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Candidate plant gene homologues in grapevine involved in *Agrobacterium* transformation

Research Article

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Abstract: The grapevine (*Vitis vinifera*) genome was analyzed *in silico* for homologues of plant genes involved in *Agrobacterium* transformation in *Arabidopsis thaliana* and *Nicotiana spp*. Grapevine homologues of the glucomannan 4-beta-mannosyltransferase 9 gene *CslA-09* involved in bacterial attachment to the cell wall, homologues of reticulon-like proteins BTI1, 2, 3 and RAB8 GTPases, both involved in T-DNA transfer to the host cell, homologues of VirE2 interacting protein VIP1 that contributes to the targeting of T-DNA into the nucleus and to its integration, and homologues of the histone protein H2A, which promotes the expression of T-DNA encoded genes, were selected. Sequences homologous to the arabinogalactan-protein AtAGP17 were not found in the grape genome. Seventeen selected candidates were tested by semiquantitative RT-PCR analysis for changes in their expression levels upon inoculation with *Agrobacterium tumefaciens* C58. Of the tested homologues, the expression of *VvRab8a*, *VvVip1a* and two histone genes (*VvHta2* and *VvHta10*) increased significantly, therefore we suppose that these might be involved in *Agrobacterium* transformation of *V. vinifera*.

Keywords: *Vitis vinifera* • *Crown gall disease* • *Pathogen-induced gene expression* • *Semiquantitative PCR*

1. Introduction

Crown gall caused by the pathogenic *Agrobacterium* species is a serious disease affecting several crop plants including fruit trees, berries, ornamental plants and grapevines. However, genetic transformation of crop plants by non-tumorigenic ("disarmed") *Agrobacterium tumefaciens* strains has became a widely used method to introduce foreign genes into plants to improve agronomical traits [1,2].

Tumorigenic agrobacteria harbour a large plasmid called tumor inducing plasmid (pTi) that carries the genes which are essential for crown gall tumor induction. During the transformation process a region of this plasmid, the transferred DNA (T-DNA) is transported into the host cell and it becomes stably integrated into the chromosomal DNA. The T-DNA transport through a type IV secretion system [3] from the prokaryotic bacterium into the eukaryotic plant cell is determined by the virulence (*vir*) genes located also on the pTi, but outside the T-DNA. The T-DNA is transported in a single-stranded form (T-strand), and this process is directed by the VirD2 protein that covalently binds to the 5' end of the T-strand [2]. Additionally, for T-DNA import the VirE2 protein forms an anion selective channel on the plasma membrane of the plant cells [4] and binds to the VirD2/T-DNA during the transport process. The VirD2/VirE2/T-strand called T-complex is targeted to the nucleus of

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the plant cell. Other bacterial virulence proteins (e. g., VirE3, VirF) are also transported into the plant cells and they are involved in the integration of T-DNA [5,6].

While the introduction and nuclear targeting of T-DNA is mediated by bacterial virulence proteins, several plant proteins contribute to its integration into the plant chromosome [5,7-13]. Methods applied to identify plant genes (proteins) involved in crown gall tumorigenesis and *Agrobacterium*-mediated plant transformation include T-DNA tagged mutagenesis of *Arabidopsis thaliana* [14], virus-induced gene silencing in tobacco [7], yeast two-hybrid system [15,16] and differential gene expression studies on *A. thaliana*, tobacco or *Ageratum conyzoides* plants [17-20]. These studies identified approximately 40 plant genes (proteins) which are involved in *Agrobacterium*-mediated transformation including, for example, the Arabinogalactan-Protein AtAGP17, the Reticulon domain proteins (BTI1-3), the VirE2 interacting proteins (Vip1 and Vip2), importins, histones and several other factors involved in the ubiquitin-proteasome complex [reviewed in 10,11]. Inactivation of such genes by insertion mutagenesis or gene silencing resulted in an attenuated tumor phenotype or even resistance to *Agrobacterium* transformation [7,8,14-16,21-23], while their overexpression in transgenic plants increased their *Agrobacterium* sensitivity [15,24,25]. Although certain contributing genes, e. g. VIP1 and CSLA9, seem to be expressed constitutively [16,21], *Agrobacterium*-infection induced the expression of a complete set of host genes involved in the transformation process [20]. For example, the histone H2A-1 gene [26], the F-box protein genes VBF [23], SKP1 and SGT-1 [8] showed elevated levels of expression following inoculation with virulent or transformation competent agrobacteria. Manipulation of these contributing genes may extend the host range of *Agrobacterium* or result in resistance to crown gall disease [1,11].

T-DNA transfer, integration and expression takes place within a relatively short period of time. For example, in tobacco cells, *gusA* transcripts could be detected 24 hrs after cocultivation [27]. In *Kalanchoe daigremontiana,* lysopine dehydrogenase (LpDH, a T-DNA encoded enzyme responsible for octopine synthesis) activity was detected 36 hrs after infection [28].

Genes involved in *Agrobacterium* transformation in grapevine (*Vitis vinifera*) have not been investigated yet. The proteomic analysis of grapevine embryogenic calli following cocultivation with agrobacteria showed that 69 of the identified 1100 proteins expressed differentially [29]. Their potential role in *Agrobacterium*-transformation was not discussed, although due to the relatively long (3 days) cocultivation period, the contributing genes might have not been expressed anymore. Expressed sequence tag analysis in *Agrobacterium*-inoculated and salicylic acid-treated 'Tanmara' grapevine focused on defence related genes that may contribute to disease resistance [30].

The objective of this study was to identify the grapevine gene homologues potentially contributing to the agrobacterial transformation and to monitor the changes in their expression following cocultivation of grapevine leaf tissues with *Agrobacterium* cells. Based on the functional evidences from the literature, six genes were considered for detailed analysis. *Arabidopsis* Lysine-Rich Arabinogalactan-Protein AtAGP17 [22] and an *Arabidopsis* glucomannan 4-beta-mannosyltransferase 9 gene AtCslA-09 [21] are both involved in the bacterial attachment to the plant cell wall. After attaching to the plant cell, the next important step of the infection is the transfer of the T-DNA into the host cell. Reticulon domain proteins BTI1 (AtRTNLB1), BTI2 (AtRTNLB2), BTI3 (AtRTNLB4), further RAB8 GTPases (AtRab8) are involved in this step from the plant side [15]. Both gene families have a high number of members in *A. thaliana*, 21 and 57 for AtRtnls [31] and AtGTPases [32], respectively. VirE2 interacting proteins (VIP1 and VIP2) play an important role in the infection process [33], especially VIP1, which promotes the targeting and entering of the T-DNA complex to the nucleus. VIP1 is also involved in the integration of the T-DNA into the host genome. The last step of the pathogenesis process is the expression of the transgenes. It has been shown that the H2A histone AtRat5 is involved in this step [24].

2. Experimental Procedures

2.1 Selection of candidate genes for screening, primer design

Candidate genes involved in different phases of the transformation process, such as bacterial attachment, T-DNA insertion, targeting and entering the nucleus and integration of the T-DNA into the host genome were selected from the literature [10,15,21]. Housekeeping genes (e. g., actin, histone H3, see ref. 7) which are essential for normal plant growth and development were not involved in our further studies. Amino acid sequences of the selected *A. thaliana* genes were received from the NCBI RefSeq database and homology searches were carried out using tblastn [34] against the blast database of the 12X version of structural annotated coding sequences (CDS) of the homozygous 'Pinot noir' genome [35]. Multiple alignment and basic phylogenetic analysis of the *A. thaliana* reference sequence and the

amino acid sequences of the best Blast results were carried out for each candidate using ClustalW [36]. Based on the topology of the phylogenetic tree, 2 to 5 gene family members were selected for each candidate for expression analysis.

Primers specific to the members of these gene families, that code the most similar grape peptides were designed using primer3 [37]. To ensure highest possible specificity of the primer pairs, mispriming libraries consisting of all further gene family members were defined during primer design. Primer design was verified in standard PCR reactions using DNA of the interspecific rootstock cultivar 'Richter 110' as template, except for primers incorporating intron-exon junction points or regions including large introns (data not shown). Primers used in sqPCR are listed in Table 1.

A. thaliana proteina	Role in the transformation	Grapevine CDSb	Grapevine genes	5'-3' primer sequencesc	PCR product length (bp)
AtCSLA-09 (NP195996) [21]	attachment to plant cell	01034719001	*VvCslA02a*	CGTCCGTGCTAGTCTCAAGG CTGGGATCAAAATGGTGAGG	290
		01033767001	*VvCslA02b*	CATCGAGCGGGTTTATATGG ACTCCAACTCGACCATCACC	278
		01031405001	*VvCslA09a*	CCCTGCACCGAACTAAGG AACAAAGGTGCCAACATAACC	299
		01025737001	*VvCslA09b*	GGTTCCTGATGTTGAGGTTCC TTTCTTGGGTGCCTTAGTGG	256
AtRAB8 (AAB65088) [15]	T-DNA transfer to plant cell	01016596001	*VvRab8a*	TGGCAAACGGATCAAATTAC AGAGCTTGACCCTTGGAGGT	261
		01025028001	*VvRab8b*	TCAGATGGGTCCTTCACCAC CAGCCTTGTTCCCTACCAGT	286
		01025619001	*VvRab8c*	GTGGGGCCATGGGTATTT TCAGTTTCTGCAAGCCTCTG	298
AtRTNLB 1/2/4 [15] (NP194094, NP192861, NP198975)		01019440001	*VvRtnl1*	ATTCCGACAACGAGAAATCG TATTGCCAGTGCGAGTATGG	245
		01027008001	*VvRtnl2*	CCGCATCTGAATCCTTGC AAACAAGGGTGAGGAGATGG	291
AtVIP1 [33] (NP564486)	T-DNA transfer to the nucleus, integration to plant genome	01034897001	*VvVip1a*	CGTCGTTTGAGGTTGAATCG GCAACCGTAGTTTGAGTTCC	291
		01032683001	*VvVip1b*	TGCATACAATTTGGGAATGC GCTTTCACTGGCAGAAATGG	184
		01024562001	*VvVip1c*	GTCATTTGACCTGGGAATGC AAAACGTGCTGCTACTCTCG	293
		01024160001	*VvVip1d*	TTGTTGACGATTTGCTCTCG AATTTTGGCGACTCATCACC	289
		01009846001	*VvVip1e*	CGCATTAGTAGACCCCAAGC TGGACTTCCAGGGTTAATGC	279
		01010836001	*VvVip1f*	ACTGGCGCACAATTTTATCC TGGTGTCTGATGCATTTTCC	262
AtH2A [24] (NP200275)	T-DNA insertion to plant genome	01014580001	*VvHta2*	GCCAAGAAGGCTACATCTCG TTGCGATCGTCACATCTCC	280
		01002735001	*VvHta10*	TGGAAGAGGAAAATCGTTGG CACCGCCAGTTGAATATGG	262

Table 1. Summary of grape coding sequences involved in the study. Names of the *Arabidopsis thaliana* reference genes and their roles in *Agrobacterium* infection are listed together with the grape homologues and the designed member-specific primers for sqPCR.

[a] *RefSeq accession numbers and references discussing the biological function of the protein are indicated*
[b] *Identifier of the CDS in the 12X 'Pinot noir' genome annotation [35]. Eleven digit identifiers refer to annotated transcripts (GSVIVT[Grapevine CDS]) at http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/.*
[c] *Sequence of the Forward primer is given in the upper, the Reverse primer in the lower row*

2.2 *Agrobacterium tumefaciens* strain and culture conditions

Cultures of *A. tumefaciens* C58 strain used for the experiments were grown overnight at 28°C in liquid AB medium [38] supplemented with 0.5% (w/v) glucose and 0.25% (w/v) yeast extract. The bacterial cells were collected by centrifugation at 5,000 rpm for 10 mins, and resuspended in Tris-buffered saline (TBS) buffer (10 mM TRIS base, 150 mM NaCl, pH=6). The cell concentration was adjusted to 10^9 cell/ml (OD_{600}=1.0).

2.3 Plant material, RNA extraction and cDNA synthesis

Leaf discs were cut from *in vitro* grown *V. vinifera* cv. 'Sauvignon blanc' plants and vacuum infiltrated with *A. tumefaciens* C58 cells suspended in TBS buffer. The leaf discs infiltrated with the bacterial suspension were incubated on sterile tapwater-agar plates at 23°C for 36 hours (Agro), which was found appropriate in similar studies in previous works [8,20,28]. Control experiments were carried out using TBS buffer without bacteria under the same conditions (Mock).

RNA was extracted from the leaf discs with PureLink plant RNA reagent (Invitrogen, LifeTechnologies) according to the instructions of the manufacturer. The extracted RNA was purified from genomic DNA by using Turbo DNA-free Kit (Ambion, Life Technologies) and the final RNA concentration was adjusted to 100 ng/µl. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies), in 20 µl reaction volume from 600 ng of total RNA, with random hexamer primers.

2.4 Semiquantitative PCR (sqPCR) and quantitation methods

Semi-quantitative PCR following reverse transcription (RT-sqPCR) was carried out in 20 µl reaction volume with 1x *Taq* buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of each dNTP, 0.4 U of *Taq* polymerase (Fermentas, Thermo Scientific) and 2 µl of cDNA. The sqPCR cycling conditions were: 94°C for 2 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, for 35 cycles. The PCR products were analyzed after agarose gel electrophoresis and ethidium bromide staining. The fluorescence intensity of Agro and Mock sample pairs binding ethidium-bromide was estimated by the ImageJ software [39]. Changes of expression levels of the investigated genes were calculated from the fluorescence intensity ratio of Agro/Mock sample pairs. The errors arising from sample preparation were corrected by normalization of target PCR product fluorescences to that of parallel ß-actin [40] samples used as a reference gene. At least 3 biological replicates were analyzed for all candidates. Changes in the level of gene expression upon *Agrobacterium* infection were statistically tested using the non parametric Kruskal-Wallis one-way analysis of variance by ranks.

3. Results

3.1 Identification of genes of interest

The first two investigated gene families are involved in attachment to the plant cell wall. *Arabidopsis* Lysine-Rich Arabinogalactan-Protein does not have any homologous sequences in the grape genome; as such AtAGP17 was excluded from further analysis. For glucomannan 4-beta-mannosyltransferase 9, phylogenetic analysis of 9 grape homologues of *AtCslA-09* and the *A. thaliana* members of the *CslA* and *CslC* families (Figure 1a) identified two grape coding sequences, which belong to the same clade as *AtCslA-09* and two further genes highly similar to *AtCslA-02* (Table 1). Four *AtCslA-09* homologues (*VvCslA-02a*, *VvCsalA-02b*, *VvCslA-09a* and *VvCslA-09b*) were selected for expression analysis.

The second group of plant genes contribute to the transfer of T-DNA to the plant cell. Based on the phylogenetic analysis of the *A. thaliana* family members of Reticulon domain proteins (AtRTNLB1, AtRTNLB2 and AtRTNLB4) and predicted grape coding sequences with high homology to the reference genes (data not shown), we selected 2 reticulon-like genes from grapevine for further testing (Table 1). Similarly, we compared AtRAB8 and homologous grape sequences and selected 3 Rab GTPases from grapevine for expression analysis (Table 1).

VIP1 is a bzip2 domain transcription factor involved in a broad range of the *Agrobacterium* infection steps. We identified 22 homologous coding sequences in the grape genome. Six of them (Table 1) were selected for expression analysis based on the phylogenetic tree drawn from AtVIP1 and the homologous grape amino acid sequences (data not shown).

Histone proteins are involved in T-DNA integration into the host genome and transgene expression. Phylogenetic analysis of 9 *Arabidopsis* H2A family members and 16 highly similar grapevine sequences (Figure 1b) identified two grapevine coding sequences which are possibly related to AtRAT5 (Table 1), which is known to be involved in the pathogenesis process.

3.2 Grapevine genes showing elevated expression after *Agrobacterium* inoculation

Although grapevines are mainly infected by various *Agrobacterium vitis* strains under natural conditions [2], we found that strains of this species cannot transform isolated plant parts (leaf discs, stem segments) *in vitro*.

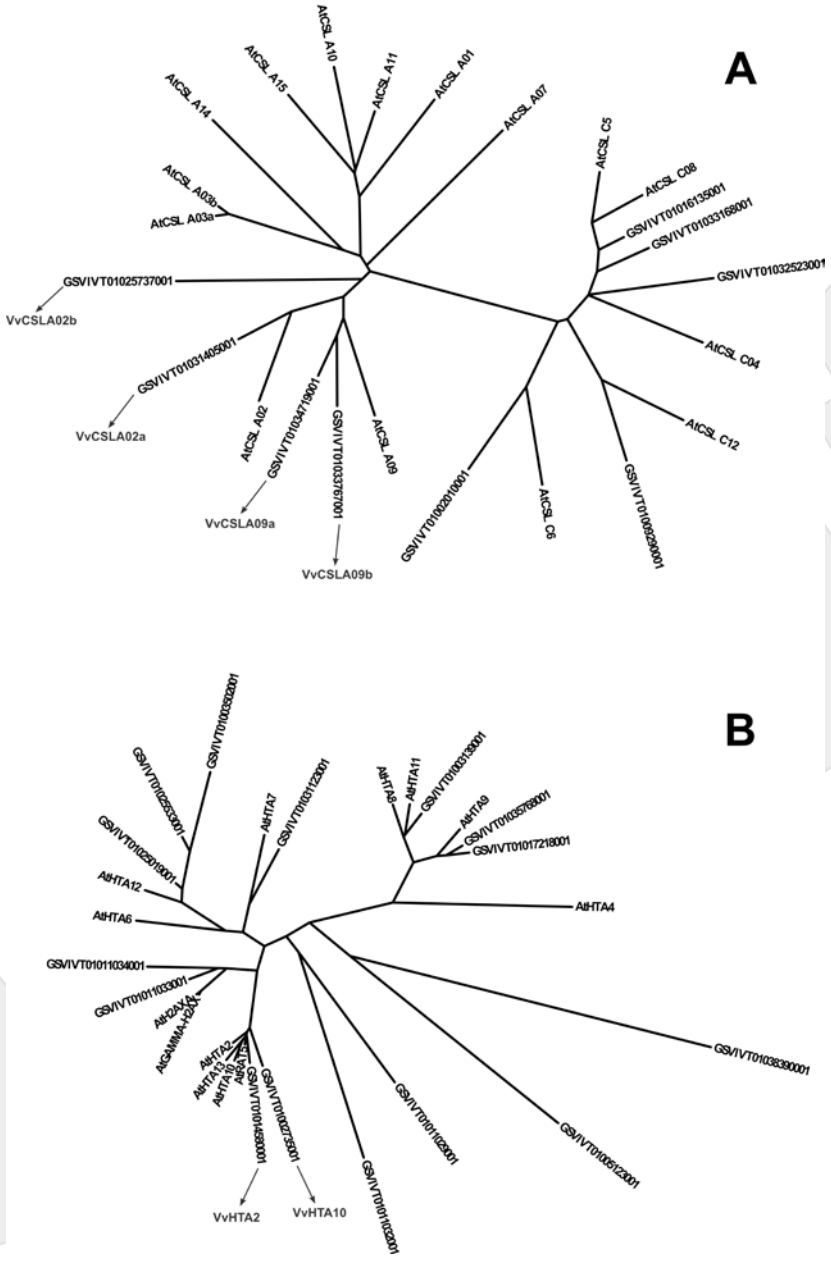

Figure 1. Unrooted neighbor-joining trees of two analyzed gene families. (A) For glucomannan 4-beta-mannosyltransferase 9 and related sequences, amino acid sequences of the CSL-A and CSL-C families from *A. thaliana* (AtCSL-A09 contributing to the *Agrobacterium*-mediated genetic transformation) and homologous grape sequences were included. VvCSLA09a, VvCSLA09b, VvCSLA02a and VvCSLA02b were selected for expression analysis. (B) *A. thaliana* H2A histones (AtRAT5 responsible for transgene expression) and peptide products of predicted grape coding sequences similar to AtH2A were included in the analysis. VvHta2 and VvHta10 were selected for expression analysis. Grapevine CDS identifiers are indicated according to the 12X 'Pinot noir' genome annotation [35] (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/).

Thus we chose *A. tumefaciens* C58 strain, which was found tumorigenic on several *V. vinifera* varieties in the greenhouse and in *in vitro* leaf disc and stem segment transformation systems as well (our unpublished data).

During the transformation process, virulent agrobacteria up-regulate the expression of several genes that contribute to crown gall tumorigenesis [8,20,23,26]. Based on these observations, we have tested 17 candidate members of five grapevine gene families by semiquantitative PCR to get data on their expression profile upon *Agrobacterium* infection. To this end, specific oligonucleotide sequences (Table 1) were designed for the analysis of candidate genes that may be involved in the *Agrobacterium*-plant interaction.

We did not detect any expression of the chosen homologues of glucomannan 4-beta-mannosyltransferase 9 gene (*CslA-02a/b*; *CslA-09a/b*) either in infected or in mock inoculated grapevine leaf discs. Selected Reticulon-like and *Rab8* genes – both families are involved in the T-DNA transfer into the plant cell – were expressed in infected and also in mock inoculated samples. No significant differences were found between treated and untreated samples of these genes (Figure 2a). In the case of *Rab8a,* the gene expression level of *Agrobacterium*-infected samples was significantly higher than that of mock inoculated

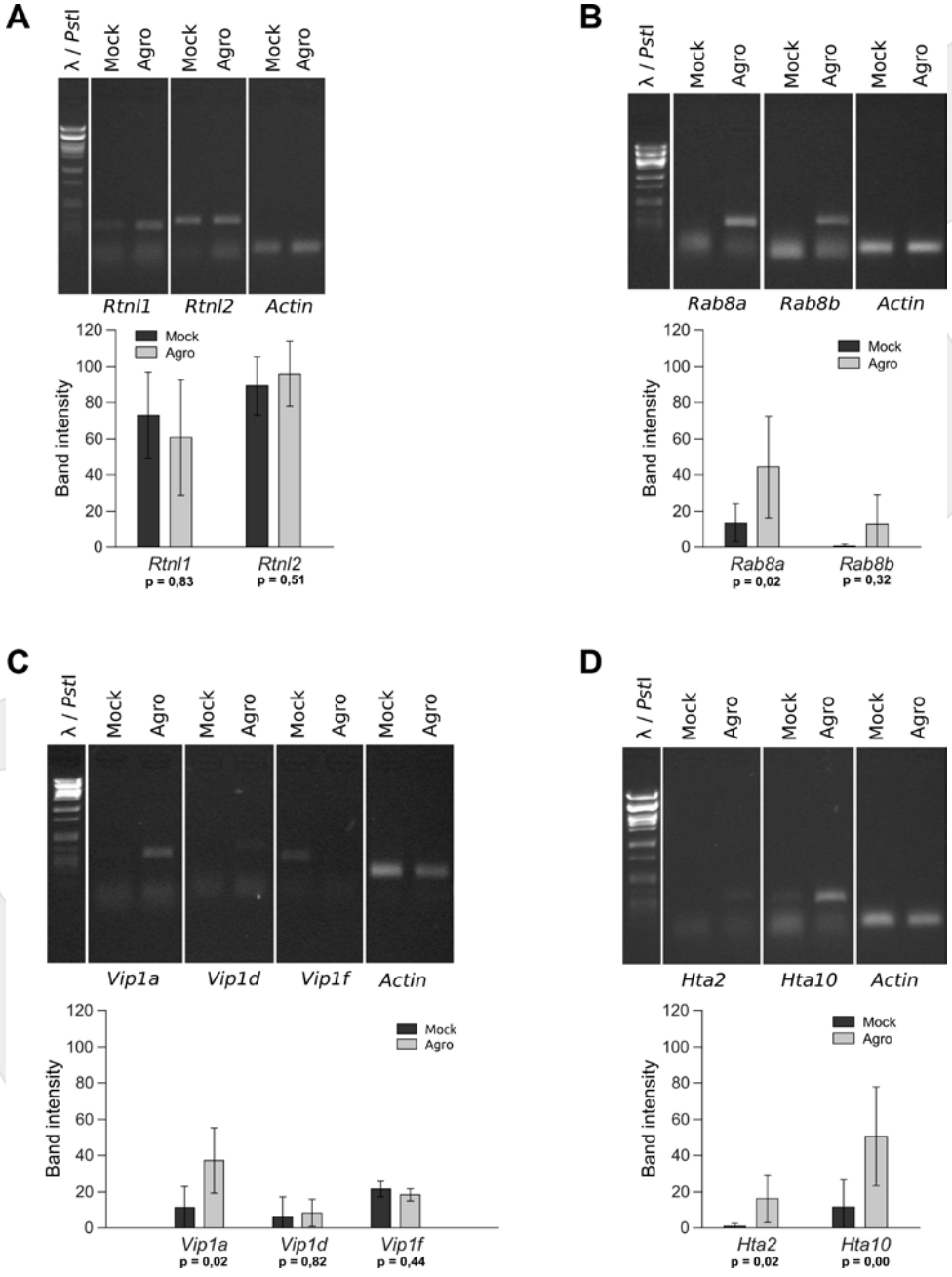

Figure 2. Changes in the expression of the candidate grapevine genes homologous to (A) Reticulon-like proteins *AtRtnl1/2/4*, (B) Rab8 GTPase *AtRab8*, (C) Vire2 Interacting protein *AtVip1* and (D) H2A histone *AtRat5*. Expression level of each candidate was compared between mock-inoculated control samples (Mock) and leaf disks infiltrated with *Agrobacterium tumefaciens* (Agro). Band intensity was normalized to the expression level of the *ß*-actin gene. Error bars represent standard deviation of biological replicates. P values of the Kruskal-Wallis one-way analysis of variance by ranks between mock and *Agrobacterium* inoculation for the expression levels of each genes are indicated.

samples (Figure 2b). Although *Rab8b* also seemed to be expressed at a higher level in treated samples, the difference was not statistically significant. Similar to *CslA* genes, expression of *VvRab8c* was not detected in the grapevine samples.

Similar differences, but at lower expression levels appeared in some members of the *Vip1* gene family. In the case of the *VvVip1a* and *VvVip1d* genes, the treatment with *Agrobacterium* resulted in the increase of gene expression. For the *Vip1f* gene an opposite effect was observed, with a weak decrease in gene expression. However, only up-regulation of *VvVip1a* proved to be statistically significant (Figure 2c). Other investigated members of the gene family (*VvVip1b/c/e*) did not show any product after sqPCR. *VvHta2* and *VvHta10* genes were both expressed in infected and mock inoculated samples, but *Agrobacterium* infection significantly increased their expression level (Figure 2d).

Altogether, 9 of the 17 investigated genes showed expression in mock or *Agrobacterium* inoculated leaf disk samples after 36 hours. For four genes (VvRab8a, VvVip1a, VvHta2, VvHta10) we were able to detect significant increase of the expression level after cocultivation with *Agrobacterium tumefaciens*.

4. Discussion

Crown gall disease induced by tumorigenic agrobacteria causes serious economic losses in several crop plants, including grapevines. The tumor formation is a rather complex process that includes the transfer of a well defined DNA segment, called T-DNA, from the prokaryote bacterium into the chromosome of the eukaryote host, its stable integration into the nuclear DNA and expression in the plant cell [2]. While the DNA transport and its nuclear targeting is mediated by bacterial virulence genes, several plant proteins contribute to the integration of T-DNA into the host genome [8,10,11]. Therefore, blocking the expression of contributing plant genes may result in resistance to *Agrobacterium* transformation [1,8,11,15,16], providing a potential strategy to engineer disease resistant crop plants.

An extensive *in silico* analysis of the grapevine genome to identify gene homologues for previously characterized contributing genes [10] was carried out. Most of these genes are members of multigenic families, thus selecting the best candidates is rather difficult. The approach of phylogenetic analysis of the homologues offers a viable solution. However, even if candidate genes were carefully selected, there is no guarantee that the process of *Agrobacterium*-mediated transformation involves the same plant genes in the model organisms and in grapevine. To further narrow down the number of candidates, expression profiles of the selected genes were analyzed.

In order to collect preliminary information about changes of expression of these genes upon *Agrobacterium* infection, leaf discs were inoculated with *A. tumefaciens* C58 and the RNA fraction was analysed by semiquantitative PCR. Four (*VvRab8a GTPase*, *VvVIP1a* and the histone genes *VvHta2* and *VvHta10*) of the tested 17 homologues showed an elevated expression following cocultivation of grapevine leaf discs with *Agrobacterium*. For the candidates involved in the early step of the transformation process (bacterial attachment and transfer of the T-DNA), we did not find any changes in the expression levels between *Agrobacterium*-treated and mock-inoculated leaf discs (except of *VvRab8a*). This may be the result of the relatively long cocultivation time. By the time of the RNA-extraction (after 36 hours cocultivation), the expression level of the genes involved in the first steps of *Agrobacterium* transformation were most probably already restored.

Genes showing elevated expression level after infection with *Agrobacterium tumefaciens* might be involved in crown gall tumorigenesis on grapevines. Further studies involving specific silencing of these genes are in progress to determine their physiological roles of in grapevine-*Agrobacterium* interaction to manipulate disease resistance.

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