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ORIGINAL PAPER

Stress-related genes define essential steps in the response of maize seedlings to smoke-water

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Abstract Smoke from burning vegetation is widely recognised as a germination cue for seed germination and recent reports suggest that smoke treatments can improve seedling vigour also. We investigated the effect of smoke-water on seedling vigour and changes of the global transcriptome in the early post-germination phase in maize. Application of smoke-water improved the germination characteristics and seedling vigour. The transcriptional response of embryos and emerging radicles 24 and 48 h after the onset of smoke treatment was investigated. The microarray study revealed a number of smoke-responsive genes amongst which stress- and abscisic acid (ABA)-related genes were over-represented. The global promoter analysis of the smoke-responsive genes revealed a tight correlation with the results obtained from Gene Ontology annotations. This concerted over-expression shows that smoke treatment induces stress and ABA-related responses in the early post-germination phase which leads to better adaptation to environmental stress factors occurring during germination, eventually resulting in greater seedling vigour.

Keywords Gene expression · Germination · Maize · Smoke · *Zea mays*

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Introduction

Smoke from burning vegetation is known to be an important environmental cue (Van Staden et al. 2000) and numerous species, mainly from South African fynbos (Brown 1993; Brown et al. 2003), Western Australian kwongan (Roche et al. 1997; Bell 1999) and Californian chaparral (Keeley and Fotheringham 1998) have shown an improved germination response to treatments using aerosol smoke or smoke-water. Many species from these fire-prone environments germinate in response to smoke treatments and several weed species, many from non-fire prone regions, respond to various smoke treatments (Adkins and Peters 2001).

Bioactivity-guided fractionation of smoke-water, using achenes of *Lactuca sativa* cv. Grand Rapids as a test system, has led to the isolation of a butenolide compound, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, from plant-derived smoke (Van Staden et al. 2004). Likewise, Flematti et al. (2004) were able to isolate the identical compound from smoke obtained from burning cellulose. This butenolide-type compound is active in promoting germination at

concentrations as low as 10^{-9} M (Flematti et al. 2004; Van Staden et al. 2004). Thus, the action of smoke in promoting the germination of seeds of many species is mainly attributed to the presence of this compound in smoke. However, it was shown that dual regulatory cues do exist in the smoke which can have either promoting or inhibitory effect on germination (Light et al. 2002) suggesting the presence of compounds with differing activities in smoke.

To date, there is relatively little documented work on the post-germination effects of smoke (Sparg et al. 2005). Baxter and Van Staden (1994) reported that the seedlings of the fire-climax grass *Themeda triandra* Forssk. from smoke-treated seeds grew more vigorously without any morphological abnormalities. A similar effect was observed for *Erica* species and species of *Asteraceae* (Brown 1993). More recently, Sparg et al. (2005) stated that although smoke treatment may not necessarily have an effect at the germination stage, it may play a role at the post-germination stage and suggested that in previous studies where many species have not responded to smoke treatments, these species may show some response at their post-germination stages, i.e. improved seedling vigour. Therefore, it may be necessary to extend germination studies to include an assessment of seedling vigour when smoke is used as a germination treatment. All these previous studies have been conducted mainly on wild species, with no evidence to suggest whether this post-germination effect of smoke can be observed in commercial crop plants.

Maize (*Zea mays* L.) is one of the most widely cultivated crops and is a large component of human and animal diets in many countries. It is considered to be a crop with the most biotechnological potential for industrial applications (McLaren 2005). Different kinds of stresses are the major constraints for maize production worldwide, since this crop is largely grown in areas in which unfavourable conditions (drought, heat and salt stress) are predominant. In these areas, seedling vigour is an important agronomic trait for the establishment of seedlings which can help young plants to overcome such adverse effects that usually result in lower yields. To date, there are only a few reports discussing the effect of smoke on the post-germination stage, although the use of fire and smoke in maize agrotechnology is not a new practice. For example, in South Africa, some rural farmers store maize seed lots over a fireplace subjecting the seeds to smoke and heat (Modi 2002). This storage method improved the germination rate, final germination in comparison with untreated seeds and produced significantly more vigorous seedlings, which were heavier and taller, in comparison with untreated seeds. Thus, smoke treatments have the potential to improve not only the percentage germination but also the seedling vigour of commercially bred maize seeds (Sparg et al. 2006).

High seedling vigour and survival rate often can be attributable to the induction of abscisic acid (ABA)-signal transduction (summarised by Chandler and Robertson 1994), stress-related events in the cell and subsequent increased stress tolerance (Khajeh-Hosseini et al. 2003; Soeda et al. 2005). During the germination process, the re-induction of the seed maturation program (Rajjou et al. 2006) and osmopriming of germinating seeds also results in the induction of stress-responsive genes (Soeda et al. 2005) which was found to correlate to seed stress tolerance and higher vigour. Smoke-water can potentially be used as germination priming agent to ensure synchronous germination and optimal seedling establishment especially under adverse conditions (Sparg et al. 2006). It was hypothesised that stress-related genes may play a major role in smoke action and the improvement in seedling vigour elicited by smoke implies huge shifts in the gene expression pattern. To achieve a better understanding of the effect of smoke on germination characteristics, seedling vigour and the molecular background of smoke action, we analysed gene expression in the early post-germination phase in an inbred maize line.

Materials and methods

Plant material

The HMV5405 inbred maize line has been developed in ARI-HAS (Martonvásár, Hungary). The line originates from the Iodent family and possesses good general and specific combining ability; therefore, the HMV5405 is widely used as parent in maize hybrid production. However, it has a low germination percentage and low seedling vigour, which makes the production of basic seed difficult. Contrary to the efforts that have been made to overcome these disadvantages, the only solution to improve seedling vigour is the application of growth stimulants and germination cues.

Growth conditions, germination and vigour tests

For the germination time course tests, kernels (seeds) of *Z. mays* L. MV5405 (130 pieces) in four replicates were decontaminated in 3% sodium hypochlorite containing Tween 20 and 70% EtOH (10 min each) and then germinated in an illuminated environmental chamber (25°C, 100 μmol m^{-2} s^{-1} light intensity) on tissue paper placed in Petri dishes. The batches of kernels were treated with 20 ml water (control), 1:500 or 1:1,000 (*v*/*v*) dilution of smoke-water, 100 μM ABA (Sigma) and 1:500 smoke-water containing 100 μM ABA. Germinated kernels were scored every 12 h for 9 days. The standard vigour test developed

for determining the viability of the seedlings of commercial seedlots ('paper roll test') was applied in the vigour experiment. Eight replicates of 25 kernels each were imbibed in distilled water or smoke-water (1:500 or 1:1,000 dilutions) for 48 h. Thereafter, the kernels were transferred to moistened filter paper and placed at 5°C for 2 days. The chilling was followed by a 7-day period during which the kernels were kept at 25°C with a light intensity of 100 μmol m^{-2} s^{-1}. This experiment was repeated with the same temperature and light regimes excluding the chilling period. Germination percentage, root and shoot length were measured after 9 days and seedling vigour was determined using the equation:

$$V = \Sigma(L_s + L_r) \times \mathrm{GP},$$

where L_s and L_r are shoot and root length in millimeters, respectively, and GP is germination percentage (Dhindwal et al. 1991). Seedling dry mass and kernel water uptake were determined gravimetrically after drying the samples in an oven set to 100°C until they reached constant weight. The smoke-water was prepared from burnt *Themeda triandra* Forssk. (Poaceae), according to the method outlined in Baxter et al. (1994).

RNA isolation

For RNA isolation from control and smoke-treated (1:500) kernels, identical conditions were applied as for the germination time course, tests and samples were harvested 24 and 48 h after treatment. Embryos in the same developmental stage and size were chosen. The embryos of kernels with ruptured testa at 24 h and embryos of kernels with emerged radicle (3–4 mm) at 48 h were harvested from both control and treated samples. Total RNA was isolated from the maize kernel embryos using TRIzol reagent (Invitrogen) and cleaned up with Qiagen RNeasy Plant Mini Kit (Qiagen) applying a few modifications. RNA was then treated with RNase-free DNase I (Promega) according to the manufacturer's instructions. The concentration of RNA was determined with a Nanodrop ND-1000 spectrophotometer (NanoDrop). The RNA integrity number (RIN) of the samples was determined using the Agilent BioAnalyzer. Only samples with a RIN≥ 8 were considered for further analysis.

Microarray platform, labelling, hybridisation and image acquisition

In the microarray study, ten individual germinating kernels from each of four replicates were chosen and the RNA samples were pooled. Three technical replicates were applied at each time point for the microarray analysis. Microarray slides (46 K) were obtained from the University of Arizona Maize Array Project (http://www.maizearray.org). The detailed array annotation and composition is available at the Internet site. All the experimental procedures were carried out according to the manufacturer of the slides, with a few modifications. In brief, 450 ng total RNA was amplified and aminoallyl-UTP was incorporated using 101 TargetAmp Kit (Epicentre) and the resulting aaRNA was labelled with Cy3 and Cy5 (Amersham). The dye-labelled probes were then cleaned up (Qiagen), mixed with the corresponding samples, concentrated, re-suspended in the hybridisation solution and incubated at 42°C overnight in a hybridisation oven. Finally, slides were washed with different concentrations of SSC at room temperature.

Scanning was performed using an Amersham Typhoon Trio+ scanner at 10 μm resolution and default settings. The detection of signal intensities and the grid adjustment were accomplished with ArrayVision software version 8.0 (Amersham). The intensity value of each spot and background region multiplied by its area was used as signal intensity for further analysis.

Microarray data normalisation and analysis

Raw intensity data were imported into the R 2.6.2 software (R Development Core Team 2008) after pre-processing it with custom made Perl scripts. Further analysis was carried out using the LIMMA (Smyth 2005) package of BIOCONDUCTOR (Gentleman et al. 2004). Background correction was done using the multi-array analysis method (Irizarry et al. 2003). Normalisation of data within arrays was done using the 'loess' method (Yang et al. 2002). To normalise the data between arrays, the 'Aquantile' method was used (Yang and Thorne 2003). Besides these, the relative reliability of each array was estimated and the data weighted accordingly, based on the method described by Ritchie et al. (2006). The microarray data for each gene were fitted to a linear model, and statistics were generated using the lmFit and eBayes functions (Smyth 2004) of the LIMMA package. The *P* values were adjusted for multiple testing using the Benjamini and Hochberg (1995) method. Genes with adjusted *P* values of <0.05 and fold change ≥2 were considered as differentially expressed.

Gene ontology and promoter analysis

The differentially expressed genes were subjected to further analysis. Based on the available chip annotation, the genes were assigned into different Gene Ontology categories (Ashburner et al. 2000), and the significant over-representation of particular categories in the 24 and 48 h up- and down-regulated gene sets were determined with a modified version of the GeneMerge software (Castillo-Davis and Hartl 2003) optimised for speed.

The cDNA sequences were searched against the available maize genome sequence (http://www.maizegenome.org) with a locally installed version of BLAST (Altschul et al. 1997) and the promoter sequences up to 1,500 bp were extracted in the case of a perfect hit. The sequences were analysed with the TRANSFAC software suite version 12.1 (Matys et al. 2006), and a search was carried out for known transcription factor binding sites. The frequencies of the binding sites up to 8 bp length in all available maize promoter sequences and the 24 and 48 h up- and down-regulated gene promoters were calculated with the compseq program of the European Molecular Biology Open Software Suite (Rice et al. 2000). The over- or under-representation of a given transcription factor binding site in the promoter regions of the smoke specific up- or down-regulated genes was calculated as the ratio of the observed frequency in the studied promoters and expected frequency in all maize promoters.

Real-time PCR

The mRNA samples (200 ng) extracted from three independent biological replicates were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with an Applied Biosystems 7500 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. The reactions were performed in quadruplicate. Confirmation of specific product amplification was done by T_m analysis using the dissociation curve option. PCR efficiency (derived from the log slope of the fluorescence versus cycle number in the exponential phase of each amplification plot) for all primer pairs ranged from 95.5% to 98.0%. Maize actin (AY103587.1) and GAPDH (NM001111944) were also selected as potential internal controls and their expression was checked using PCR and microarray data (data not shown). Based on the preliminary findings, actin was selected and used in further experiments. The relative ratio of threshold cycle (C_t) values between the actin and the specific gene and their standard deviations were calculated for each sample.

Results

Germination and imbibition characteristics

Germination curves were recorded for control and smoke-treated (1:500 and 1:1,000 dilutions) maize kernels to determine the most effective concentration of the smoke-water. In general, smoke-water improved the germination of maize (Fig. 1a). Compared with the control, smoke-water at a concentration of 1:500 resulted in significantly higher germination with a mean of 64±1.45% whilst the 1:1,000 concentration only had a slight effect. Besides, ruptured kernels were first observed in smoke-treated kernels (1:500 and 1:1,000 smoke-water treatments). Since the microarray data (presented later) suggested a possible involvement of ABA-related pathways in smoke action, ABA (100 μM) and ABA (100 μM) + smoke-water (1:500) were also applied to maize kernels. ABA treatment caused a slower germination rate in the first half of the experiment but later on, the germination percentage was the same as for controls. However, smoke-water applied simultaneously with ABA resulted in significantly lower germination percentage throughout the course of the experiment.

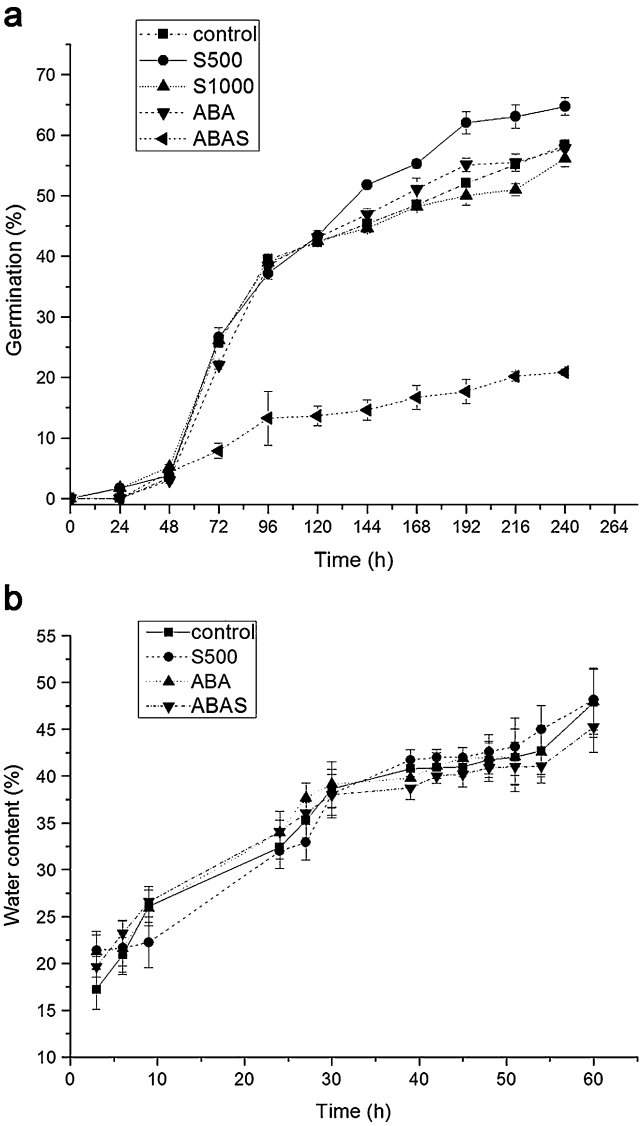

Fig. 1 Germination characteristics of MV5405 inbred maize kernels. **a** Germination time course of control and treated kernels. Treatments as follows: smoke-water 1:500 dilution (*S500*) and 1:1,000 dilution (*S1000*), ABA at 100 μM (*ABA*) and 100 μM ABA + 1:500 smoke-water (*ABAS*). **b** Relative water content of germinating maize kernels. Treatments, as above excluding the 1:1,000 smoke-water treatment

The water uptake of kernels treated with smoke-water (1:500 and 1:1,000 dilutions), ABA (100 μM) and ABA (100 μM) + smoke-water (1:500) were recorded to outline the early post-germination phase (or phase III of germination). Imbibition of the kernels was not affected by any of the treatments used, as water content was quite similar in all cases during the course of the experiment (Fig. 1b). The rapid initial imbibition ended after 24 h, when the coat started to rupture. Thereafter, the water content reached a plateau phase, and 48 h onwards, a massive increase in water uptake was observed which coincided with radicle elongation and emergence.

Effect of smoke-water on seedling vigour

Seedling vigour tests were carried out under two different temperature regimes. Applying smoke-water at both concentrations resulted in higher vigour compared to the control during both regimes (data not shown). When the chilling period was abandoned, the joint application of smoke and ABA slightly decreased the seedling vigour (data not shown). The effect of the smoke-water was, however, more pronounced if a short chilling period was introduced. The highest seedling vigour was observed in this experiment after treating the kernels with smoke-water at 1:500 dilution (Fig. 2a). These seedlings had significantly longer roots and shoots, more roots and they produced significantly higher dry mass after 9 days compared to the control (Fig. 2 b1–b4). If the smoke-water was applied together with 100 μM ABA, the vigour decreased compared to water or ABA alone. This negative effect on vigour was mainly attributed to the lower germination percentage and shorter root length.

Transcriptome profile of smoke response in the early post-germination phase

Based on the germination characteristics and imbibition tests, 24 and 48 h were chosen as the time points to collect samples for the microarray study (i.e. the earliest time points at the beginning of phase III of germination). The main objective of choosing these time points was that smoke-responsive genes would be difficult to ascertain at later stages following radicle emergence. In order to obtain homogenic samples in terms of developmental stage, embryos were collected either at 24 h when the embryos just ruptured the testa or at 48 h when radicles had reached a length of 3–4 mm. During this stage, the kernels had just entered phase III of germination which is characterised by rapid and pronounced water uptake. Genome-wide detection of smoke-responsive genes was performed by comparing smoke-treated kernels with control samples. The microarray platform used covers nearly 98% of maize genes. Thus, the transcriptome profile obtained represents approximately all the genes potentially involved in smoke action. According to the stringency levels (adjusted P value <0.05 and fold change ≥2), a total of 1,842 genes (721 up- and 1,121 down-regulated) showed differential

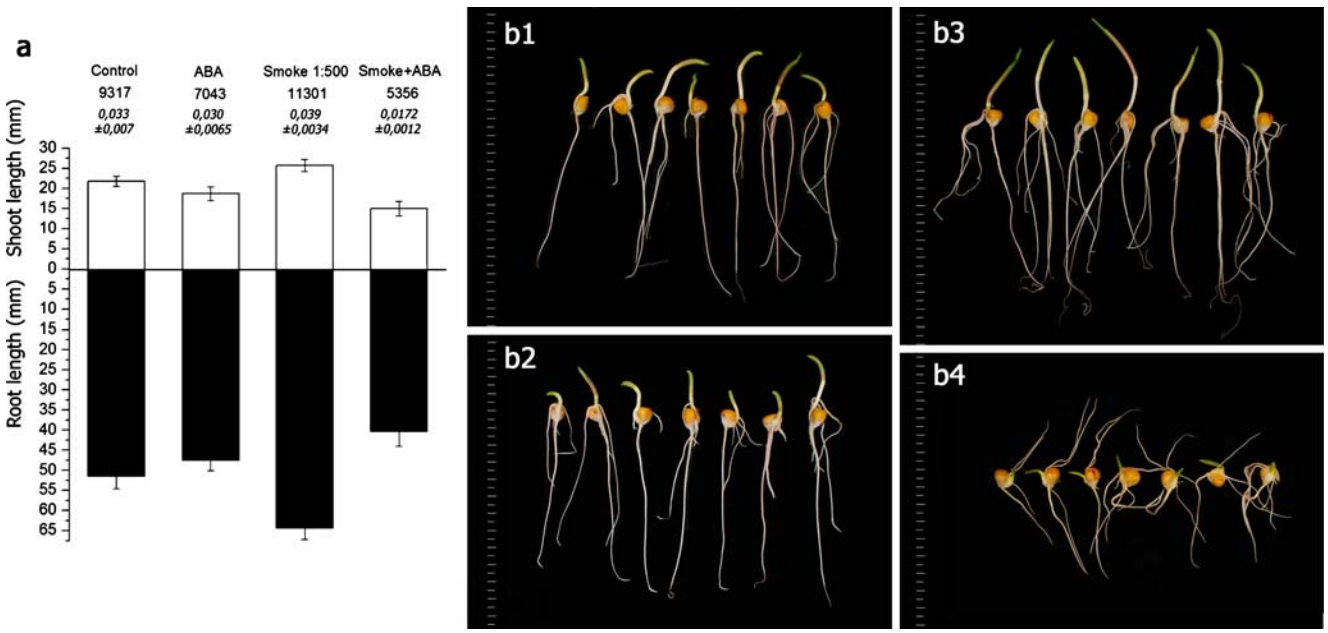

Fig. 2 Vigour of control and treated MV5405 inbred maize seedlings. **a** The vigour index is indicated below the treatment name. The seedling dry mass (g/seedling without kernel) and SE are in *italics*. *b1–b4* Morphology of 9-day-old control and treated seedlings. *b1* Water control; *b2* 100 μM ABA; *b3* 1:500 dilution smoke-water; *b4* 100 μM ABA + 1:500 dilution smoke-water

expression at 24 h. Likewise, 1,652 genes (887 up- and 765 down-regulated) showed differential expression at 48 h (see Supplementary Table S1). Tables 1 and 2 show the 20 most strongly up- and down-regulated genes in the 24 and 48 h experiments, respectively. At 24 h after smoke-water treatment, the transcript abundance of a sulfiredoxin-like protein (MZ00020514), putative LRR receptor-like kinase 2 (MZ00000704), ubiquitin-activating enzyme E1 1.

Table 1 Smoke-responsive genes in the 24 h experiment

Maize array ID	Fold change	Annotation
MZ00020514	27.9	Sulfiredoxin-like protein
MZ00000704	25.3	Putative LRR receptor-like kinase 2
MZ00041434	20.1	Ubiquitin-activating enzyme E1 1.
MZ00044608	10.8	DIE/ALG protein
MZ00021574	9.4	None
MZ00014903	9.4	Putative deoxyuridine triphosphatase
MZ00025151	9.0	Putative heat shock transcription factor
MZ00042191	8.5	*Zea mays* NAS2 like protein
MZ00048436	7.9	Transcription factor AP2D23-like
MZ00018324	6.5	None
MZ00014843	6.4	Putative methyl-binding domain protein MBD111
MZ00018343	6.4	None
MZ00041286	5.9	Putative actin-depolymerising factor
MZ00032953	5.9	Putative transcription factor E2F/dimerisation partner
MZ00004981	5.8	None
MZ00023856	5.7	Ribosomal protein L7Ae-like
MZ00027074	5.7	Copine III-like
MZ00032624	5.6	Glutathione S-transferase (GST) 39
MZ00023897	5.5	Putative DNA-binding protein GBP16
MZ00041376	5.5	Floral organ regulator 1
MZ00044400	−10.0	Cyclin type B-like
MZ00004143	−8.0	None
MZ00036429	−5.8	Parathymosin-like
MZ00035829	−5.7	None
MZ00033434	−5.7	Putative cellulase
MZ00042427	−5.1	None
MZ00046452	−4.9	Myb-like protein
MZ00032728	−4.5	None
MZ00024244	−4.5	Similar to splicing factor/activator protein
MZ00030105	−4.4	Contains tetratricopeptide repeat
MZ00004850	−4.4	Mini-chromosome maintenance protein
MZ00007375	−4.4	Putative 40S ribosomal protein S15
MZ00047595	−4.3	None
MZ00056286	−4.2	Poly-pyrimidine tract-binding protein-like
MZ00026860	−3.9	Fatty aldehyde dehydrogenase 1
MZ00005353	−3.9	None
MZ00015740	−3.9	None
MZ00032962	−3.8	Putative cyclic nucleotide-binding transporter 1
MZ00044097	−3.8	Putative holocarboxylase synthetase
MZ00020180	−3.8	None

Top 20 up- and down-regulated genes were selected and displayed

Table 2 Smoke-responsive genes in the 48 h experiment

Maize array ID	Fold change	Annotation
MZ00026223	19.6	Contains leucine zipper
MZ00033282	18.9	Expressed protein
MZ00035162	15.4	None
MZ00055869	11.6	None
MZ00036882	11.3	None
MZ00024571	10.9	Auxin-regulated protein
MZ00019209	8.8	Putative zinc finger protein
MZ00001222	8.3	None
MZ00047597	8.3	None
MZ00055822	8.2	None
MZ00048220	8.1	AP domain containing transcription factor
MZ00001329	8.1	None
MZ00041357	8.0	None
MZ00043490	7.6	Transport protein particle component Bet3-like protein
MZ00014903	7.5	Putative deoxyuridine triphosphatase
MZ00042879	7.5	Putative 60S ribosomal protein L37a
MZ00022728	7.4	None
MZ00036981	7.2	Transcription factor, MADS-box
MZ00018793	7.1	None
MZ00035697	7.1	Expressed protein
MZ00044747	−12.3	None
MZ00030535	−11.6	None
MZ00016593	−10.3	Putative vicilin
MZ00007309	−10.0	Putative calcium-dependent protein kinase
MZ00022141	−9.8	Receptor protein kinase PERK1-like protein
MZ00005478	−9.8	Deoxyribodipyrimidine photolyase
MZ00032617	−9.5	Putative Sm protein F
MZ00029468	−9.3	Putative geranylgeranyl diphosphate synthase
MZ00017582	−9.2	None
MZ00027351	−8.8	None
MZ00044410	−8.5	Putative glycerol 3-phosphate permease
MZ00031823	−7.5	NPK1-related protein kinase-like protein
MZ00005032	−7.2	Putative oxidoreductase, FAD-binding
MZ00019288	−7.2	Putative Rab geranylgeranyl transferase, a subunit
MZ00013558	−7.1	Protein kinase-like
MZ00043179	−6.7	Putative protein-L-isoaspartate *O*-methyltransferase
MZ00034227	−6.7	Putative NEC1
MZ00051069	−6.4	None
MZ00001125	−6.4	Putative peptide transporter
MZ00028057	−6.1	Putative TATA box binding protein-associated factor

Top 20 up- and down-regulated genes were selected and displayed

(MZ00041434), DIE2/ALG10 family protein (MZ00044608), heat shock factor RHSF3 (MZ00025151) and several unknown genes were amongst the highest up-regulated genes. Genes that were down-regulated at 24 h included a B-type cyclin homologue (MZ00044400), a parathymosin-like pro-

tein (MZ00036429) and a tetratricopeptide repeat containing protein (MZ00030105). The transcription profile of 48 h samples showed very few overlaps with the 24-h data. Amongst others, leucine zipper (MZ00026223) and zinc finger (MZ00019209) proteins, AP domain containing transcription factor (MZ00048220), auxin induced protein (MZ00024571) and genes with unknown function were the most abundant transcripts. Conversely, a putative vicilin (MZ00016593), a putative calcium-dependent protein kinase (MZ00007309), a putative glycerol 3-phosphate permease (MZ00044410) and other unknown genes were down-regulated at 48 h.

Validation of microarray data by quantitative real-time RT-PCR

To confirm the reliability of the microarray results, differential expression was corroborated for 12 genes using quantitative real-time reverse transcriptase PCR. The expression pattern observed in the microarray experiments was consistent with the genes analysed by real-time PCR (Fig. 3; Operon oligo identifiers are in brackets). As suggested by microarray results, the relative transcript abundance of an unknown gene similar to DIE/ALG protein (MZ00044608), the putative LRR receptor-like kinase 2 (MZ00000704), the cytochrome P450 gene (MZ00022704), sulfiredoxin-like protein (MZ00020514) and ubiquitin-activating enzyme E1 1. (MZ00041434) were up-regulated after 24 h of smoke-water treatment. The expression of the putative zinc finger protein (MZ00019209), the gene similar to basic leucine zipper family protein (MZ00026223), was up-regulated after 48 h whilst the unknown gene (MZ00030105) which contains a tetratricopeptide repeat was down-regulated. We tested the transcript abundance of several known stress or ABA-related genes, such as a putative MYB transcription factor (MZ00004101), calcineurin B-like protein 9 (MZ00024064), putative protein kinase (MZ00047075) and unknown gene (MZ00051069), which were up- or down-regulated in line with the expression pattern obtained from the microarray study.

Gene ontology analysis of differentially expressed genes

The genes up-regulated by more or equal to twofold due to smoke-water treatment (Supplementary Table S1) were associated with different Gene Ontology terms based on the Gene Ontology assignments available (Supplementary Table S3). As the 'biological function' functional category seemed to be the most informative in this case, the other two (i.e. 'molecular function' and 'cellular localisation') were excluded from further analysis. As revealed by the Gene Ontology annotation, the most pronounced differences in the gene expression pattern after smoke-water treatment correspond to translation and categories of

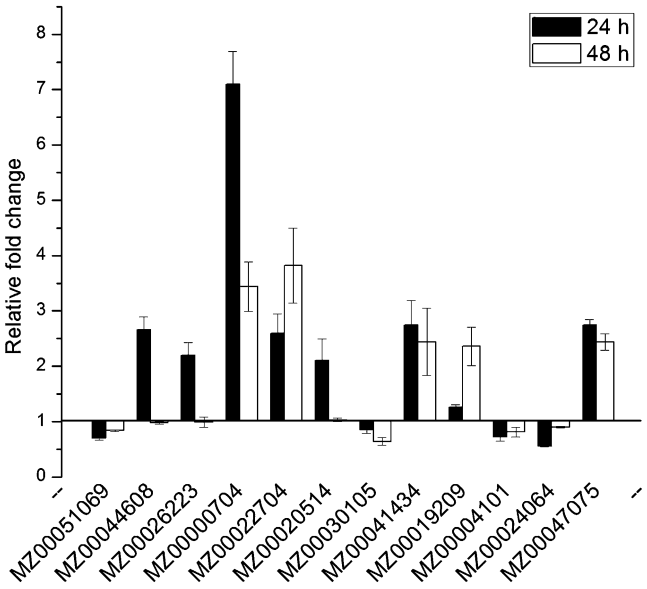

Fig. 3 Expression analysis of some selected genes in response to smoke treatment after 24 and 48 h. Relative transcript abundance (RQ, relative quantification number) was calculated and normalised with respect to the actin transcript level. At each time point, the control was set as the calibrator. Data shown represent the mean values obtained from four independent amplification reactions (n=4). The experiment was repeated three times with similar results. *Error bars* represent standard deviation. Operon oligo identifiers are shown: MZ00051069—unknown {*Oryza sativa* (japonica cultivar group)}; MZ00044608—unknown {*O. sativa* (japonica cultivar group)}, similar to DIE/ALG protein; MZ00026223—NA, similar to basic leucine zipper family protein; MZ00000704—putative LRR receptor-like kinase 2 {*O. sativa* (japonica cultivar group)}; MZ00022704—cytochrome P450; MZ00020514—sulfiredoxin-like protein {*O. sativa* (japonica cultivar group)}; MZ00030105—unknown protein {*O. sativa* (japonica cultivar group)}, contains tetratricopeptide repeat; MZ00041434—ubiquitin-activating enzyme E1 1. {*Triticum aestivum*}; MZ00019209—putative zinc finger protein {*O. sativa* (japonica cultivar group)}; MZ00004101—putative MYB transcription factor {*O. sativa* (japonica cultivar group)}; MZ00024064—calcineurin B-like protein 9 {*O. sativa* (japonica cultivar group)}; MZ00047075—putative protein kinase {*O. sativa* (japonica cultivar group)}

'embryonic development ending in seed dormancy', 'cell growth' and 'seed germination'. A number of Gene Ontology terms involved in stress and ABA responsiveness were highly enriched in the smoke-treated gene list. Gene Ontology terms such as 'response to cold', 'response to water deprivation', 'response to salt stress' and 'response to heat' were ranked the highest amongst smoke-responsive genes. The up-regulation of stress-related genes was robust and extensive amongst the responses identified in this work. Amongst the annotated genes, the stress-responsive and hormone-related genes accounted for the significant proportion of the smoke-responsive genes. At 24 h, genes involved in stress responses (cold, water deprivation, salt and osmotic stresses) were up-regulated and this tendency continued at 48 h, although to a lower extent. After 48 h, the enrichment of Gene Ontology terms such as 'electron

transport' and 'ribosome biogenesis and assembly' were the most prevalent but categories related to perception of environmental stimuli and abiotic stress like 'red and far-red signaling pathway', 'response to ozone' and 'response to hypoxia' were also over-represented. Surprisingly, various biotic stress-related terms such as 'detection of bacteria', 'response to insect', 'response to chitin', 'defense response signaling pathway' and 'systemic acquired resistance' were significantly enriched in the up-regulated gene list. However, the terms 'response to fungus' and 'response to wounding' were over-represented in the down-regulated gene list. In terms of hormone action, most genes showing up-regulation in both time points were related to ABA and ABA-mediated stress responses, whilst other hormone-responsive genes (such as auxin, ethylene, jasmonic acid-related genes) showed altered expression patterns. After 48 h, genes involved in the auxin-mediated signaling pathway were more prevalent in the Gene Ontology terms list. Ethylene and salicylic acid dependent systemic resistance-related genes were also abundant. However, salicylic acid-responsive genes were up-regulated after 48 h. As a general rule, fewer Gene Ontology terms were significantly enriched in the 48 h data, compared to the 24 h data. This might be attributed to the fact that at 48 h, a large number of genes and biological processes are in operation, and the microarray results contained more false positive and false negative hits, causing the Gene Ontology analysis to be less sensitive.

Promoter analysis of smoke-responsive genes

Cis-acting regulatory elements are the key points for the regulation of gene expression and the determination of a comprehensive set of smoke-related responses in the early post-germination phase provided an opportunity to search for elements common to their promoter region. These known promoter motifs were searched for in 1,500-bp regions upstream of the predicted start codon of the smoke-regulated genes. The *cis*-acting element composition (Supplementary Table S2) of smoke-responsive genes reflects similar characteristics and involvement of biological processes similar to that observed through the Gene Ontology annotation. As expected, motifs and binding sites related to organogenesis, meristem development and housekeeping functions were the most pronounced in the promoters of differentially expressed genes of smoke-treated maize. The Cab140 and the seed specific CANNTG motif occurred in a large proportion of the promoters investigated. Different stress motifs, especially biotic stress, cold- and dehydration-responsive elements (DREs), ABA/glucose signalling and ABA-related motifs, present the second largest group of the found motifs. Dehydration-responsive elements, ABA-responsive elements (ABREs) and ABRE/MYC recognition sites, Gt-box (pathogenesis related), Sp8b (at both time points), Sph and Sph-box motifs (at 48 h) are all over-represented after application of smoke-water. Similarly to Gene Ontology annotation, the incidence of stress and ABA-related *cis*-acting elements were higher at 24 h than at 48 h, but these motifs were very well represented at this time point also. At 48 h, WRKY1 was one of the most pronounced whilst at 24 h, ABA-related motifs were more abundant.

Discussion

Many reports have been published in the past few years describing the physiological effects of smoke treatments on seed germination, but only a few have discussed the possible mode of action (Van Staden et al. 2000). This is the first report in which the effect of smoke on improving seedling vigour and the post-germination phase are assessed with respect to the molecular background of the phenomena. In agreement with a previous investigation (Sparg et al. 2006), our results show that smoke can considerably increase seedling vigour and dry mass in the MV5405 maize inbred line. A similar improvement of seedling vigour was observed when smoke was applied to *Erica* species and species of *Asteraceae* (Brown 1993), South African indigenous medicinal plants (Sparg et al. 2005) and arable weeds (Daws et al. 2007). In addition, smoke treatment resulted in earlier germination, higher germination percentage in line with the findings of the vast majority of the publications discussing the effect of smoke on seed germination.

Smoke-water treatment considerably affected the transcription profile of young seedlings just entering the early post-germination stage. The common genes that were up-regulated by smoke-water have been described previously as stress-related genes, some of which have been well characterised in this regard (Zhu 2002; Kirch et al. 2005; Dreher and Callis 2007). Plant response to smoke in the early post-germination phase, according to the present microarray results, may be caused by reactions that are similar to those occurring during abiotic and biotic stress. Stress-related genes (cold, heat, drought, salt, wounding) are up-regulated and the abundance of ABA-stimulated transcripts were also pronounced. The proposed crosstalk between stress response pathways and the smoke response may be mediated by ABA. This finding is supported by the fact that besides the occurrence of promoter motifs involved in developmental regulation and stress-related events, the most frequent motifs were the ABA-related elements such as ABRE, ABRE/MYC recognition sites, Sph and Sph-box (at 48 h). Although these motifs can be found widespread in the promoter region of stress-related genes, this high

Table 3 The most significant Gene Ontology terms in the different gene lists

24 h				48 h			
Terms in up-regulated genes		Terms in down-regulated genes		Terms in up-regulated genes		Terms in down-regulated genes	
Nucleosome assembly	5	Response to salicylic acid stimulus	18	Negative regulation of gene expression, epigenetic	2	Mitochondrial fission	4
Translation	25	Response to absence of light	2	Ribosome biogenesis and assembly	7	*Pollen maturation*	*14*
Response to virus	10	Cellular response to water deprivation	3	Phloem histogenesis	2	*Defense response to fungus*	*18*
Nuclear mRNA splicing, via spliceosome	5	Polysaccharid biosynthetic process	3	Nucleosome assembly	3	*Protein amino acid auto-phosphorylation*	*19*
RNA-dependent DNA replication	3	Starch biosynthetic process	3	Nucleotide-excision repair	2	*Cell growth*	*16*
Chromosome organisation and biogenesis	3	Cell morphogenesis	3	Negative regulation of transcription by glucose	2	*Embryonic development ending in seed dormancy*	*25*
DNA replication initiation	3	Trichome morphogenesis	4	Developmental growth	2	*Transmembrane receptor protein tyrosine kinase signaling pathway*	*19*
Embryonic pattern specification	3	Translational elongation	3	Pollen wall formation	2	*Response to wounding*	*28*
N-terminal protein myristoylation	15	Sugar-mediated signaling	5	Translational elongation	3	*Brassinosteroid-mediated signaling*	*15*
Regulation of translation	3	Gamma-aminobutyric acid catabolic process	2	Base-excision repair	2	*Root hair cell differentiation*	*4*
Galactolipid biosynthetic process	2	Response to high light intensity	3	Red light signaling pathway	2	*Nucleotide metabolic process*	*2*
Response to desiccation	9	Plant-type cell wall biogenesis	6	Red or far red light signaling pathway	3	*Regulation of membrane potential*	*2*
Response to cold	33	Toxin catabolic process	3	*Focal adhesion formation*	*2*	*Cell morphogenesis*	*4*
Trehalose biosynthetic process	3	Response to cadmium ion	8	*Detection of bacterium*	*3*	*Cytokinesis by cell plate formation*	*6*
Oxygen and reactive oxygen species metabolic process	10	Thylakoid membrane organisation and biogenesis	3	*Response to hypoxia*	*2*	*Proteolysis*	*15*
Response to light stimulus	22	Abscisic acid biosynthetic process	2	*Response to insect*	*2*	*Defense response to bacterium, incompatible interaction*	*13*
Seed germination	10	Plant-type primary cell wall biogenesis	2	*Defense response signaling pathway, resistance gene independent*	*2*	*Response to high light intensity*	*4*
Histone phosphorylation	7	Protein ubiquitination during ubiquitin-dependent protein catabolic process	2	*Auxin-mediated signaling pathway*	*6*	*Protein amino acid phosphorylation*	*26*
Potassium ion import	5	Protein ubiquitination	4	*Cation transport*	*2*	*Growth*	*3*
Lipid metabolic process	4	Actin filament-based process	2	*Response to ozone*	*2*	*Cell proliferation*	*4*
Early endosome to late endosome transport	3	Protein neddylation	2	*Nitrate transport*	*2*	*Oligopeptide transport*	*5*
Protein targeting	2	*Cell cycle*	*2*	*Cell division*	*4*	*Actin cytoskeleton organisation and biogenesis*	*5*
Actin filament-based movement	2	*Anthocyanin biosynthetic process*	*4*	*Response to chitin*	*3*	*Actin filament-based process*	*3*
Response to water deprivation	23	*Protein modification process*	*3*	*Detection of ethylene stimulus*	*2*	*Cytoskeleton organisation and biogenesis*	*5*
Base-excision repair	2	*Response to reactive oxygen species*	*3*	*Salicylic acid-mediated signaling pathway*	*2*	*Root epidermal cell differentiation*	*5*

Table 3 (continued)

24 h				48 h			
Terms in up-regulated genes		Terms in down-regulated genes		Terms in up-regulated genes		Terms in down-regulated genes	
Phenylpropanoid biosynthetic process	9	*Regulation of transcription, DNA dependent*	*15*	*Abaxial cell fate specification*	*2*	*Response to fungus*	*21*
ATP-dependent proteolysis	5	*Regulation of meristem organisation*	*5*	*Proton transport*	*2*	*Coumarin biosynthetic process*	*4*
Indole phytoalexin biosynthetic process	3	*Ubiquitin-dependent protein catabolic process*	*10*	*Systemic acquired resistance, salicylic acid-mediated signaling pathway*	*3*	*Actin filament organisation*	*4*
Inter-Golgi cisterna vesicle-mediated transport	3	*Response to nutrient*	*2*	*Response to high light intensity*	*2*	*Sulphate assimilation*	*2*
Negative regulation of abscisic acid-mediated signaling	*6*	*Trichome branching*	*5*	*Circadian rhythm*	*4*	*Anastral spindle assembly involved in male meiosis*	*2*
Response to heat	*16*	*Proanthocyanidin biosynthetic process*	*5*	*Jasmonic acid and ethylene-dependent systemic resistance*	*3*	*Mitochondrion organisation and biogenesis*	*3*
Response to other organism	*12*	*Actin cytoskeleton organisation and biogenesis*	*3*	*DNA endoreduplication*	*3*	*MAPKKK cascade*	*4*

The terms in italic forms are statistically less significant but seem to be relevant

number of occurrence reflects the possible involvement of ABA-pathways in the smoke response. The co-suppressive effect of ABA and smoke-water on maize germination characteristics and seedling vigour further highlights this proposed crosstalk (Figs. 1a and 2). It is established that a single copy of ABRE is not sufficient for ABA-mediated induction of transcription, but multiple ABREs or the combination with coupling elements such as DRE can, together with ABREs, establish a minimal ABA-responsive complex (Narusaka et al. 2003) which is essential for ABA-related gene expression. DRE element was also over-represented after smoke-water treatment. DRE-related motifs have been reported in promoter regions of cold- and drought-inducible genes such as *KIN*, *COR6* and *RAB17* (Wang et al. 1995). The present results suggest that DRE-related motifs are involved in drought- and cold-responsive but ABA-independent gene expression. The Sp8b motif found in the promoters of genes involved in glucose/ABA signaling interplay (Rook et al. 2001) was also over-represented. Other motifs, such as the pathogenesis-related Gt-box and MYB binding site type I confer to biotic stress response which term could be found in the Gene Ontology annotation list. However, the results of the promoter analysis should be interpreted with caution and we used it as a guideline for additional experiments.

Our data demonstrated that in the early post-germination phase, smoke-water treatment resulted in the over-expression of stress and ABA-related genes (Table 3). It has been shown that a short time window exists during which the germinating seeds are supposed to recapitulate at least a part of a maturation program of seed development (Lopez-Molina et al. 2002; Rajjou et al. 2004; Holdsworth et al. 2008). This post-germination developmental arrest checkpoint is mediated by ABA and requires the ABI5 transcription factor in *Arabidopsis* (Lopez-Molina et al. 2002). The maturation drawback implies the expression of ABA-related genes (Rajjou et al. 2006) allowing the young plantlets to reinforce the capacity to cope with environmental stress factors. Besides, ABA-treated germinating seeds accumulate high levels of sucrose from the breakdown of storage lipid suggesting also that ABA may have a large effect on seedling development and vigour (Pritchard et al. 2002). ABA is known as a stress hormone which integrates cell stress responses in plant cells and as an essential mediator in triggering plant responses to adverse environmental stimuli (Chandler and Robertson 1994). This role is further emphasised by the fact that exogenous application of ABA is able to increase plant adaptive responses to various environmental conditions (Smith-Espinoza et al. 2005). Osmopriming of young tomato seedlings promoted vegetative growth during seedling establishment, which was reflected in plant vigour, crop yield and seed quantity (Albacete et al. 2008). Although ABA is recognised as a growth retardant, some studies indicate that application of exogenous ABA initially inhibits growth but after a short latency period, it increases the growth rate (Hall and McWha 1981). There are specific situations in which ABA is associated with growth (Barrero et al. 2005) and

this is especially true when the growth is an aspect of a stress response (De Smet et al. 2006). *Tagetes erecta* plants exposed to ABA exhibited reduced growth (leaves, stem, roots) but soon after plants exhibited increased growth in leaves and stem diameter which finally resulted in improved plant morphology and increased field survival (Aguilar et al. 2000).

Smoke not only promotes germination, but its effect extends beyond germination stimulation and can also act to enhance seedling vigour and survival after stresses (Sparg et al. 2005, 2006). These results, together with previous findings, suggest that smoke has a dual effect. On the one hand, it promotes a higher germination rate in an unknown way, and on the other hand, the concerted over-expression shows that smoke could induce stress and ABA-related stress-like responses in the early post-germination phase. This change in transcriptome is very similar to cold, drought and several biotic responses which are mediated by ABA and which may contribute to the higher seedling vigour. Presumably, smoke application on young seedlings may lead to better adaptation to environmental stress factors occurring during radicle emergence resulting in higher seedling vigour. Most of the transcripts affected by the smoke-water are regarded as stress-related messages showing that smoke indeed has a direct effect on gene expression which is quite similar to the pattern experienced following different stresses.

The hypothesis that formed the basis of the present study was that smoke interferes with stress and/or ABA-related signal transduction. This hypothesis is supported by the fact that the joint application of smoke and ABA on chilled stressed kernels resulted in decreased seedling vigour. The high incidence of ABA-related promoter motifs in the smoke responsive gene list also supported this finding. A key conclusion is that smoke can act as a hardening factor and its application can eventually lead to increased stress tolerance and growth. Master genes orchestrating smoke action in the early post-germination phase are still unknown. In the near future, extensive transcriptome analysis of kernels treated with smoke, ABA and exposed to different stresses will be carried out in our laboratory to draw the map of possible interactions of smoke- and stress-related pathways. In addition, if the presumptive active compounds will be available in sufficient quantity, an extensive transcriptome analysis will be carried out to distinguish the effects of the butenolide versus other compounds.

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