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Activation of gene expression during hypersensitive response (HR) induced by auxin in the grapevine rootstock cultivar 'Börner'

A. Dietrich, T. Wolf, K. Eimert and M. B. Schröder

Department of Botany, Geisenheim Research Center, Geisenheim, Germany

Summary

The cultivar 'Börner' is one of the very few grape phylloxera (*Daktulosphaira vitifoliae* Fitch) resistant rootstocks commercially available. In contrast to tolerant or sensitive rootstocks, 'Börner' roots react to grape phylloxera attack with a hypersensitive response leading to necroses around the puncture sites. In this study, we identified genes differentially up-regulated during the HR. HR was chemically induced in root cells by the application of indol-acetic-acid (IAA). After a cDNA subtraction of induced and non-induced material, the subtracted cDNA-samples were also hybridized to *Arabidopsis* microarray chips to identify differentially expressed candidate genes. The microarray data were analyzed and differentially expressed genes were grouped into different functional categories, e.g. signal transduction pathways, transcription factors, defence associated genes. Primers were designed to target genes of interest putatively involved in the HR. So far, 38 ESTs induced in 'Börner' roots undergoing a HR have been sequenced and annotated.

K e y w o r d s : *Vitis*; phylloxera; hypersensitive response (HR); suppression subtractive hybridization (SSH), DNA-microarray.

Introduction

In the middle of the 19th century grape phylloxera (*Daktulosphaira vitifoliae* Fitch) was introduced into Europe together with North American grape species causing essential damage to the European wine growing industry. To manage the growing problem of the spreading infestation, plant material bred from North American grape species, thought to be phylloxera resistant, was introduced as rootstocks for the European varieties. Due to the seemingly successful management of the phylloxera threat by usage of those apparently resistant rootstocks, no significant research was done on the subject until the last couple of decades.

Increasing damage was then reported from vineyards, only hesitantly at first attributed to a phylloxera "comeback". From this development we know that the majority of rootstock varieties currently in use are phylloxera tolerant only, rather than resistant. Usually, the vigorous root system of tolerant rootstock varieties can compensate for the damage caused by phylloxera feeding on their roots. Nevertheless, in the presence of concomitant adverse conditions, such as negative weather conditions or increasing damage by mechanization, the tolerant rootstocks can collapse under a severe phylloxera attack.

To our knowledge, there are only three truly resistant rootstocks commercially available – 'Börner' and, more recently, its siblings 'Rici' and 'Cina'. 'Börner' is a hybrid between *Vitis riparia* and *V. cinerea*. Upon phylloxera attack this variety responds with a hypersensitive reaction (HR) (Anders 1957).

Since the phylloxera come-back, quite some work has been done on the genetics, population structure and diversity of the pathogen (Forneck *et al.* 2000, Corrie and Hoffmann 2004, Downie 2004, Chitkowski and Fisher 2005, Lin *et al*. 2006, Powell *et al.* 2007, Vorwerk and Forneck 2007, Fong *et al.* 2008), and a few papers were published on pathogen/host interactions in susceptible varieties (Omer *et al.* 2000, Kellow *et al.* 2004, Blanchfield *et al.* 2006). But, despite the economic importance of resistant rootstocks, the mechanism(s) underlying the defence reaction and its regulation is still poorly understood.

Recently, El-Nadi and Schröder (2003) described the accumulation of oxidized phenolic compounds around the puncture area, a feature frequently occurring in hypersensitive pathogen response (for review, see Heath 2000). El-Nadi and Schröder (2003) also identified features associated with programmed cell death, like fragmentation of the nucleolus and blistering of the plasma membrane (Greenberg 1997, Lamb and Dixon 1997, Greenberg and Yao 2004). They could also show that auxin, one of the main components of the phylloxera saliva (Schäller 1968), is cecidogenic in susceptible cultivars and triggers the HR in resistant 'Börner' (El-Nadi and Schröder 2003). The aim of this study was to investigate the molecular features of the HR chemically induced in 'Börner'.

Material and Methods

R o o t p r e p a r a t i o n a n d i n d u c t i o n : Plant material came from the Department of Grapevine Breeding and Grafting, Geisenheim Research Center. Roots of 'Börner' plants grown in greenhouse were used for this study. Root material was grown and induced as described by El-Nady and Schröder (2003). In short, root material

Correspondence to: Dr. K. Eimert, Department of Botany, Geisenheim Research Center, Von-Lade-Str. 1, 65366 Geisenheim, Germany. Fax.: +49-6722-502-469. E-mail: K.Eimert@fa-gm.de

(pieces about 5 cm in length and 2-3 mm in diameter) was harvested and sterilized with 70 % ethanol and 1 % sodium hypochloride. The basal end of each root piece was then wrapped with sterile cotton and incubated at 28 °C in Petri dishes in the dark for three weeks. HR on adventitious roots was then induced with 0.1 % IAA for 2.5 or 4.5 h. After induction, roots were frozen in liquid nitrogen. Root material from several induction experiments was collected and pooled (150 to 200 rootlets of each treatment).

RNA extraction and cDNA synthesis: RNA-extraction was carried out using the RNeasy® Mini Kit (QIAGEN GmbH, Hilden, Germany) following the instructions of the manufacturer. cDNA synthesis was carried out using the SMART™ PCR cDNA synthesis Kit from Clontech (Takara Bio Europe, SAS) and an amount of total RNA ranging from 0.1 to 0.5 µg per reaction.

cDNA subtraction: The synthesized cDNA was cleaned up using Chroma-SpinTM -1000 Columns (Clontech) essentially following the manufacturers protocol, except for the use of TE instead of TNE buffer and three additional elution steps with 150 µL TE buffer. For cDNA subtraction the PCR-SelectTM cDNA subtraction Kit from Clontech was used following the instructions of the manufacturer.

DNA microarray chip hybridization: DNA microarray chips spotted with the *Arabidopsis* genome oligo set 1.0 (QUIAGEN) were obtained from Galbraith *et al.* (http://www.ag.arizona.edu/microarray). Version 1.0 contained 26090 oligonucleotides (70mere) per slide. The microarray chips were rehydrated and UV-cross linked. Both subtracted cDNA samples were divided in two samples and labelled with the nucleotides Cy3 and Cy5 (PERKIN ELMER LIFE SCIENCES, Boston, USA), respectively. The cDNA samples were amplified with the AdvantageTM 2 Polymerase Mix (Clontech, Takara Bio Europe, SAS) using nested primers of the PCR-SelectTM cDNA subtraction Kit (Clontech). Subsequently, the labelled cDNA samples were purified using the QIAquick Kit (QIAGEN).

Two hybridization experiments were carried out with 1.6 µg of each labelled cDNA sample. In the first hybridization, the Cy3-labelled 2.5 h sample and the Cy5-labelled 4.5 h sample were combined, mixed with 20x SSC, Liquid BlockTM, 2 % SDS, topped up with sterile water to a total volume of 250 µL and hybridized to the microarray chip following the instructions of Galbraith *et al.* The hybridization reaction was mixed well, heated for 2 min at 94 °C degrees and immediately placed on ice. The microarray chip was heated to 65 °C degrees and the total hybridization reaction was applied to the chip, which was then covered with a cover slip. In the second hybridization the corresponding flip-dye experiment was carried out in an identical manner. The microarray chips were incubated in a hybridization chamber at 55 °C degrees for 15 h. After incubation, the microarray chips were washed and dried according to the instructions. The dry microarray chips were scanned with an Affymetrix 428TM Array Scanner (Affymetrix, Santa Clara, USA) using the software JaguarTM 2.0 from Affymetrix. Cy3 was measured at a wavelength of 570 nm and Cy5 at 670 nm.

Spots were detected and quantified with the software ImaGeneTM Standard Edition from BIODISCOVERY (El Segundo, USA). Data evaluation was accomplished with the software GeneSightTM 4.1 Lite Edition from BIODISCOVERY. The Cy3 and Cy5 datasets of each subtracted sample were combined and analyzed as replicates. Therefore, arithmetic median of the signal value was used for each spot and the following transformation sequence was carried out: background correction, elimination of negative and poor spots, substitution of eliminated values by the arithmetic median over all signal values, global and linear LOWESS-normalization. Only genes which were beyond the defined threshold (fold change cut-off of 2) were used for further analysis.

Validation of the DNA microarray chip experiments: Primers pairs were designed for a set of candidate genes selected from the 2.5 h sample. The *Arabidopsis* sequences were checked against grape ESTs in the TIGR grape gene index database (http://www.tigr.org). Primer pairs were synthesized to target the corresponding grape ESTs (Tab. 1). These genes were amplified using the 2.5 hours induced cDNA sample as a template. PCR products were recovered from a 1.3 % agarose gel, ligated into pGEM-T Easy vector (PROMEGA GmbH, Mannheim, Germany) and transformed into XL1-Blue E. coli competent cells (STRATAGENE Europe). Inserts were sequenced (GENTERPRISE, Mainz, Germany), analyzed and annotated in NCBI (http://www.ncbi.nih.gov). Induction of transcription and successful subtraction was proven by semi-quantitative amplification with sense and anti-sense primers in induced and non-induced cDNA populations.

Sequence alignment: DNA sequences were analyzed using the unweighted pair group method with arithmetic mean (UPGMA, Sneath and Sokal, 1973) of the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

Results

DNA subtraction: cDNA subtraction was always carried out for the IAA induced samples against the non-induced samples, respectively. Simultaneously, a control subtraction was carried out as recommended by the manufacturer. First cDNA subtractions were conducted 4.5 h after induction (hai). From these subtractions we could clone, sequence and identify 5 ESTs (Tab. 1). Due to the low number of ESTs obtained, additional subtractions were conducted at 2.5 hai. In the 2.5 h sample 25 ESTs could be identified, also including all of those from the 4.5 h sample (Tab. 1). Only technical repeats were conducted as we used pooled biological material.

The transcription of six stilbene synthases (STS) was found up-regulated during the induced HR. As resveratrol has previously been described to be involved in plant defence in grape (Adrian *et al.* 1997, Bais *et al.* 2000, Bézier *et al.* 2002) we decided to clone and further analyze those sequences. A sequence alignment (UPGMA) of all six identified STS mRNAs revealed three distinct clusters

Table 1

Annotation of ESTs identified by cDNA subtraction

hai	Gene accession number of the Börner ESTs	Homology with NCBI GenBank accessions using BLASTn
4.5	CK986230	98 %, cyclase, *Vitis pseudoreticulata*, DQ336281
4.5	CK986231	76 %, auxin and ethylene responsive GH3-like protein (GH3), *Capsicum chinense*, AY525089
4.5	CK986232	84 %, non-cell-autonomous heat shock cognate protein 70 (Hsc70-2), *Cucurbita maxima*, AF527794
4.5	DV466767	99 %, labst1 stilbene synthase, *Vitis labrusca*, AB046374
4.5	DV466768	97 %, ripst1 stilbene synthase, *Vitis riparia*, AB046373
2.5	CK906352	74 %, rooting related oxygenase ARRO-1, *Malus domestica*, AJ225045
2.5	CK986215	75 %, StMKP1 MAP kinase phosphatase 1, *Solanum tuberosum*, AB206784
2.5	CK986216	68 %, putative reticuline oxidase-like protein, *Arabidopsis thaliana*, AY140079
2.5	CK986217	80 %, CmGal3 beta-galactosidase, *Cucumis melo*, AB270925
2.5	CK986218	78 %, stress-responsive zinc-finger protein, *Camellia sinensis*, DQ869863
2.5	CK986219	84 %, phragmoplastin (dynamin-like protein), *Camellia sinensis*, DQ444295
2.5	CK986220	96 %, NAD(H) glutamate dehydrogenase, *Vitis vinifera*, X86924
2.5	CK986221	* 47 % (69 %), lectin protein kinase family protein, *Arabidopsis thaliana*, NP196292
2.5	CK986222	89 %, elongation factor 1 alpha (efalpha gene), *Platanus* x *acerifolia*, AM293616
2.5	CK986223	88 %, blp3 luminal binding protein (BiP), *Nicotiana tabacum*, X60061
2.5	CK986224	78 %, proline iminopeptidase (PIP), *Arabidopsis thaliana*, NM201725;
2.5	CK986225	84 %, HbAACT acetyl-CoA C-acetyltransferase, *Hevea brasiliensis*, AB294687
2.5	CK986226	74 %, quinone reductase-like protein, *Arabidopsis thaliana*, AK226382
2.5	CK986227	77 %, arabinoxylan arabinofuranohydrolase isoenzyme AXAH-I, *Hordeum vulgare*, AF320324
2.5	CK986228	73 %, zinc finger an1-like family protein, *Brassica rapa*, EU186377
2.5	CK986229	67 %, SIEP1L protein (secreted membrane associated glycoprotein), *Beta vulgaris*, X87931
2.5	DV466766	98 %, stilbene synthase, *Vitis vinifera*, S63225
2.5	DV466769	96 %, resveratrol synthase f3-like, *Vitis vinifera*, AY656726
2.5	DV466770	93 %, resveratrol synthase f3-like, *Vitis vinifera*, AY656726
2.5	DV466771	99 %, stilbene synthase, *Vitis vinifera*, AY670232

* BLASTx was used when the BLASTn algorithm did not identify significantly homologous expressed sequences – results are presented as % identity and similarity (in parentheses), respectively.

at the cDNA level (Fig. 1; alignment data available upon request). One cluster (I) includes sequences DV466769 and DV466770 with 95.62 % identity to each other. This branch separates very clearly at 64.98 ± 1.92 % similarity from clusters II and III, consisting of sequences DV466771, DV466766 and DV466768, DV466767, respectively. Sequences in cluster II and III share 99.03 % and 97.31 % homology, respectively. These branches separate later, but still significantly distinct. While all STS were up-regulated at 2.5 hai, only the two included in cluster III could also be found in the 4.5 hai sample. It might be worth mentioning, that the latter 2 showed higher homology to STS genes of American wild grapevine varieties, while the first 4 were closer to those of *V.vinifera*.

DNA microarrays: Subtracted libraries from 2.5 and 4.5 hai were hybridized to the whole genome *Arabidopsis* microarrays. As in the cDNA subtraction, more genes were found to be up-regulated in the 2.5 hai sample than in the 4.5 hai sample (845 vs. 611), with 92 % of the identified sequences from the 4.5 hai sample also being present in the 2.5 hai sample (a full list is available upon request). 19 of the genes identified by the cDNA subtraction could also be identified by the DNA microarray. Only the six stilbene/resveratrol synthases were not found. The identified sequences can be classified into 10 functional categories (Tab. 2). No significant differences in classification were observed between the two samples.

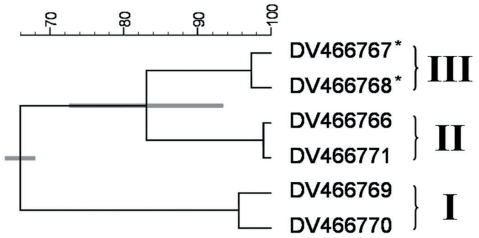

Fig. 1: UPGMA cluster analysis of pairwise aligned sequences of 6 stilbene synthases (STS) derived from SSH. * indicates sequences found at 2.5 and also at 4.5 hai; shaded bars represent SD values.

Validation of the DNA microarray chip experiments: To clone the according cDNAs from the original mRNA populations of the 2.5 hai sample, primer pairs were designed from identified *Arabidopsis* sequences. Primers were then tested to be specific and to specifically amplify differentially expressed sequences in 'Börner' (Fig. 2). So far, 11 specific primer pairs yielded 13 distinct cDNAs (Tab. 3).

Discussion

Grapevine rootstocks usually considered resistant to grape phylloxera do develop nodosities and show dam-

Table 2

Classification of identified sequences into functional categories

category	4.5 hai	2.5 hai
signal transduction	15 %	14%
DNA/RNA transcription associated proteins	19 %	18%
metabolism	12 %	14%
transport	8 %	10%
protein metabolism	14 %	14%
auxin and growth	4 %	4%
photosynthesis	1 %	1%
cell wall and cytoskeleton associated genes	8 %	7%
defence	9 %	9%
genes with unknown function	10 %	9%

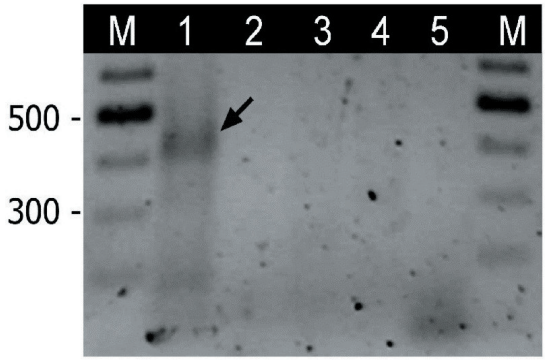

Fig. 2: RT-PCR of EST DR831575 (Hypersensitive induced response protein). M - molecular weight markers; 1- induced 2.5 hai with sense primers (arrow indicates the amplified 420bp product); 2 - induced 2.5 hai with antisense primers; 3 - non-induced with sense primers; 4 - non-induced with antisense primers; 5 - negative control without RNA.

Table 3

Annotation of ESTs identified by microarray

hai	Arabidopsis gene accession number and annotation	Gene accession number of the Börner ESTs	Homology with NCBI GenBank accessions using BLASTn
2.5	At5g12140 cystatin	DR784731	79 %, cysteine protease inhibitor, EF016117, *Populus tomentosa*
2.5	At5g12140 cystatin	DR784732	72 %, cysteine proteinase inhibitor, EU284927, *Elaeis guineensis*
2.5	At5g41680 Pto kinase interactor	DR784733	86 %, putative serine/threonine protein kinase, *Arabidopsis thaliana*, NM129885
2.5	At3g22840 Early light-induced protein	DR784734	83 %, early light-induced protein 4, *Rhododendron catawbiense*, EF527265
2.5	At3g52880 Monodehydroascorbate reductase	DR784735	99 %, monodehydroascorbate reductase, *Vitis vinifera*, EF554360
2.5	At5g51570 Hypersensitive induced response protein	DR831574	79 %, hypersensitive-induced reaction protein 4, *Oryza sativa*, EF576167
2.5	At5g51570 Hypersensitive induced response protein	DR831575	82 %, phytochrome A, *Populus tremula* x *Populus tremuloides*, AJ001318
2.5	At5g62740 Hypersensitive induced response protein	DR831576	87 %, hypersensitive-induced response protein, *Carica papaya*, EF512303
2.5	At5g62740 Hypersensitive induced response protein	DR831577	81 %, hypersensitive induced response protein 3 (HIR3), *Triticum aestivum*, EU908213
2.5	At4g37000 Accelerated cell death 2 (ACD2)	DR831578	75 %, senescence-associated protein, *Elaeis guineensis*, EU284887
2.5	At5g14450 Lipase/hydrolase family protein, similar to early nodulin ENOD8	DV111307	100 %, pathogene-related protein PR-1, *Vitis vinifera*, AJ003113
2.5	At5g46700 Senescence-associated protein 5-like protein	DV111308	72 %, eukaryotic initiation factor eIF-4 gamma like protein, *Ipomoea nil*, AB277195
2.5	At5g61640 Peptide methionine sulfoxide reductase	DV111309	93 %, elongation factor 1-alpha (eEF1A), *Prunus persica*, FJ267653

age under certain conditions. For that reason, they should more accurately be described as 'tolerant'. There are a few hybrids, one of them being the rootstock 'Börner' used in our experiments, showing a qualitatively different reaction. In case of grape phylloxera attacks, it reacts with a hypersensitive reaction (HR) causing local necroses on leaves and roots which prevents feeding and breeding of the insect (Anders 1958; Börner 1943; Niklowitz 1955). Although, reduction of 'resistance' in tolerant varieties has been described (Forneck *et al.* 1998; Corrie *et al.* 2003), which may be due to the rather high genotypic diversity reported for grape phylloxera (Forneck *et al.* 2000, Corrie *et al.* 2002), no breach of resistance has yet been reported in 'Börner'. Of course, that does not exclude the possibility of an adaptation of grape phylloxera biotypes to 'Börner' in the future, but we feel that the situation stresses the dif-

ferences between the quantitative variation of a tolerance (which often has been overcome) in comparison to the qualitatively different HR-like resistance, yet to be broken.

Phylloxera forages on susceptible grapevine roots inserting its proboscis in different places before settling in one place for feeding and breeding. On the other hand, it often rests in one position not inserting its proboscis at all. On 'Börner' roots necroses develop in the insertion areas within 6 to 24 hours depending on the developmental age of the tissue (El-Nadi and Schröder 2003). It proved impossible to determine whether insects pausing on the root surface had actually inserted their proboscis until the necroses appeared. This unpredictable feeding pattern and also the minuscule size of the necrotic target tissue, often less than 0.5 mm in diameter, made it practically impossible to use the insects themselves for controlled induction in order to produce sufficient quantities of samples. Necessarily, we had to rely on a chemical induction of the necrosis. Not much is known about the triggering factor(s) of the HR in 'Börner' upon grape phylloxera attack. However, it has been assumed that the plant hormone IAA, which is present in phylloxera saliva at high concentrations, is the component triggering the resistance mechanism (Anders 1958, Schäller 1960, 1965, 1968 a and b). Our previous studies demonstrated that externally applied IAA suffices to induce necrosis formation in resistant cultivars, such as 'Börner', and gall or nodosity formation in susceptible ones. These reactions were morphologically and histochemically virtually indistinguishable from those triggered by grape phylloxera (El-Nadi and Schröder 2003). Therefore, we choose to use the auxin IAA for timed and controlled induction of the HR despite the obvious shortcomings of this system. While Granett (1990) reported that, for some rootstock varieties other than 'Börner', swellings caused by IAA and phylloxera are not identical, that is not in conflict with our observations, since the varieties used as resistant controls in those experiments are now considered tolerant, only. Therefore, the differences described there seem to represent quantitative changes between sensitive and (moderately) tolerant varieties rather than the qualitative ones between sensitive and truly resistant (as marked by a HR) varieties.

Suppression subtractive hybridization (SSH, Diatchenko et al. 1996) was used in the first part of the attempt to identify genes differentially regulated in the induced HR in 'Börner'. This technique has recently been successfully used to identify differentially expressed genes in plants (Geng *et al*. 2008, Mi *et al.* 2008), including those involved in pathogen responses (Mahalingam *et al.* 2003, Vieira Dos Santos *et al.* 2003, Lin *et al.* 2007, Amaral *et al.* 2008). One of the main advantages of SSH is that it normalizes the amounts of cDNAs allowing for the identification of infrequently but differentially expressed genes (Diatchenko *et al.* 1996, Kürkcüoglu *et al.* 2007). The combination of SSH with the SMART technique allowed for undertaking the attempt despite minuscule amounts of initial RNAs. Even using these techniques it was necessary to induce and collect samples over several months. Pools of samples were used for RNA extraction for the cDNAs. For this reason only technical replicates were used in the experiments. Due to the limited amount of induced RNA populations the SSH could only be driven in one direction since it requires a large excess of driver cDNA. So, in this setup we could only identify genes activated during the HR. Down-regulated genes cannot be identified thus. Since first necroses could be seen on 'Börner' earliest within 6 h after phylloxera attack or induction by auxin we decided to sample the roots after 4.5 hours to identify what we thought early genes involved in the process. Despite the stated limits we could identify five genes activated 4.5 hai. Having successfully identified those genes we opted for an earlier sampling at 2.5 hai. Surprisingly, many more genes appeared to be activated at the earlier time point. As all genes from the 4.5 hai sample were also present in the 2.5 hai sample we assume that gene activation in HR is happening earlier in the process and, partially, being terminated later.

Because of their known role in plant defence (phytoalexin synthesis) the 6 stilbene synthase sequences derived from SSH were subjected to pairwise alignment and cluster analysis. While three clearly separated clusters could be identified it is difficult to classify those sequences functionally. Despite the similarities in biosynthesis of stilbenes the enzymology of stilbene synthases differs considerably (Supp 2001). They also exhibit a strong homology to other polyketide synthases like chalcone synthase (Tropf *et al.* 1994) rendering a decision about substrate specificity and enzymology based on the primary sequence alone impossible (Ferrer *et al.* 1999). We refrain from speculation and only state at this point that there seems to be a differential regulation of different stilbene synthase classes during the HR in 'Börner'.

Apart from the main bands visible after subtraction, which were used for the cloning and identification of activated genes, there is a multitude of weaker bands. Those bands are difficult to excise separably for cloning and sequencing. For this reason we decided to use the subtracted cDNA libraries for hybridization to a DNA microarray. At the time of the experiments the only available *Vitis* DNA microarray included only about 15,000 genes, many identified from developing berries. Also, these commercially available microarrays are exclusively based on *Vitis vinifera*, for which, to our knowledge, no hypersensitive pathogen response has been reported. Therefore, beside the prohibiting cost, we decided to use a heterologous DNA microarray covering the whole genome of *Arabidopsis*, which is known to display hypersensitive responses (for review, see Morel and Dangl 1997). The use of heterologous systems has been described before (Horvath *et al.* 2003, Bar-Or *et al.* 2007) and proof of concept was presented recently (Bagnaresi *et al.* 2008). We could successfully employ the *Arabidopsis* microarray to identify several hundred genes up-regulated during the HR in 'Börner' at different time points. The feasibility of this approach was also demonstrated by verifying all sequences identified by SSH in the microarrays. Missing were only the six stilbene synthase genes. This is in agreement with *Arabidopsis* not having according homologous sequences despite the suggested presence of unknown stilbene isomerase activities in *Arabidopsis* (Yu *et al.* 2006). The classification of the identi-

fied genes into functional groups indicates that, during the HR in 'Börner', there occurs an activation of primary metabolism pathways, together with the induction of several signalling pathways for the expression of defence-related genes. That is in accordance with earlier reports (Mahalingam *et al*. 2003, Hammond-Kosack and Jones 1996, Birch *et al.* 1999).

Concluding, we can say that by SSH and heterologous DNA microarray we could identify many genes activated during the auxin induced HR in 'Börner'. 38 of those genes were sequenced, annotated and verified for differential expression. Apart from demonstrating the general applicability of these methods, the results give us a first insight into the processes occurring during the defence reaction of 'Börner'. Nevertheless, one has to be aware of the limits of the described methods. So far, only activated or strongly up-regulated genes could be targeted. Genes down-regulated or genes unique to *Vitis* and absent in heterologous microarrays would avoid detection.

Further work is planned with custom-made *Vitis* chips drawing on the growing sequence data from *Vitis* genome (IASMA Genomics, http://genomics.research.iasma.it) and related projects. Furthermore, earlier time points after induction will be included into the analyses to narrow down early regulation events.

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