68E-04
VIT_02s0012g01040	NAC domain-containing protein 71	NAC071	AT4G17980	1,24E-25	148,03	267,02	29,05	0,11	-3,2	3,07E-28
VIT_04s0008g00220	auxin-responsive protein IAA16	IAA6	AT3G16500	9,18E-08	27,41	48,81	6	0,12	-3,02	2,54E-09
VIT_04s0008g06000	Ethylene-responsive transcription factor 3	ERF003_2	AT5G25190	6,20E-04	442,24	697,07	187,42	0,27	-1,9	4,51E-05
VIT_04s0023g01660	Scarecrow-like transcription factor 7	SCL7	AT5G66770	1,58E-02	13,42	20,4	6,45	0,32	-1,66	2,02E-03
VIT_04s0023g03680	Histidine kinase 1 (AHK1)	ATHK1	AT2G17820	8,52E-04	16,97	27,82	6,12	0,22	-2,18	6,52E-05
VIT_05s0049g01010	Myb domain protein 14	MYB14_4	AT3G23250	5,12E-05	22,98	37,37	8,59	0,23	-2,12	2,78E-06
VIT_06s0004g02800	Homeodomain-leucine zipper protein Revoluta	REV_2	AT5G60690	1,03E-05	91,83	138,45	45,2	0,33	-1,61	4,67E-07
VIT_06s0004g03130	Auxin response factor 4	ARF4	AT5G60450	1,17E-08	124,19	194,04	54,34	0,28	-1,84	2,74E-10
VIT_06s0061g00470	Myb domain protein 36	MYB36_1	AT5G57620	2,31E-05	106,16	163,37	48,95	0,3	-1,74	1,15E-06
VIT_07s0031g01840	WRKY DNA-binding protein 13	WRKY13	AT4G39410	6,63E-20	415,27	700,87	129,67	0,19	-2,43	3,11E-22
VIT_07s0129g00030	Short-root transcription factor (SHR)	SGR7_4	AT4G37650	2,44E-07	39,09	64,38	13,8	0,21	-2,22	7,52E-09
VIT_07s0129g01000	F-box family protein	SLY1	ND	1,38E-13	540,99	837,58	244,41	0,29	-1,78	1,46E-15
VIT_07s0141g00270	Auxin-induced protein 22D	IAA22D_1	AT5G43700	302,5E-4	13,33	21,97	4,68	0,21	-2,23	4,46E-03
VIT_07s0141g00290	IAA16	IAA16_2	AT3G04730	5,40E-14	766,69	1320,96	212,42	0,16	-2,64	5,31E-16
VIT_07s0255g00020	OBF binding protein 1	OBP1_3	AT3G50410	756,7E-10	124,98	191,04	58,92	0,31	-1,7	2,04E-09
VIT_08s0007g04150	RKL1 (Receptor-like kinase 1)	RKL1	AT2G36570	649,2E-21	758,28	1235,8	280,76	0,23	-2,14	3,61E-21
VIT_08s0032g00150	F-box family protein	F-box	ND	1,51E-05	29,94	49,9	9,97	0,2	-2,32	7,12E-07
VIT_08s0058g01390	WRKY DNA-binding protein 70	WRKY70_2	AT3G56400	611,7E-6	916,7	1361,76	471,64	0,35	-1,53	4,44E-05
VIT_09s0002g03750	GATA transcription factor 11	GAT11_2	AT5G25830	454,7E-6	20,26	32,4	8,13	0,25	-1,99	3,16E-05
VIT_09s0002g04080	IAA9	IAA9_2	AT2G22670	217,2E-36	625,25	1144,78	105,72	0,09	-3,44	2,40E-37
VIT_09s0002g05150	IAA19	IAA19_1	AT3G15540	234,2E-10	25,92	46,64	5,21	0,11	-3,16	5,88E-10
VIT_10s0003g00130	ERF Domain protein 12	ERF12_2	ND	110,5E-7	487,26	728,08	246,44	0,34	-1,56	5,02E-07
VIT_10s0071g00870	High mobility group B 6	HMGB6_2	AT5G23420	1,67E-03	96,93	162,42	31,44	0,19	-2,37	1,42E-04
VIT_10s0116g00190	KNOX/ELK homeobox transcription factor	KNOX	AT1G62360.1	1,57E-06	38,25	62,97	13,53	0,21	-2,22	5,80E-08
VIT_11s0016g03540	Auxin-responsive protein IAA27	IAA27_1	AT4G29080	215,2E-15	696,34	1076,5	316,18	0,29	-1,77	2,34E-15
VIT_11s0052g00100	Basic helix-loop-helix (bHLH) family	BHLHDOM_7	AT5G57150	346,3E-13	112,12	187,09	37,15	0,2	-2,33	5,35E-13
VIT_13s0019g02120	Zinc finger (C2H2 type) family	C2H2FAM_21	AT2G37740	580,3E-5	12,45	20,11	4,78	0,24	-2,07	6,16E-04
VIT_13s0073g00140	Ovate family protein 13 OFP13	OFP13_2	AT1G73220	132,4E-4	33,47	54,41	12,52	0,23	-2,12	1,64E-03
VIT_14s0006g02730	Zinc finger protein 6	ZFP6_1	AT1G68360	474,7E-6	93,59	141,8	45,37	0,32	-1,64	3,32E-05
VIT_14s0083g00700	BZIP transcription factor BZIP42	BZIP42	AT3G30530	822,8E-30	171,2	320,69	21,7	0,07	-3,89	1,59E-30
VIT_15s0048g02660	NAC Secondary wall thickening promoting factor1	NST1_1	AT2G46770	264,1E-7	21,01	35,16	6,86	0,2	-2,36	1,33E-06
VIT_15s0048g02820	Basic helix-loop-helix (bHLH) family	BHLHDOM_3	AT4G00870	143,5E-5	12,8	21,24	4,36	0,21	-2,29	1,18E-04
VIT_16s0013g00890	Ethylene-responsive element binding factor	ERF1A	AT2G44840	373,4E-10	239,65	369,17	110,13	0,3	-1,75	9,67E-10
VIT_17s0000g06200	Mini zinc finger 1 MIF1	MIF1_2	AT1G74660	164,3E-9	380,48	562,93	198,02	0,35	-1,51	4,81E-09
VIT_18s0001g02540	ARR9 typeA	ARR9_4	AT3G57040	3,64E-12	323,72	564,6	82,84	0,15	-2,77	4,88E-14
VIT_18s0001g06430	Homeobox-leucine zipper protein ATHB-6	ATHB6_2	AT2G22430	252,7E-16	356,19	604,48	107,9	0,18	-2,49	2,42E-16
VIT_18s0001g08610	AP2-like AINTEGUMENTA-like	ANT_4	AT4G37750	192,9E-13	251,77	384,87	118,67	0,31	-1,7	2,85E-13
VIT_18s0001g10160	Wuschel homeobox 4	WOX4	AT1G46480	724,6E-18	1273,44	2048,41	498,47	0,24	-2,04	5,64E-18
VIT_19s0014g04670	Basic helix-loop-helix (bHLH) family	BHLHDOM_42	AT3G20640	101,4E-5	26,95	41,38	12,52	0,3	-1,72	7,97E-05
VIT_19s0027g01120	Lateral organ boundaries protein 4	LBD4_1	AT1G31320	3,47E-05	467,92	713,58	222,26	0,31	-1,68	1,81E-06
VIT_08s0007g07670	NAC domain containing protein 47	NAC047_1	ND	1,53E-32	626,24	115,32	1137,15	9,86	3,3	2,12E-35
VIT_03s0038g03410	NAC domain containing protein 36	NAC036_2	AT2G17040	3,15E-05	280,46	381,63	179,29	0,47	-1,09	1,62E-06
VIT_19s0014g03290	NAC domain containing protein 19	NAC019_1	AT4G27410	4,11E-24	1789,58	452,11	3127,05	6,92	2,79	1,23E-26
VIT_17s0000g01230	MADS-box protein AGL20	SOC1_2	AT2G45660	3,13E-04	1173,15	1729,18	617,12	0,36	-1,49	2,08E-05
VIT_01s0026g02710	NAC domain-containing protein 29	NAC029	AT1G69490	6,30E-15	4726,68	1396,86	8056,5	5,77	2,53	5,45E-17

Table S4. MACE Results and Annotation of identified TFs in **BI vs BII** Library.

Sequence	Annotation	Symbol	Best arabidopsis match	FDR	baseMean	baseMean BI	baseMean BII	Fold Change	log2FoldChange	pvalue
VIT_18s0001g08610	AP2-like AINTEGUMENTA-like	ANT_4	AT4G37750	1,88E-07	249,72	395,28	104,16	0,26	-1,92	1,79E-09
VIT_18s0001g02540	ARR9 typeA	ARR9_4	AT3G57040	8,75E-15	382,61	659,44	105,78	0,16	-2,64	7,42E-18
VIT_06s0004g03130	Auxin response factor 4	ARF4	AT5G60450	2,61E-06	129,45	209,77	49,13	0,23	-2,09	3,64E-08
VIT_11s0016g03540	Auxin-responsive protein IAA27	IAA27_1	AT4G29080	6,21E-04	881,32	1449,88	312,75	0,22	-2,21	2,44E-05
VIT_11s0052g00100	Basic helix-loop-helix (bHLH) family	BHLHDOM_7	AT5G57150	3,93E-08	83,62	140,58	26,66	0,19	-2,4	2,88E-10
VIT_00s0541g00020	BZIP transcription factor 6	BZIP6_2	AT2G22850.2	8,63E-07	205,12	331,15	79,09	0,24	-2,07	9,47E-09
VIT_14s0083g00700	BZIP transcription factor BZIP42	BZIP42	AT3G30530	1,44E-03	207,29	386,5	28,09	0,07	-3,78	6,65E-05
VIT_01s0010g00930	BZIP transcription factor bZIP58	BZIP58	AT1G13600	8,48E-13	55	104,64	5,35	0,05	-4,29	1,48E-15
VIT_04s0008g03400	DREB sub A-4 of ERF/AP2 transcription factor	TINY2_2	AT5G11590	9,53E-07	99,13	157,03	41,22	0,26	-1,93	1,07E-08
VIT_19s0014g02240	Ethylene responsive element binding factor 4	ERF4_1	ND	6,12E-08	249,16	407,95	90,38	0,22	-2,17	4,75E-10
VIT_16s0013g00890	Ethylene-responsive element binding factor	ERF1A	AT2G44840	3,96E-15	201,63	359,38	43,89	0,12	-3,03	3,18E-18
VIT_04s0008g06000	Ethylene-responsive transcription factor 3	ERF003_2	AT5G25190	2,34E-04	399,44	631,97	166,91	0,26	-1,92	7,57E-06
VIT_10s0071g00870	High mobility group B 6	HMGB6_2	AT5G23420	3,33E-09	74,64	127,13	22,15	0,17	-2,52	1,75E-11
VIT_18s0001g06430	Homeobox-leucine zipper protein ATHB-6	ATHB6_2	AT2G22430	4,53E-13	325,91	542,91	108,91	0,2	-2,32	7,67E-16
VIT_07s0141g00290	IAA16	IAA16_2	AT3G04730	6,19E-06	1019,76	1760,05	279,47	0,16	-2,65	9,86E-08
VIT_09s0002g04080	IAA9	IAA9_2	AT2G22670	1,26E-07	697,35	1274,69	120,02	0,09	-3,41	1,12E-09
VIT_17s0000g01230	MADS-box protein AGL20	SOC1_2	AT2G45660	6,82E-06	1825,79	2926,18	725,4	0,25	-2,01	1,13E-07
VIT_19s0014g03290	NAC domain containing protein 19	NAC019_1	AT4G27410	2,06E-05	2155,88	625,18	3686,59	5,9	2,56	4,09E-07
VIT_03s0038g03410	NAC domain containing protein 36	NAC036_2	AT2G17040	1,31E-08	233,82	370,66	96,99	0,26	-1,93	8,20E-11
VIT_08s0007g07670	NAC domain containing protein 47	NAC047_1	ND	1,50E-03	664,13	147,04	1181,22	8,03	3,01	7,01E-05
VIT_01s0026g02710	NAC domain-containing protein 29	NAC029	AT1G69490	2,20E-05	5151,71	1765,79	8537,63	4,84	2,27	4,49E-07
VIT_02s0012g01040	NAC domain-containing protein 71	NAC071	AT4G17980	4,11E-10	152,25	278,56	25,94	0,09	-3,42	1,63E-12
VIT_07s0031g01840	WRKY DNA-binding protein 13	WRKY13	AT4G39410	1,24E-09	365,31	627,66	102,96	0,16	-2,61	5,52E-12
VIT_04s0008g05760	WRKY DNA-binding protein 18	WRKY18_2	AT4G31800	1,20E-08	429,65	734,45	124,85	0,17	-2,56	7,33E-11
VIT_08s0058g01390	WRKY DNA-binding protein 70	WRKY70_2	AT3G56400	1,01E-09	582,22	959,62	204,83	0,21	-2,23	4,28E-12
VIT_06s0004g06240	Zinc finger (C2H2 type) family	C2H2FAM_31	AT2G29660	2,90E-05	274,51	454,64	94,38	0,21	-2,27	6,27E-07
VIT_07s0129g00030	Short-root transcription factor (SHR)	SGR7_4	AT4G37650	1,84E-02	44,48	67,55	21,41	0,32	-1,66	1,56E-03
VIT_04s0023g01660	Scarecrow-like transcription factor 7	SCL7	AT5G66770	1,06E-01	15,97	23,22	8,72	0,38	-1,41	1,52E-02
VIT_06s0004g02800	Homeodomain-leucine zipper protein Revoluta	REV_2	AT5G60690	6,69E-03	137,82	199,73	75,91	0,38	-1,4	4,41E-04
VIT_04s0023g03680 *	Histidine kinase 1 (AHK1)	ATHK1	AT2G17820	*6,42E-01*	17,96	25,2	10,72	0,43	-1,23	*2,48E-01*
VIT_18s0001g10160	Wuschel homeobox 4	WOX4	AT1G46480	1,82E-05	1666,3	2481,17	851,42	0,34	-1,54	3,53E-07
VIT_19s0027g01120	Lateral organ boundaries protein 4	LBD4_1	AT1G31320	3,07E-05	511,83	739,42	284,23	0,38	-1,38	6,76E-07
VIT_04s0008g00220	auxin-responsive protein IAA16	IAA6	AT3G16500	1,36E-02	14,78	23,27	6,28	0,27	-1,89	1,07E-03
VIT_09s0002g05150	IAA19	IAA19_1	AT3G15540	2,38E-09	26,1	49,53	2,68	0,05	-4,21	1,19E-11
VIT_07s0141g00270	Auxin-induced protein 22D	IAA22D_1	AT5G43700	5,06E-05	20,26	36,73	3,79	0,1	-3,28	1,20E-06
VIT_07s0129g01000	F-box family protein	SLY1	ND	4,94E-05	438,35	640,98	235,73	0,37	-1,44	1,16E-06
VIT_08s0007g04150	RKL1 (Receptor-like kinase 1)	RKL1	AT2G36570	1,00E-02	1004,72	1593,94	415,49	0,26	-1,94	7,30E-04

(*) FDR value>0,5