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RESEARCH PAPER

Role of a respiratory burst oxidase of *Lepidium sativum* (cress) seedlings in root development and auxin signalling

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Abstract

Reactive oxygen species are increasingly perceived as players in plant development and plant hormone signalling pathways. One of these species, superoxide, is produced in the apoplast by respiratory burst oxidase homologues (rbohs), a family of proteins that is conserved throughout the plant kingdom. Because of the availability of mutants, the focus of research into plant rbohs has been on *Arabidopsis thaliana*, mainly on AtrbohD and AtrbohF. This study investigates: (i) a different member of the Atrboh family, AtrbohB, and (ii) several rbohs from the close relative of *A. thaliana*, *Lepidium sativum* ('cress'). Five cress rbohs (Lesarbohs) were sequenced and it was found that their expression patterns were similar to their *Arabidopsis* orthologues throughout the life cycle. Cress plants in which *LesarbohB* expression was knocked down showed a strong seedling root phenotype that resembles phenotypes associated with defective auxin-related genes. These transgenic plants further displayed altered expression of auxin marker genes including those encoding the auxin responsive proteins 14 and 5 (IAA14 and IAA5), and LBD16 (LATERAL ORGAN BOUNDARIES DOMAIN16), an auxin-responsive protein implicated in lateral root initiation. It is speculated that ROS produced by rbohs play a role in root development via auxin signalling.

Key words: AtrbohB, auxin, *Lepidium sativum*, Rboh, reactive oxygen species, RNAi, root development, superoxide.

Introduction

Over the last decade, widespread developmental roles for the reactive oxygen species (ROS) superoxide, hydrogen peroxide, and hydroxyl radicals throughout the plant life cycle have emerged (reviewed by Gapper and Dolan, 2006; Swanson and Gilroy, 2010). All three of these ROS are known to be produced in the apoplast of plant cells where they can participate in pathogen responses (Torres *et al*., 2006), biophysical changes in cell walls (i.e. weakening or strengthening) (Schopfer *et al*., 2002; Müller *et al*., 2009*a*), and as mediators of systemic signalling (Miller *et al*., 2009).

Apoplastic superoxide can be produced by respiratory burst oxidase homologues (rbohs). These proteins are named after the homology of their catalytic domain to the mammalian respiratory burst oxidase subunit gp91^{phox} (Keller *et al*., 1998). Electron transport and superoxide production occur when gp91^{phox} accepts electrons intracellularly from NADH or NADPH and transfers them to extracellular O_2. Rbohs consist of only one polypeptide with a gp91^{phox}-homologous domain as a catalytic centre. They also contain six conserved transmembrane domains, a cytoplasmic FAD-binding domain and an N-terminal cytoplasmic domain with Ca^{2+}-binding EF-hand motifs (Keller *et al*., 1998).

The first role to be characterized for rbohs was in ROS production during pathogen defence (reviewed by Torres *et al*., 2006). Since then, multiple roles of rboh activity have been identified in a variety of processes, including seedling elongation growth (Frahry and Schopfer, 2001), tip growth of root hairs (Foreman

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et al., 2003; Monshausen *et al*., 2007) and pollen tubes (Potocky *et al*., 2007), seed germination (Müller *et al*., 2009*b*), and mechanosensing (Monshausen *et al*., 2009). Recently, the rbohs of *Medicago truncatula* have been linked to symbiotic nodule formation (Marino *et al*., 2011), and apoplastic superoxide as their reaction product has been linked to environmentally regulated allelopathic plant–plant interactions (Oracz *et al*., 2012). Rbohs thus seem to be involved in a large variety of developmental processes and responses to internal and external stimuli.

To date, ten rbohs (AtrbohA–AtrbohJ) have been identified in *Arabidopsis*. Of those, only three, namely AtrbohC, AtrbohD, and AtrbohF, have been formally characterized. *AtrbohC* was discovered as being allelic to *Root Hair Deficient2* (*RHD2*). The *rhd2* mutant exhibits impaired root hair growth, a phenotype associated with a reduced production of apoplastic superoxide (Foreman *et al*., 2003). The exact function of the apoplastic superoxide in root hair growth is unclear. However, a complex pattern of oscillating Ca^{2+}, pH, and ROS pulses accompanies the growth process (Monshausen *et al*., 2007). AtrbohD and F have functions in plant defence responses to biotic stresses (Torres *et al*., 2002; Torres and Dangl, 2005). They also function in guard cells (Kwak *et al*., 2003); *atrbohf/atrbohd* double mutants display an inhibition of the ABA-induced increase in ROS and cytosolic Ca^{2+} and subsequent stomatal closure. The protein encoded by *AtrbohD*, which is ubiquitously expressed in *Arabidopsis* plants, is involved in systemic signalling by apoplastic ROS, which is triggered by wounding as well as abiotic stresses such as heat, cold, high-intensity light, and high salinity (Miller *et al*., 2009).

AtrbohC and AtrbohD, together with AtrbohA and AtrbohG, belong to the same phylogenetic group as determined by the construction of a phylogenetic tree based on rboh protein sequences from five species (*Glycine max*, *Lotus japonicas*, *Oryza sativa, Arabidopsis thaliana*, and *Medicago sativa*) (Marino *et al*., 2011). According to the tree, AtrbohF belongs to a second group together with AtrbohI, while AtrbohB is part of a separate group and is the only *Arabidopsis* rboh in its group, as is AtrbohE. Finally, AtrbohH and AtrbohJ are in the last group. It is of interest to characterize the rbohs from different phylogenetic groups in order to get a more complete picture of rboh functions, and to determine if the different groups have acquired different functions during evolution.

Müller *et al*. (2009*b*) elucidated a function of AtrbohB in seed after-ripening. *AtrbohB* is alternatively spliced in seeds depending on the after-ripening status of the seeds and the presence or absence of exogenous ABA. Our studies of *AtrbohB* are now extended to a characterization of its expression in other tissues and in other plant species. In order to explore the evolutionary conservation of *rboh* sequences and expression patterns, we looked at a close relative of *Arabidopsis* in our study: the Brassicaceae *Lepidium sativum* (garden cress, 'cress'). Cress has several strategic advantages over *Arabidopsis*, such as seeds that are 40-50 times larger. Cress seedlings can easily be separated into their individual organs and tissues (Müller *et al*., 2009*a*; Linkies *et al*., 2009). Five cress *Rbohs* (*Lesarbohs*) from separate phylogenetic groups were completely or partially cloned and sequenced and RNAi-lines were created for which *LesarbohB* expression is knocked down. Our phenotypic analysis of the transgenic lines points to a role for RbohB in cress seedling root development and in fertility.

Materials and methods

Plant materials

Arabidopsis plants (Columbia wild type, WT), enhancer-trap line AtrbohB-GUS (line CS24365 in Liu *et al*., 2005), and *atrbohB,* (all lines described in Müller *et al*., 2009*b*) were grown in soil at 22 ºC under a 16-h photoperiod.

To generate the seedlings for stress treatments, mature seeds were plated on half-strength Murashige-Skoog (MS) media, pH 6.5, solidified with 1% agar and the plates were placed in germination conditions (22 ºC under a 16 h photoperiod). After 48 h, when all seeds had completed germination, the young seedlings were transferred to plates containing 2.5 mM H_2O_2 (oxidative stress), 150 mM NaCl (salt stress), or 6% mannitol (osmotic stress) for a duration of 48 h. As controls, seedlings were maintained for the 48 h on solid half-strength MS medium.

Lepidium sativum FR 14 and the *lesarbohB*-RNAi lines were grown in soil at 22 ºC under a 16-h photoperiod. Mature seeds were collected and plated on Petri dishes containing filter paper soaked with 6 ml of sterile water and the plates were placed in germination conditions (22 ºC under a 16-h photoperiod) to obtain seedlings.

GUS histochemical staining

AtrbohB-GUS and WT *Arabidopsis* materials at different stages in the life cycle were washed with deionized water, vacuum infiltrated with GUS staining solution (2 mM X-Gluc in 20 mM phosphate buffer, pH 6.2, 0.1% Triton X-100), and incubated overnight at 37 ºC with slow rotation. In some cases where low GUS activities occurred incubations were conducted for up to 3 d. The plant/seedling tissues were then transferred to 80% ethanol for several hours for destaining. The ethanol was exchanged as required. Destained plants were photographed on a lightbox with a Nikon Coolpix 4500 digital camera. WT plant and seedling tissues served as a negative control.

Cloning of Lesarbohs

Primers for the cloning of *Lesarboh* cDNAs were designed with the primer3 (Rozen and Skaletsky, 2000) tool in Geneious 4.8.5 based on *Arabidopsis* sequences as well as conserved domains of rbohs from different species. RT-PCRs were conducted on RNA extracted from cress seedlings and seeds. PCR fragments were cloned into pGEM-Teasy-vectors (Promega, Karlsruhe, Germany), propagated in *E. coli*, extracted with a miniprep kit (Fermentas/ThermoScientific) and sequenced. Geneious software was used for alignments of sequences. 5'- and 3'-RACE was conducted with the corresponding Invitrogen kits (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Sequences have been submitted to Genebank under the following accession numbers: *LesarbohA* JX312066, *LesarbohB* JX312067, *LesarbohD* JX312068, *LesarbohF* JX312069, and *LesarbohH* JX312070.

Generation of transgenic plants

Two fragments of the *LesarbohB* cDNA were amplified using the primer pairs RNAiB1for (5'-GGGG-attB1-GATGCAAAGCCACTGGTTCA-3')/RNAiB1rev (5'-GGGG-attB1-CCAAAATTCTGATAATTCAGTTTT-3'), fragment length 437 bp, and RNAiB2for (5'-GGGG-attB1-CCCTAAATCCAAATTTGGACAT-3')/RNAiB2rev (5'-GGGG-attB1-GGATCGAGTTCTTCCATGATT-3'), fragment length 330 bp. The fragments were cloned into Gateway vector pB7GWIWG2(I),0 via pDONR221 using the BP and LR cloning system (Invitrogen, Karlsruhe, Germany) as described in the manufacturer's manual. The vectors were sequenced and cress plants were transformed by the floral dip method as described for *Arabidopsis* plants (Clough and Bent, 1998), but with

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only 0.01% (v/v) Silwet in the dipping solution. Transgenic T_1 plants were selected by spraying BASTA on 7-d-old seedlings and repeating the spraying twice more after 4 d and 7 d.

Characterization of the fertility of transgenic plants

Reciprocal crosses between WT plants and transgenic *lesarbohb-RNAiB1* and *lesarbohB-RNAiB2* plants, respectively, were performed by transferring the pollen with a thin brush to the stigma of plants whose anthers had been removed to prevent self-fertilization. All flowers of eight WT-plants were pollinated with pollen from *lesarbohb-RNAiB1* and *lesarbohB-RNAiB2* plants, respectively. Reciprocally, all flowers on five *lesarbohb-RNAiB1* and 5 *lesarbohB-RNAiB2* plants were pollinated with WT pollen. The seed yield was counted after natural drying of the siliques on the plants.

RNA extraction and cDNA synthesis

Tissues of *Arabidopsis* or cress were ground in liquid nitrogen and total RNA was extracted as described in Chang *et al*. (1993) with the following modifications: After the addition of CTAB buffer (2% hexadecyl trimethyl-ammonium bromide [CTAB], 2% polyvinylpyrrolidone [MW=40 000/K30], 100 mM TRIS-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 2 M NaCl, 2% β-mercaptoethanol), the extracts were kept at 65 °C for 10 min. All chloroform:isoamylalcohol extractions were repeated twice. RNA was treated with DNase-I (Fermentas, Burlington ON, Canada) to remove any remaining genomic DNA. RNA was run on an agarose gel to check for degradation, and the quantity and purity of the RNA samples were determined with a nanodrop spectrophotometer (ND-2000C, ThermoScientific, Mississauga ON, Canada). Two micrograms of RNA were reverse transcribed using the EasyScript Plus kit (abmgood, Richmond BC, Canada) with a mixture of random hexamers and oligo-dT primers. cDNA from four biological replicate RNA samples was used for qRT-PCR.

qRT-PCR

qRT-PCRs were run in 15 µl reactions on an ABI7900HT machine (Applied Biosystems, Carlsbad CA, USA) using the PerfeCTa Sybr Green Supermix with ROX (Qanta Biosciences, Gaithersburg MD, USA). Primers were designed with the primer3 (Rozen and Skaletsky, 2000) tool in Geneious 4.8.5. The reaction mixture consisted of 150 ng cDNA (RNA equivalent), 7.5 µl supermix and 140 nM of each primer and were subjected to a temperature regime of 3 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A dissociation curve was run after each qPCR to validate that only one product had been amplified in each well.

The efficiency *E* of the primer pairs was calculated as the average of the *E*s of the individual reactions by using raw fluorescence data with the publicly available PCR Miner tool (http://www.miner.ewindup.info/version2) (Zhao and Fernald, 2005). The efficiency was then used to calculate transcript abundance for the individual samples as $(1+E)(-CT)$. No-template-controls were included for each primer pair to check for contamination of the reaction solutions, and no-RT-controls were used to check for genomic DNA contamination in the RNA. Actin 7 (ACT7; At5g09810) and elongation factor 1-α (EF1α; At5g60390) were used as reference genes. For both *Arabidopsis thaliana* and *Lepidium sativum*, species-specific gene primers were created and used as described in Graeber *et al*. (2011).

Determination of superoxide production in seedling roots

Superoxide production was measured by photometric determination of the reduction of XTT (3'-[1-phenylamino-carbonyl]-3,4-tetrazolium]-*bis*[4-methoxy-6-nitro] benzenesulphonic acid hydrate) (Polysciences Inc., Eppelheim, Germany) as described in Müller *et al*. (2009*b*) with the following modification: eight whole seedlings were collected for each sample in 10 mM phosphate buffer, pH 6.5. As the hypocotyls and cotyledons are covered by a cuticle, only the superoxide production from the root was measured. This was confirmed by visual inspection of the samples, which only showed red staining (indicative of XTT-reduction) in the roots.

Results

Cloning and expression analyses of Rbohs of cress

Using primers based on consensus sequences of conserved regions of *Rboh* genes from different species, or on the *Arabidopsis* gene sequences, two complete cress *Rboh* cDNAs were cloned. These were named *LesarbohB* (JX312067) and *LesarbohF* (JX312069) after their closest *A. thaliana* orthologues (Fig. 1). The predicted protein based on the *LesarbohB* cDNA shares 88% of its amino acid sequence with AtrbohB, which is also the highest hit in a pBLAST search. On the nucleotide level, there is 87% similarity between the two sequences. The closest pBLAST match for LesarbohF was a protein from *Arabidopsis lyrata*. By contrast, the highest BLAST hit on the nucleotide level was for rbohF of *A. thaliana,* in which there was 90% similarity. All of the

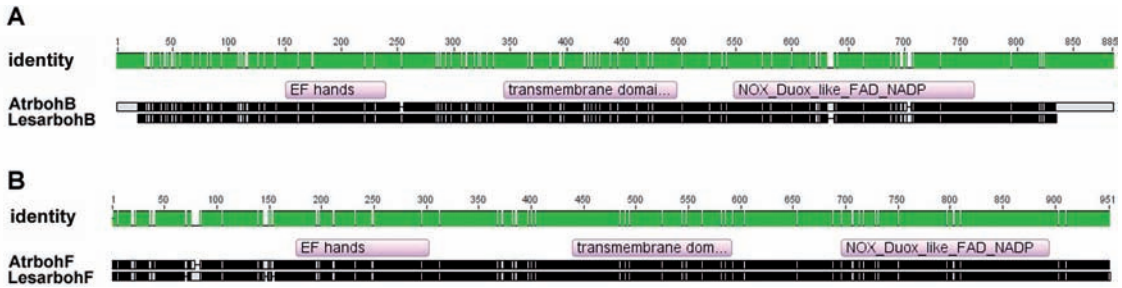

Fig. 1. Alignment of the deduced amino acid sequences of the AtbohB and LesarbohB cDNAs. (A) Alignment of the AtrbohB amino acid sequence with that of its putative ortholog LesarbohB. (B) Alignment of the AtrbohF amino acid sequence with that of its putative orthologue LesarbohF. EF hand, transmembrane, and NOX/Duox like FAD/NADP binding domains of RbohB and RbohF of the two plant species were determined with the algorithm DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST) and are indicated in the alignment. Note that the amino acids are missing at the LesarbohB termini due to missing bases in the sequencing. Both the green and black sections in the sequences represent identical amino acids between the two proteins. The top panel with green filling also shows amino acid numbers. For an enlarged alignment detailing the specific amino acid residues, see Supplementary Fig. S1 for RbohB and Supplementary Fig. S2 for RbohF at *JXB* online.

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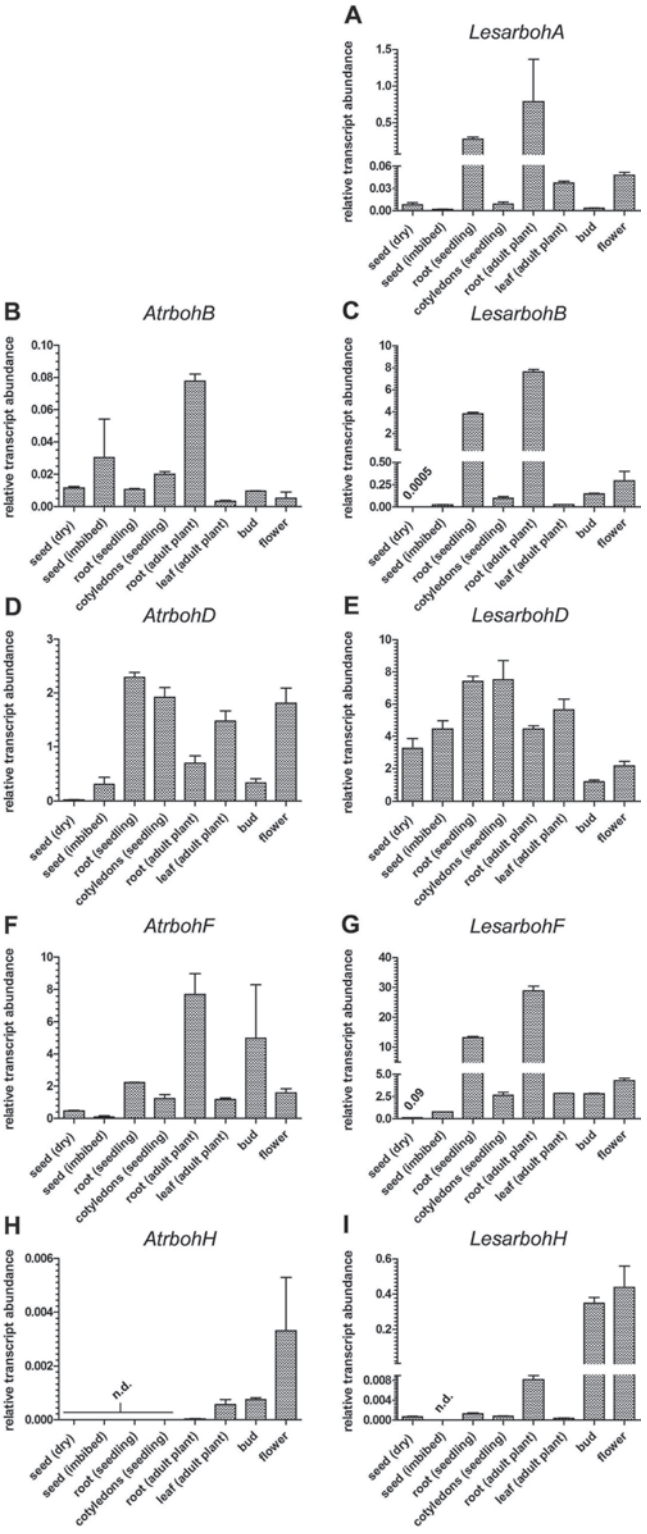

Fig. 2. Expression analysis of Lesarbohs and their *Arabidopsis* respiratory burst oxidase orthologues. Transcript abundance analysis for five members of the gene family determined by qRT-PCR in different plant tissues of *L. sativum* and *A. thaliana*. Note the similarities in intensity and expression profile between the two species. Averages of three biological replicates ± SE are presented. For the imbibed seed samples, seeds were harvested after 24 h on water in continuous light. Other samples included 5-d-old seedlings (5 d post-imbibition), and mature adult plants harvested 4 weeks post-germination. No *AtrbohA* transcript was found in any of the stages tested. Transcript abundance was corrected to the abundance of transcripts associated with the standard genes—the constitutive genes *Actin 7* (*ACT7*; At5g09810) and *Elongation Factor 1α* (*EF1α*; At5g60390) for *Arabidopsis* and the corresponding genes (*ACT7*, HQ436350.1 and *EF1α*, HS981853.1) for *L. sativum*. n.d.=not detectable.

major domains that rbohs are known to possess could be identified: EF-hands, transmembrane domains, and the catalytic site with its FAD and NADH/NADPH-binding pockets (Fig. 1; see Supplementary Figs. S1 and S2 at *JXB* online).

Partial sequences for *LesarbohA* (JX312066), *LesarbohD* (JX312068), and *LesarbohH* (JX312070) were also obtained; these served as the basis for conducting qPCR analyses of gene expression. The five Lesarbohs (A, B, D, F, and H) belong to four of the five different groups on the phylogenetic tree of Marino *et al*. (2011) based on their sequence similarity to Atrbohs. Transcripts of these *Lesarbohs* were quantified by qRT-PCR analyses to examine their expression patterns and were compared with those exhibited by their *Arabidopsis* orthologues at different stages of the life cycle (Fig. 2). The orthologous genes of cress showed similar expression profiles: the *RbohD* and *F* orthologues were most strongly expressed in both species, followed by that corresponding to *RbohB*. In *Arabidopsis*, expression intensity of the transcripts differed by two orders of magnitude; differences were less pronounced in cress. *RbohH* showed weak or no expression in the various organs of both species. *RbohA* showed weak expression in cress; *RbohA* transcripts were undetectable in all tissues of *Arabidopsis* examined. This is in agreement with expression data published in the developmental map of the *Arabidopsis* eFP browser (Winter *et al*., 2007), which indicates that expression is exclusively in developing seeds.

The expression patterns throughout the life cycle were also very similar between the two species (Fig. 2): *RbohD* showed strong constitutive expression, while *RbohF* was expressed strongly during all post-germinative stages and moderately in early-imbibed mature seeds. *RbohH* transcripts were found at low levels in roots and buds; maximum expression occurred in flowers. *RbohB* expression was low in mature seeds, but increased upon imbibition, and was most strongly expressed in seedlings and roots of adult plants in both species. *Arabidopsis*, but not cress, showed strong expression of *RbohB* in seedling cotyledons.

The expression patterns of *AtrbohB* throughout the life cycle of *Arabidopsis* was confirmed by histochemical analysis of an enhancer trap line, with a GUS coding sequence inserted 260 bp upstream of the start codon for AtrbohB (Müller *et al*., 2009*b*). GUS staining showed that expression was limited to mature embryos (Müller *et al*., 2009b), seedling hypocotyls and cotyledons, as well as to seedling root tips (Fig. 3). Within root tips, staining occurred mostly in the root cap and meristem areas, where cells do not undergo any major elongation (Fig. 3). GUS histochemical staining patterns corresponded well with the qRT-PCR data (Fig. 2). An exception was in relation to *AtrbohB* expression in adult roots (Fig. 2), which was not

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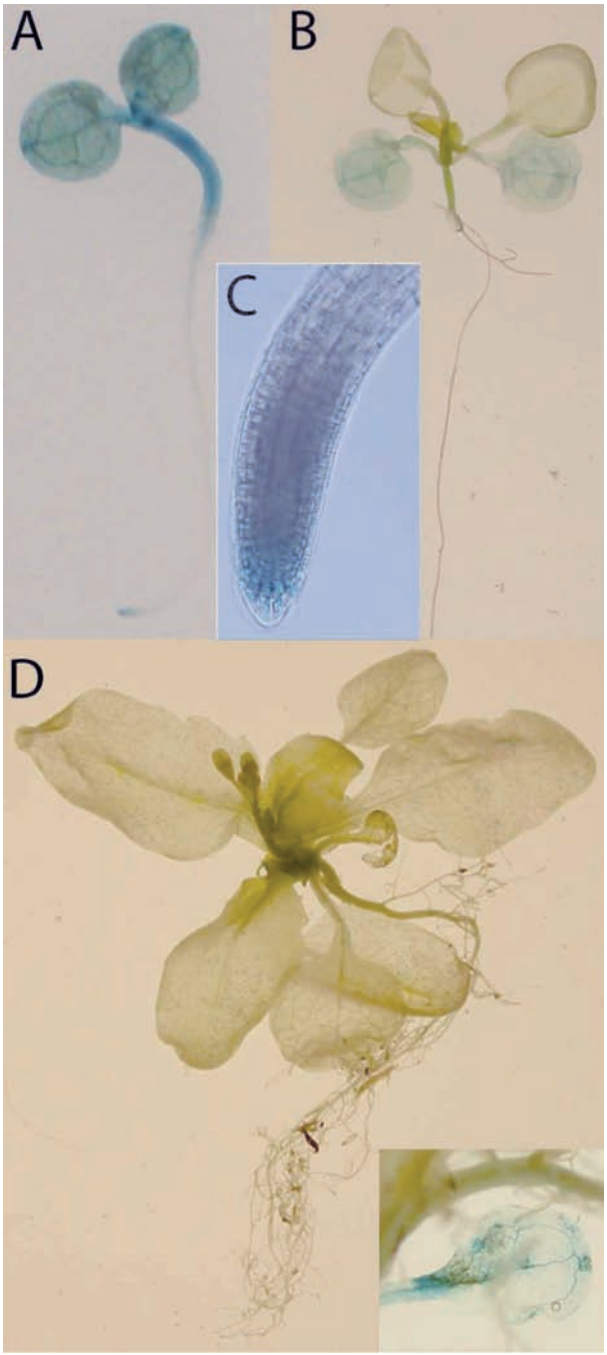

Fig. 3. GUS histochemical staining of seedlings and plants derived from an enhancer trap line with an insertion 300 bp upstream of the AtrbohB start codon. (A) Four-day-old seedling with staining in cotyledons, hypocotyl, and root tip. (B) Ten-day-old plantlet. Staining remains visible in cotyledons. (C) Enlarged image of root tip of 4-d-old seedling. Note that staining is confined to the cells which do not undergo elongation. (D) Plant at 24 d. No major areas of the adult plant show staining. The inset image shows that the cotyledons are still stained.

evident by histochemical staining (Fig. 3). Seedlings showed GUS-expression in the cotyledons even after several true leaves had been formed (Fig. 3).

Intron retention in the *AtrbohB* gene is regulated by developmental and hormonal cues in *Arabidopsis* seeds (Müller *et al*., 2009*b*). Therefore it was investigated if alternative splicing was evident in seedling tissues in which *AtrbohB* gene expression occurred (Figs 2,3). Alternative splicing of *AtrbohB* and *LesarbohB* transcripts was examined in response to ABA treatment of seedlings and seeds, and in response to stress treatments of seedlings (salt stress: 150 mM NaCl; osmotic stress: 6% mannitol, and oxidative stress: 2.5 mM H_2O_2) compared with the controls. Analyses conducted on *Arabidopsis* confirmed the phenomenon of alternative splicing of *AtrbohB* in the ABA-treated seeds; however no alternative splicing was observed in stress-exposed *Arabidopsis* seedlings. Further, no alternative splicing of the *LesarbohB* transcripts occurred in cress seeds and seedlings under any of the conditions.

Knock-down of Lesarboh B expression leads to reduced fertility and abnormal seedling root development in cress

In order to elucidate putative developmental roles of LesarbohB, the expression of the *LesarbohB* gene in cress was knocked down using two different RNAi-constructs, RNAiB1 and RNAiB2. Although different in length, both constructs target the sequences specifying a unique portion of the N-terminus of LesarbohB. Expression of both constructs had the same developmental effects. Following *Agrobacterium*-mediated floral dip transformation and BASTA selection, several independent seed-bearing lines were generated for each construct. BASTA-resistant T_1-plants for both constructs produced less seeds than WT plants grown in parallel, with the transgenic lines producing an average of 19±4 seeds per plant as compared to 54±9 for the WT. Twenty-two per cent of the BASTA-resistant plants flowered, but produced no seeds; these were not included in the seed number calculation. In order to narrow down the possible cause of the reduced fertility, reciprocal crosses were made between the transgenic T_1 plants, which are expected to be hemizygous, and WT plants. Pollinating a transgenic plant with WT pollen significantly increased the seed number compared with plants that were allowed to self-pollinate, and yielded an average of 35±7 seeds per plant. When WT plants were fertilized with pollen derived from transgenic plants, the seed numbers were fewer (24±8). It was therefore hypothesized that the pollen of the transgenic plants carrying the RNAi-construct may be impaired in its ability to effect pollination or fertilization. No other visible phenotype was identified in the adult plants and the dormancy and germination of seeds produced on plants displaying reduced fertility did not differ from the WT.

Strikingly, many of the T_2 seedlings from transgenic lines of *lesarbohb-RNAiB1* and *lesarbohB-RNAiB2* showed severe developmental defects of their roots that appeared at about 5 d after germination: the lower part of the root was defective in tissue organization and turned into a thin thread-like structure with a thickened root tip (‘stage 1’; Fig. 4B). The thickened root tip fell off after a few days, and the root developed irregular lateral roots and callus-like tissues (‘stage 2’; Fig. 4C-E). Most of the affected seedlings dried out and died after transfer to soil.

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A comparison of the *LesarbohB* transcript level between WT-seedlings versus T_2 seedlings with WT-like roots versus those with severely affected roots showed that expression was strongly reduced in the latter (Fig. 4G). This was associated with reduced superoxide production in the affected seedlings: superoxide production as measured by an XTT assay was significantly reduced in stage 1 seedlings. The generation of this ROS was higher once the unstructured lateral root growth set in and stage 2 was reached, but did not reach WT levels (Fig. 4F). There is no phenotype of comparable severity in any *atrboh* single mutant (Müller *et al*., 2009*b*). The expression of the four other *Lesarbohs*, for which the respective sequences were cloned, was therefore tested. No significant changes in *LesarbohA*, *LesarbohD*, and *LesarbohH* transcript levels occurred; however, seedlings with defective root development showed a lower expression of *LesarbohF* than WT and unaffected seedlings (Fig. 4G). The effect on *LesarbohF* expression was much less pronounced than the effect on *LesarbohB* expression.

The root phenotype associated with the RNAi lines strongly resembled that connected with defective auxin-modulated processes (see Discussion). Therefore it was tested if *Lesarboh-B* and *-F* expression could be stimulated by auxin in WT seedlings. However, no statistically significant (P <0.05) change of expression was observed in seedling roots or shoots in response to 1 h or 24 h exposure to the synthetic auxin 2,4-D (data not shown). *LesarbohB* and *LesarbohF* are therefore likely not directly regulated by auxin. It was also of interest to determine if the expression of auxin-marker genes (Paponov *et al*., 2008) was altered in the roots and shoots of seedlings of the RNAi lines that displayed the abnormal phenotypes. The expression of the marker genes was compared in the RNAi seedlings whose roots were in stages 1 and 2 of the phenotype (RNAiB stage1/2), with transgenic

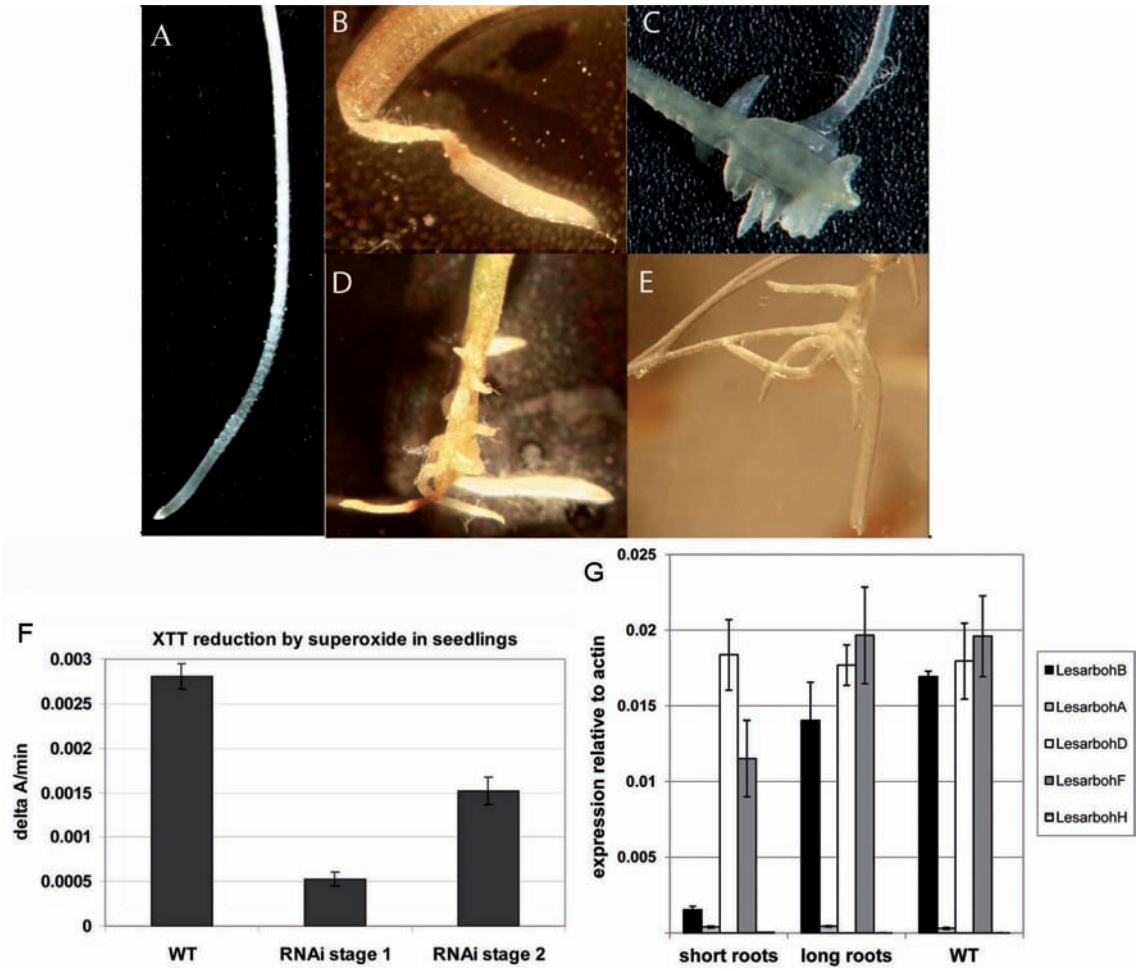

Fig. 4. Root phenotype of seedlings derived from *Lesarboh*-RNAi-knock-down lines. (A) Root of WT seedling. (B) Root of *lesarbohB-RNAiB1* seedling 5 d after completion of germination: representative of phenotype stage 1. The lower part of the root is defective in tissue organization and turned into a thin thread-like structure with a thickened root tip. (C–E) Roots of *lesarbohB-RNAiB1* seedlings, 10 d after completion of germination: representative of phenotype stage 2. The thickened root tips have fallen off, and the roots have developed irregular lateral roots and callus-like tissues. (F) Superoxide production as measured by XTT-reduction in WT seedlings and in the RNAi-seedlings at stages 1 and 2 of the root phenotype as indicated. (G) Expression of *Lesarbohs* in T_2 seedlings that showed the root phenotype ("short roots") versus seedlings that have WT-like roots ('long roots'). Transcript abundance was corrected to the abundance of transcripts associated with the *L. sativum* standard genes *Actin 7* (*ACT7*, HQ436350.1) and *Elongation Factor 1*α (*EF1*α, HS981853.1). Averages of 5 biological replicates ± SE are shown.

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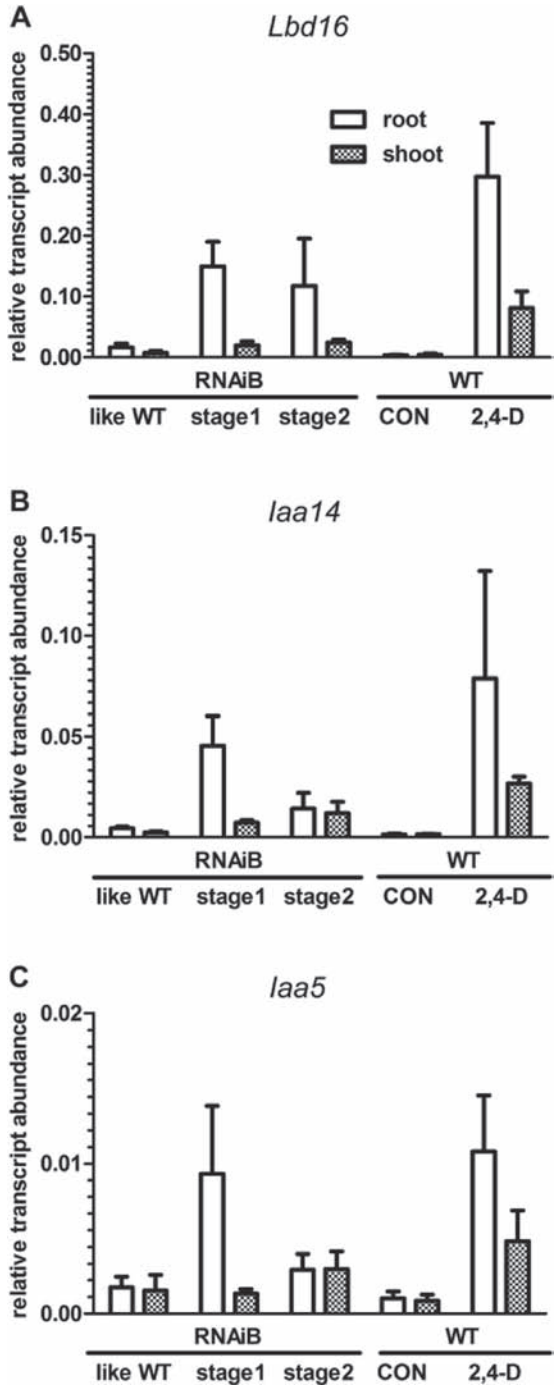

Fig. 5. Expression of auxin-related genes in roots and shoots of *L. sativum* seedlings of wild-type and *Lesarboh*-RNAi knockdown lines. Transcript abundance for the genes encoding the LOB-domain containing protein (LBD) 16 (A), the auxin-responsive-protein, IAA14 (B) and the auxin-responsive protein IAA5 (C) as determined by qRT-PCR. Stage 1 corresponds to the phenotype shown in Fig. 4B, while stage 2 corresponds to the phenotype shown in Fig. 4C–E. Transcripts in wild-type seedlings were examined under 2,4-D treatment (24 h on 1 μM 2,4-D) and control conditions. Transcript abundance was corrected to the abundance of transcripts associated with the standard genes – the constitutive genes *Actin 7* (*ACT7*; At5g09810) and *Elongation Factor 1α* (*EF1α*; At5g60390). Averages of three biological replicates ± SE are presented.

seedlings of equivalent age whose roots did not show a phenotype (RNAiB-likeWT), and with WT seedlings of equivalent age (Fig. 5). All transcripts were auxin-inducible as tested by the exposure of seedlings to 1 μM of the synthetic auxin 2,4-D for 24 h (Fig. 5, and data not shown). No differences in expression between WT and RNAi-lines were observed for the genes encoding the auxin transporter PIN1, the auxin-response factor ARF5, and the downstream effectors CKX6 (a cytokinin oxidase), and LBD29 (LATERAL ORGAN BOUNDARIES DOMAIN29). However, there were clear differences in the expression levels of genes encoding other auxin signalling components—auxin responsive proteins 14 and 5 (IAA-14 and -5), and LBD16, an auxin-responsive protein implicated in lateral root initiation (Okushima *et al*., 2007). *IAA5* and *IAA14* transcripts were induced in roots and shoots of the RNAi-seedlings in stages 1 and 2, with *IAA5* showing strong induction particularly during stage 1 (Fig. 5C). *LBD16* was induced strongly in roots of the *lesarbohB-RNAi* line (stages 1 and 2) compared with its control counterparts (WT- and RNAiB-like WT-seedlings); induction in shoots was less marked (Fig. 5A). The addition of exogenous auxin (0.5–2.5 μM 2,4-D or IAA) to the growth medium of WT *Lepidium* seedlings did not mimic the *lesarbohB RNAi* phenotype: exogenous auxin led to shortened roots, but did not produce the impaired root development that was observed in our RNAi-lines (data not shown). It was hypothesized that this is caused by the fact that the RNAi-effect is specific to the cells where *RbohB* is usually expressed, while exogenous auxin affects all cells in the root.

Discussion

Rbohs have been identified and described in a number of angiosperm species such as those belonging to the Brassicaceae (*Arabidopsis thaliana*) (Keller *et al*., 1998; Torres *et al*., 1998), the Solanaceae (*Solanum tuberosum* and *S. lycopersicum*; potato and tomato) (Kobayashi *et al*., 2006; Sagi *et al*., 2004), and in various other dicotyledonous and monocotyledonous plants such as *Medicago truncatula* (Marino *et al*., 2011), *Citrullus colocynthis* (Si *et al*., 2010), *Nicotiana benthamiana* (Yoshioka *et al*., 2003), *Oryza sativa* (Groom *et al*., 1996), *Hordeum vulgare* (Trijillo *et al*., 2006), and *Zea mays* (Lin *et al*., 2009). Sequence information retrieved from the NCBI databases shows that rbohs also exist in a number of other angiosperms, as well as in other evolutionary groups such as gymnosperms, the lycophyte *Selaginella moellendorffii* and the moss *Physcomitrella patens*.

While very similar expression patterns were observed between the orthologuous *rbohs* of cress and *Arabidopsis*, the expression patterns of *rbohs* from other more evolutionarily distant species (e.g. rice, alfalfa, and soybean) cannot be predicted based on their phylogenetic groups classified by Marino *et al*. (2010). For example, the rice *Rboh* genes, *OsrbohD* and *OsrbohE*, which are normally grouped with *AtrbohH* (an *Rboh* with an expression maximum in mature flowers of *Arabidopsis*), exhibit strong expression peaks in seeds and leaves, respectively, and only *OsrbohE* shows expression in flowers according to the rice eFP-browser (data from Jain *et al*., 2007).

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It was of interest to elucidate the putative physiological function of LesarbohB by knocking-down the expression of the corresponding gene, a process which appeared to down-regulate at least one additional *Lesarboh* gene. The two major phenotypes that were observed in our RNAi-lines were reduced fertilization success and abnormal seedling root development. The flowers of the transgenic plants were anatomically normal, but their pollen was markedly less successful than WT pollen in terms of its ability to effect pollination or fertilization. This might be due to a reduced expression of *rbohs* in the pollen, as ROS play a role in pollen tube initiation (Speranza *et al*., 2012) and elongation (Potocky *et al*., 2007). In fact, an antisense construct against *rboh*s leads to decreased ROS production and pollen tube elongation in *Nicotiana tabacum* (Potocky *et al*., 2007), and reduced pollen tube initiation and elongation would indirectly affect fertilization success by preventing the pollen from reaching the egg cell. It is speculated that this is the case in our RNAi lines.

The root phenotype that we observed in the RNAi transgenic seedlings has not been described so far for a plant affected in *Rboh*-expression. Two stages in the defective root phenotype were noted: the first stage was characterized by the disintegration of the root leaving a thickened root tip that eventually died. This resembles the phenotype described for over-expression of genes encoding the serine/threonine kinase PINOID (Benjamins *et al*., 2001), so named in turn for its resemblance to the *pin* mutants, which are affected in polar auxin transport. PINOID is a positive regulator of polar auxin transport to the root tip.

The second stage of the root phenotype – the unstructured lateral root emergence – is somewhat reminiscent of the phenotype observed by Pasternak *et al*. (2005) in roots of seedlings that have been exposed to chemicals that cause oxidative stress, as well as in auxin-connected phenotypes. Over-expression of the auxin-response factor ARF5 in whole seedling roots or pericycle cells of a *solitary-root* (*slr)*-mutant background is associated with an inability of cells to initiate lateral roots because of a decreased auxin sensitivity; this leads to irregularly spaced and fused lateral roots (De Smet *et al*., 2010). Indeed these root characteristics were also observed in our RNAi transgenic lines. Irregular lateral root formation also occurs in the *superroot* (*sur1)*-mutants, mutants associated with increased endogenous auxin levels (Boerjan *et al*., 1995). Our transgenics thus exhibit a phenotype that resembles an enhanced response of the root to auxin.

The known functions of AtrbohD and AtrbohF include ABA-, methyl jasmonate-, and ethylene-regulated guard cell closure (Kwak *et al*., 2003; Suhita *et al*., 2004; Desikan *et al*., 2006). These studies were the first to suggest a role for rboh-mediated superoxide production in hormone signalling pathways (reviewed by Kwak *et al*., 2006). The notion that ROS play important roles in a variety of hormone signalling pathways as well as in the integration of hormone signals was confirmed by a number of subsequent studies (reviewed by Mori *et al*., 2009). Interestingly, apoplastic ROS down-regulates auxin signalling in *Arabidopsis* (Blomster *et al*., 2011). Given that the RNAi-transgenic plants of the present study have reduced apoplastic superoxide production, it is possible that the normal processes that down-regulate auxin signalling are affected, which might explain the similarity of the RNAi lines to phenotypes found in auxin over-producers. In conjunction with this an up-regulation of specific auxin-responsive genes occurred in the roots of the RNAi-lines, although this did not include the entire spectrum of auxin signalling components investigated.

While many *Rboh* genes are expressed in roots, the precise physiological functions of their encoded proteins in the roots are unclear. Root cells originate from the root meristem; these cells then go through a process of elongation and differentiation. Root tips are sites of active ROS production, particularly in the elongation zone of the root (Liszkay *et al*., 2004; Dunand *et al*., 2007; Tsukagoshi *et al*., 2010). Apoplastic ROS can participate in cell elongation (Liszkay *et al*., 2004; Müller *et al*., 2009*a*), and in addition to aberrant root development, our RNAi transgenic seedlings also exhibited significantly shorter roots than those of WT seedlings. Thus, the transgenic lines might be affected in both elongation growth and in patterning. ROS have been connected to root cell differentiation in *Arabidopsis*: Vernoux *et al*. (2000) identified the gene *ROOT MERISTEMLESS 1* that encodes an enzyme involved in glutathione biosynthesis, thus linking root meristem identity and activity to the redox state of the cells. The transition from cell division in the meristem to differentiation involves transcriptional up-regulation of a number of genes encoding peroxidases and is also accompanied by changes in ROS concentrations (Tsukagoshi *et al*., 2010). This fits with the failure of seedling root tips of the RNAi-lines to maintain a functional architecture. It was found that, in *Arabidopsis*, *AtrbohB* is expressed specifically in the root tip below the elongation zone. It is speculated that RbohB is responsible for the ROS production in this zone.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Alignment of the deduced amino acid sequences of the AtrbohB and LesarbohB cDNAs.

Supplementary Fig. S2. Alignment of the deduced amino acid sequences of the AtrbohF and LesarbohF cDNAs. sequences.

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