Expression of putative expansin genes in phylloxera (*Daktulosphaira vitifoliae* Fitch) induced root galls of *Vitis* spp.

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Abstract Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is a serious global pest in viticulture. The insects are sedentary feeders and require a gall to feed and reproduce. The insects induce their feeding site within the meristematic zone of the root tip, where they stay attached, feeding both intra- and intercellularly, and causing damage by reducing plant vigour. Several changes in cell structure and composition, including increased cell division and tissue swelling close to the feeding site, cause an organoid gall called a nodosity to develop. Because alpha expansin genes are involved in cell enlargement and cell wall loosening in many plant tissues it may be anticipated that they are also involved in nodosity formation. To identify expansin genes in *Vitis vinifera* cv. *Pinot noir*, we mined for orthologues genes in a comparative analysis. Eleven putative expansin genes were identified and shown to be present in the rootstock Teleki 5C (*V. berlandieri* Planch. x *V. riparia* Michx.) using specific PCR followed by DNA sequencing. Expression analysis of young and mature nodosities and uninfested root tips were conducted via quantitative real time PCR (qRT-PCR). Up-regulation was measured for three putative expansin genes (VvEXPA15, -A17 and partly -A20) or down-regulation for three other putative genes (VvEXPA7, -A12, -A20) in nodosities. The present study clearly shows the involvement of putative expansin genes in the phylloxera–root interaction.

Keywords phylloxera · *Vitis berlandieri* × *V. riparia* · *V. vinifera* · qRT-PCR · root · nodosity · gall

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Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) (Homoptera: Phylloxeridae) cause substantial damage to susceptible varieties of *Vitis* spp. by organoid root-gall formation, known as nodosities and/or tuberosities. The insects induce a feeding site within the meristematic zone of the root tip, where they stay attached to the root, feeding both inter- and intracellularly (Hofmann 1957) and inducing changes in the uptake and transportation of water, minerals and assimilates (Porten and Huber 2003). Damage is caused by reducing plant vigour, although secondary soil-borne infections by saprophytes are also thought to cause substantial harm to

plants so attacked (Hoffmann et al. 2011). The majority of commercial rootstocks employed in viticulture tolerate phylloxera populations on nodosities; however, due to changed environmental conditions and extensive vineyard management, increasing phylloxera densities are seen to cause grapevine stress in viticulture regions worldwide (Kocsis et al. 1999; Granett et al. 2001; Forneck et al. 2001a). Nodosity induction is thought to be triggered by the phylloxera's saliva (Miles 1968) which induces, besides several changes in cell structure (Niklowitz 1955), composition (Steffan and Rilling 1981; Schaefer 1985) and volatile metabolomics (Lawo et al. 2011a), an increased cell growth resulting in hook-shaped tissue swelling close to the feeding site (Forneck et al. 2002; Kellow et al. 2004).

Involvement of expansin genes in cell enlargement and cell wall loosening via non-enzymatic mechanisms has been reported in many plant tissues, for example, during fruit softening, pollen tube invasion, root hair growth and organ abscission (Cosgrove 2000). Expansin genes are also involved in nematode feeding site formation upon cyst (*Globodera* spp., *Heterodera* spp.) and root-knot nematode (*Meloidogyne* spp.) attack in tomato (Gal et al. 2006; Griesser and Grundler 2008), *Arabidopsis thaliana* (Jammes et al. 2005; Wieczorek et al. 2006), soybean (Ibrahim et al. 2011) and peanut (Tirumalaraju et al. 2011). In tomato the expansin genes LeEXPA2, -A5 and -A11 were up-regulated upon cyst nematode attack (Griesser and Grundler 2008), whereas in *A. thaliana*, up-regulation of expansin genes was observed for AtEXPA1, -A15 and -B3 genes upon root-knot nematode attack (Jammes et al. 2005) and for AtEXPA1, -A3, -A6, -A8, -A10, -A16 and -B3 genes upon cyst nematode attack (Wieczorek et al. 2006). Down-regulation of expansin genes expression has also been shown for AtEXPA7 and -A18 genes in the feeding structure (syncytia) of cyst nematodes (Wieczorek et al. 2006). Two major gene families of expansin genes are described in the literature: the α-expansins (EXPA) and the β-expansins (EXPB) (Kende et al. 2004). For *A. thaliana*, 26 EXPA, five EXPB and four expansin-like proteins have now been characterized (Sampedro and Cosgrove 2005).

For *Vitis* spp., no extended analysis of expansin gene families has been conducted to date. Studies only refer to expansin genes in *Vitis* spp. during grape berry development (Deluc et al. 2007; Ishimaru et al. 2007; Schlosser et al. 2008) or during the ripening phase (Chervin et al. 2008). Knowledge on the regulation

of expansin genes is still scarce, but in many cases expression is regulated by plant hormones (e.g. auxins) or abiotic stress (e.g. Bray 2004; Cosgrove 2005; Lee et al. 2005). It has been recently demonstrated that nematodes secrete proteins similar to plant expansin gene products (Wieczorek et al. 2006) and that the saliva of aphids contain proteins (enzymes) able to break down cell walls (Carolan et al. 2011). Whilst phylloxera saliva studies have not yet been performed, aphid secretions do nevertheless contain expansin-like and other cell-wall-loosening proteins that may complement the establishment of the feeding site developed by the host plant.

In the present study, we investigated whether the expression of putative expansin genes are specifically regulated during nodosity formation in roots of a commercial rootstock Teleki 5C (*V. berlandieri* Planch. x *V. riparia* Michx.) and thus are an essential part of the herbivore–plant interaction. To illustrate the role of expansin genes in this interaction, the following steps were performed: (1) in silico analysis to identify putative expansin genes in *Vitis vinifera* cv. Pinot; (2) similarity studies to analyze candidate expansin gene sequences of *V. vinifera* cv. Pinot noir and the rootstock Teleki 5C (*V. berlandieri* x *V. riparia*); and (3), quantitative real-time PCR (qRT-PCR) studies to gain detailed understanding of the expression of candidate expansin genes during nodosity formation.

Material and methods

Biological material

Phylloxera (Daktulosphaira vitifoliae)

Insects were collected in Grosshoeflein, Austria in 2007. A single asexually reproducing founder phylloxera lineage was established and subsequently maintained in a greenhouse without further addition of field-collected material. The line was reared on leaves and roots of caged potted plants of the rootstock Teleki 5C (*V. berlandieri* Planch. x *V. riparia* Michx.) and confirmed through microsatellite genotyping according to Vorwerk and Forneck (2006) and Vorwerk et al. (2007) (data not shown). Plants were watered and fertilized [5 ml/3 L; FERTY Spezial Mega (PLANTAN, Germany)] as required.

Root tissue collection

Young nodosities [infested by one 3rd nymphal stage phylloxera using the nomenclature for phylloxera as suggested by Forneck and Huber (2009)] were collected from greenhouse cultures during June 2009 to verify the expression of putative expansin genes in the rootstock Teleki 5C (for details see Lawo et al. 2011b).

For quantitative real-time (qRT-) PCR studies, young nodosities (infested by one 2nd or 3rd nymphal stage phylloxera; Ny), mature nodosities (infested by one adult phylloxera producing eggs; Nm) and uninfested root tips (length *ca.* 1.5 cm) from uninfested plants were collected from the rootstock Teleki 5C from June–October 2010 produced from several independent inoculations of the same asexual phylloxera lineage in the greenhouse. A rearing system was performed according to Lawo et al. (2011b). Prior to freezing, the galls were separated into the basipetal body of the gall/root and the apical tip. Samples were stored at −80 °C until extraction of RNA.

Identification and verification of putative expansin genes

According to the current scientific literature, four expansin genes have been identified in *Vitis* spp. [Vexp-1 (*Q84UT0*), Vexp-2 (*Q84US9*), Vexp-3 (*Q84US8*), Vexp-4 (*Q84US7*)], whilst a fifth gene is essentially similar to an expansin gene [tam_exps (*E3WHD4*)]. A BLAST search with the above gene sequences revealed three different predicted expansin genes from *A. thaliana*, namely AtEXPA2, -A8 and -A15 (score: 394–332; E-value: $1e^{-91}$–$3e^{-93}$) (data not shown). To identify further putative expansin genes in *Vitis* spp., the amino acid sequences of expansin genes from *A. thaliana* were taken as a template (Cosgrove 2006) and BLAST search within the *V. vinifera* genome database Genoscope performed (Jaillon et al. 2007). Orthologous genes for AtEXPA4, -A7, -A8, -A11, -A12, -A15, -A17, -A20, -A23, -B2 and -B3 were identified. A phylogenetic tree produced in order to give an overview of the genetic relationship among the genes identified is given in the supplemental material (Supplementary Figure 1). For the verification of the expression of identified putative expansin genes in the rootstock Teleki 5C, a specific polymerase chain reaction (PCR) followed by sequencing (Agowa, Berlin, Germany) was performed. DNA from *V. vinifera Pinot noir Clone 18Gm* served as a control to ensure primer binding.

Gene expression analyzes

Total RNA was extracted from individual samples of young or mature nodosity bodies or uninfested root tips and from samples of mature nodosity apex vs. root apex, using a modified RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as described in Lawo et al. (2011b), including an additional step for DNA digestion (RNase-Free DNase Set, Qiagen, Germany). Each individual sample included between 16 and 24 tissue pieces. Concentration and integrity of the isolated RNA was determined using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, USA). Samples with a 260/230 ratio below 1.4 were checked on a gel to exclude any RNA degradation. A subsequent reverse transcription was performed using a QuantiTec Reverse Transcription kit (Qiagen, Hilden, Germany). 500 ng RNA was used for reverse transcription and qRT-PCR reactions were performed using the Rotorgene cycler (Qiagen, Hilden, Germany) employing KAPA SYBR FAST qPCR Universal (Peqlab, Erlangen, Germany) as a detector agent. All samples were pipetted in duplicates, whilst all primers were tested for their efficiency prior to analysis by conducting standard curves for four step template dilutions. Efficiencies were calculated using the formula: $E = \left[10^{(-1/\text{slope})}\right] - 1$. Primers with efficiencies $\ll 87$ % were discarded for further studies. A concentration of 200 nM was used for all primers (Table 1). Cycling conditions were: one cycle for 4 min at 95 °C, 35 cycles for 5 s at 95 °C, 20 s at 60 °C, 5 s at 72 °C and 10 s at 75 °C, where the fluorescence signal was measured. Dissociation curves were performed with continuous fluorescence acquisition between 70 °C to 95 °C. Blanks were accomplished for each primer pair per run. Quantitative fold changes (FC) were calculated using the $2^{-\text{ddCt}}$ formula (Livak and Schmittgen 2001). Thus a FC of 1.0 indicates no gene regulation, whether a FC of two indicates a two-fold expression and one of 0.5 half of the gene expression. To determine putative expansin gene expression, three to four independent samples were analyzed as biological replicates. The relative expression (fold change) of putative expansin genes in nodosities compared to uninfested root tips was normalized to the expression of two reference genes as suggested by Pfaffl (2001) [actin (GSVIVT00034893001) and ubiquitin 1 (GSVIVT00037199001)] chosen in preliminary studies (data not shown). Pretests were performed with two (mature nodosities and uninfested root tips) or one

Table 1 Primer used for reference genes [actin and ubiquitin 1 (UBI1)] and putative expansin identification in qRT-PCR studies, their efficiency and RNA input

[a]Gene of interest	Forward 5′–3′	Reverse 5′–3′	Product size (bp)	RNA input (ng/μl)	[b]effi-ciency (%)
Ubiquitin 1	TTGATGCAATTGGCTAGGAA	TGTAACACTGCATGCACCAA	185	14 or 28	87
Actin	TGTGCTTAGTGGTGGGTCAA	ATCTGCTGGAAGGTGCTGAG	174	14 or 28	93
VvEXPA4	CAATCGCTGTCGTTCAGAGT	TATAAAGGCGGGAAAAATTCG	164	14	89
VvEXPA7	TAACGGTTACGGGACAGACA	GCACTTGACTGAACACACTTGA	102	14	92
VvEXPA8	ATCCATTGTTTGGGAGAAGG	CTATCAACAAAAGGCGACCA	178	14	88
VvEXPA11	TTTTGCCCTCCAAACTATGC	CGATCCCGCCTCTGTAAATA	121	14	91
VvEXPA12	ACACTGGAATTCTATAACGTCGTG	AGTGAACTGATTCTCTGAAGCAAA	75	28	96
VvEXPA15	GGAGGCATCAGGTTTACCAT	CCAGTCCTTGACCCTTTGAT	113	14	91
VvEXPA17	GCGCTGAGCACGGCTTTGTT	GAGGATTGCACCACCCGCCA	181	14	100
VvEXPA20	GCTGGACTCAGTACCATGTTGT	GGCTTCGGACATCTCAAAAT	210	14	91
VvEXPB2	GAGTCTACTCATTTTGACCTAAGTGG	TACTCGCACTCAACCCTTCTATATT	122	28	91
VvEXPB3	GTATGGGTCGCTAGTAGATGTGAA	TACTATGATTGTAACGGCTCTCCTT	154	14	95

[a] As a high sequence similarity (98–100 %) was observed between Teleki 5C and *V. vinifera* it seemed justified to use the expression VvEXPxxx for putative expansin genes

[b] Efficiencies are shown as mean value of two qRT-PCR runs. R^2 for all studies was >98 %

(young nodosities) sample. Additional analyses (resulting in four biological replicates in total) were performed with genes showing a FC either ≪ 0.4 or ≫ 2.5 in at least two of the four treatments.

Data analysis

FC-values from qRT-PCR data were calculated to determine changes in expression. *T*-tests were used for exploratory assessment of changes from 1.0 following calculation of unadjusted two-sided 95 % confidence intervals and respective *p*-values. Statistical analyses were conducted using the software package SPSS, version 16 (SPSS Incorporation). As the sample size (*N*) was limited, data was not corrected; thus the study has an exploratory character.

Results

Identification and verification of putative expansin genes

Following in silico studies, eleven putative expansin genes were identified in *V. vinifera* cv. *P. noir* homologous in terms of sequence with those from *thaliana* (Supplementary Figure 1). Nine of these genes were verified in the rootstock Teleki 5C via PCR-based sequencing (VvEXPA4, -A7, -A8, -11, -A12, -A15, -A17, -A23, -B2). Sequences showed 98–100 % similarity when compared to the databank from Genoscope (Jaillon et al. 2007) (data not shown). In the following (as below), putative expansin genes from the rootstock Teleki 5C were pooled under the abbreviation "VvEXP" due to the fact that no further functional or genomic studies on this genes have been performed to indicate the similarity (if any) to *V. vinifera* expansin genes.

Expression of putative expansin genes

Our qRT-PCR study revealed an involvement of the putative expansin genes VvEXPA7, -A12, -A15, -A17, -A20 and -B2 in phylloxera-root interaction expressed either via up- (quantitative FC >2) or down-regulation (quantitative FC < 0.5 [a schematic overview of the regulation is given in Fig. 1]). Significant up-regulation was observed for the putative expansin genes VvEXPA15 (*P*=0.035) and -A17 (*P*=0.005) in the bodies of mature nodosity as well as for the putative expansin gene VvEXPA20 at the tips of mature nodosities (*P*=0.036). Significant down-regulation was calculated for the putative expansin genes VvEXPA7 in all treatments (body of young nodosities: *P*=0.045; body of mature nodosities: *P*=
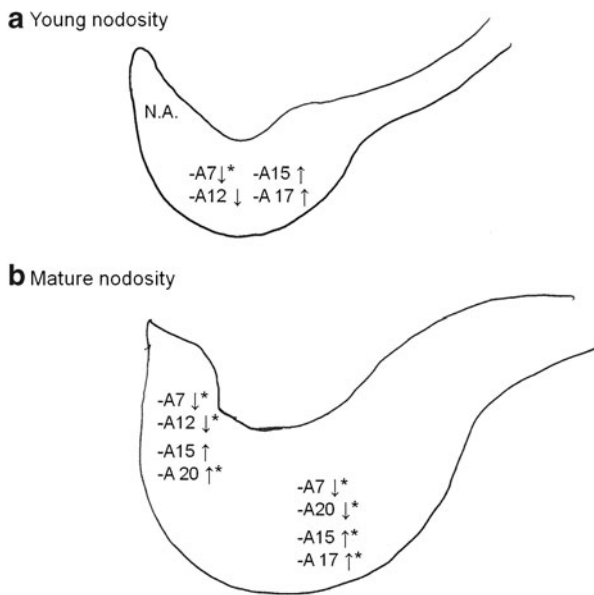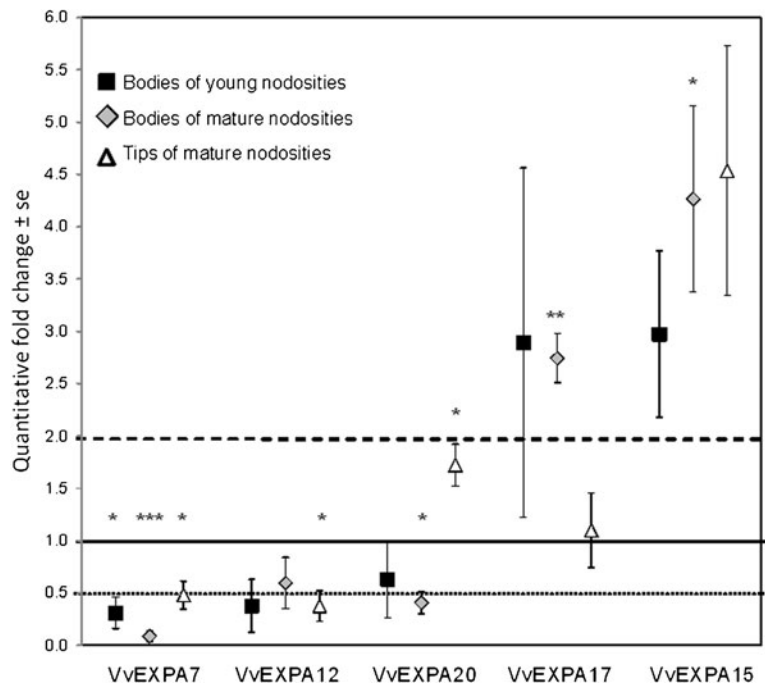
Fig. 1 Schematic drawing of young (a) and mature (b) nodosities showing which putative expansin genes were up- or down-regulated after a phylloxera attack and whether this occurred in the nodosity body and/or the root tip. All putative expansin genes start with VvEXP-. *Stars* indicate significance for a certain putative expansin gene. No putative expansin genes were measured in the tips of young nodosities (N.A.)

0.0004, tips of mature nodosities: $P=0.030$) and further down-regulation was observed for the putative

expansin gene VvEXPA20 in the body of mature nodosities ($P=0.011$) in addition to VvEXPA12 in mature nodosity tips ($P=0.024$).

As gene expression for VvEXPB2 was either up- or down-regulated within the same treatment and a high standard error was calculated for those data points, results are not discussed further for this gene. An overview excluding VvEXPB2 is given in Fig. 2 based on the calculated FCs.

Discussion

This study clearly shows that putative expansin genes are involved in grape root phylloxera-interaction, with up- as well as down-regulated genes. Three putative expansin genes (VvEXPA15, -A17 and partly -A20) were up-regulated in nodosities whilst three putative genes were partly down-regulated in young or mature nodosity bodies or mature nodosity tips (VvEXPA7, -A12, -A20).

Expression profiles in nodosities

Initiation and development of nodosities involves a combination of hypertrophy of cortical cells distal to the feeding site and lack of radial expansion of cortical cells proximal to the feeding site. The active feeding

Fig. 2 Expression changes of putative expansin genes [relative fold change (FC)± se] in young and mature nodosity bodies and mature nodosity tips. At a FC of 1.0, no gene regulation occurs (*black line*), an FC of 2.0 indicates that a doubling of gene expression occurs (*dashed line*), whilst an FC of 0.5 indicates that only half of the potential gene expression occurs (*dotted line*). Values above 1.0 indicate up-regulation, values below 1.0 down-regulation in gene expression. Treatments are in the same order for all genes. $N=3$–4

site is located in the upper layers of the root cortex whilst the stylet sheath ends intracellularly apart from the endodermis (Forneck et al. 2002). The nodosity apical end retains the meristematic cell profiles and calyptra but does not show major histological cell modifications, which are mainly hypertrophic due to phylloxera feeding.

By studying young vs. mature nodosities and dividing the latter into basipetal vs. apical ends of the galls, our aim was to search for information regarding the expansin regulation during nodosity growth. Comparing our results between young and mature nodosity bodies, it is clear that more putative expansin genes are expressed over time, resulting in up- as well as down-regulation (young nodosities: down-regulated: VvEXPA7; mature nodosities: down-regulated: VvEXPA7, and A20, up-regulated: VvEXPA15 and -A17). As the body of the root galls enlarge via cell growth (Niklowitz 1955; Forneck et al. 2002), up-regulation in case of mature nodosity bodies seemingly indicates cell-wall loosening and modification—characteristics well known to be associated with expansin genes. A stronger down-regulation of putative expansin genes in mature nodosity bodies compared to young ones is probably a result of plant defence. Indications of plant defence mechanisms in compatible phylloxera-plant reactions were earlier shown by Lawo et al. (2011a) who found that mevalonate and/or alternative isopentenyl pyrophosphate-, the lipoxygenase- (LOX) as well as the phenylpropanoid pathway, are induced as a direct response to phylloxera attack in the nodosity. In this particular paper, it was also shown that volatile profile in young nodosities was less clearly expressed compared to mature nodosities.

The comparison of expression levels of putative expansin genes in the basipetal ends of mature nodosities versus their apical ends showed similar up-regulation only for VvEXPA15 (FC: 4.3±0.89 vs. 4.54±1.19). A significantly stronger down-regulation of the putative expansin gene VvEXPA7 occurs at the base of the root compared to its tip (FC: 0.1±0.05 vs. 0.5±0.13). Selective sampling further also showed that VvEXPA17 was mainly expressed in the basipetal main part of the gall (FC: 2.7±0.23 vs. 1.1±0.36) and for the putative expansin gene VvEXPA20, a down-regulation in nodosity main parts was observed, whereas significant up-regulation was measured in the tips (FC: 0.4±0.01 vs. 1.7±0.20).

Comparison of root galls between nematodes and phylloxera

Gall formation of nematodes and insects has been contrasted for a long time, although gall induction in plants through pathogen involvement may involve similar host pathways or that host responses involve common plant regulatory cascades (e.g. Bird and Kaloshian 2003). The majority of studies on expansin expression in plants were performed using nematodes, either root-knot nematodes which form galls or cyst nematodes which form syncytia. Whether cyst nematodes establish their feeding site via a single root cell which then fuses with neighbouring cells is still not clear (Wyss and Grundler 1992), although root-knot nematodes seem to use 5–7 parenchymatic cells to establish their so-called giant cells (Wyss et al. 1992). In case of nodosity formation, root cells close to the phylloxera's feeding site become polyploid, whereas those at opposite to the feeding site enlarge and are filled with starch granules (summarised by Forneck et al. 2002).

Several previous studies are in agreement with our findings in reporting up-regulation of the expansin gene AtEXPA15 (referring to VvEXPA15) in syncytium formation (Wieczorek et al. 2006) as well as upon root-knot nematode attack (Jammes et al. 2005). Down regulation was observed for the putative expansin genes VvEXPA7 and partly VvEXPA12 and -A20 in nodosity bodies and/or tips (these results). Similar results were shown upon cyst nematode attack in syncytia only for AtEXPA7 in *A. thaliana* (Wieczorek et al. 2006; Szakasits et al. 2009).

Furthermore, upon comparing our findings with a phylogenetic tree based on the protein sequences of *A. thaliana* and tomato (Griesser and Grundler 2008), none of the putative expansin genes involved in nodosity formation in tomato were found to be regulated under root-knot nematode attack. This, together with the difference in gall formation as discussed above, provides strong evidence that differently regulated expansin genes are triggered due to different herbivore–plant interactions. However, an up-regulation of expansin genes is not only limited to gall-forming pests. Thus studies by Divol et al. (2005) have reported an increased expression of two expansin genes in celery after phloem feeding of the peach-potato aphid, *Myzus persicae* (Sulzer). In contrast, Kempema et al. (2007) reported the down-regulation

of three expansin genes due to phloem-feeding white-fly nymphs (*Bemesia tabaci* Gennadius) on *A. thaliana*. Such examples infer that expansin genes are not only associated with cell growth and expansion but that their function is much more complex.

Several studies show that besides expansin genes, other genes involved in cell wall modification such as glucanases, pectinases or cellulases are up-regulated as a result of root-knot nematode attack (Caillaud et al. 2008) as well as the gene family of endo-1,4-β-glucanases upon cyst nematode attack (Wieczorek et al. 2008). So far no detail studies related to this field have been conducted in relation to nodosity formation by phylloxera. Even so, earlier studies have provided some evidence of physiological changes in nodosities upon attack by this insect (e.g. Forneck et al. 2001b; Kellow et al. 2004; Du et al. 2011) arguing that not only genes involved in cell wall modification are affected. Thus, further enzymatic changes expressed in increased activity of peroxidase, acidic phosphatase and leucine aminopeptidase have also been shown upon phylloxera attack (Forneck et al. 2002). Other studies have reported the accumulation of free amino acids and amides in nodosities compared to uninfested roots (Kellow et al. 2004) and recently a change in endogenous hormone levels in nodosities over time was measured (Du et al. 2011).

In this study we have shown that expansin genes are specifically regulated in the phylloxera–root interaction. The studies of Lawo et al. (2011b) are consistent with our present findings of an up-regulation of the putative expansin gene VvEXPA15 as well as a down-regulation of genes VvEXPA7 and -A12. In our present study, even when complete nodosities of different age were compared to control roots, a similar expression pattern was revealed. Based on this plus the involvement of these genes upon nematode attack, we conclude that the putative expansin gene VvEXPA15 is essential during gall formation processes and further, that the genes VvEXPA7, -12, -20 and -17 are involved in nodosity formation. To further analyze and complement gene function, studies are necessary with adequate transgenic grapevines (since there are no knock-out mutants available) to prove expansin—effects on the gall formation upon phylloxera infection. To localize the expression of expansin genes in nodosities by in situ hybridization (e.g. Vorwerk et al. 2008) has been shown to be efficient and may further enhance our knowledge on the nodosity development.

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