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Roothairless5, which functions in maize (*Zea mays* L.) root hair initiation and elongation encodes a monocot-specific NADPH oxidase

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SUMMARY

Root hairs are instrumental for nutrient uptake in monocot cereals. The maize (*Zea mays* L.) *roothairless5* (*rth5*) mutant displays defects in root hair initiation and elongation manifested by a reduced density and length of root hairs. Map-based cloning revealed that the *rth5* gene encodes a monocot-specific NADPH oxidase. RNA-Seq, *in situ* hybridization and qRT-PCR experiments demonstrated that the *rth5* gene displays preferential expression in root hairs but also accumulates to low levels in other tissues. Immunolocalization detected RTH5 proteins in the epidermis of the elongation and differentiation zone of primary roots. Because superoxide and hydrogen peroxide levels are reduced in the tips of growing *rth5* mutant root hairs as compared with wild-type, and Reactive oxygen species (ROS) is known to be involved in tip growth, we hypothesize that the RTH5 protein is responsible for establishing the high levels of ROS in the tips of growing root hairs required for elongation. Consistent with this hypothesis, a comparative RNA-Seq analysis of 6-day-old *rth5* versus wild-type primary roots revealed significant over-representation of only two gene ontology (GO) classes related to the biological functions (i.e. oxidation/reduction and carbohydrate metabolism) among 893 differentially expressed genes (FDR <5%). Within these two classes the subgroups 'response to oxidative stress' and 'cellulose biosynthesis' were most prominently represented.

Keywords: maize, root hairs, *rth5*, NADPH oxidase, RNA-Seq.

INTRODUCTION

Root hairs are tubular extensions of epidermis cells and comprise up to 77% of the root surface of cereals (Parker *et al.*, 2000). They are, therefore, considered to be instrumental for nutrient uptake and to support plant growth and development (Gilroy and Jones, 2000), a view that is supported by replicated field trials involving near-isogenic lines of the maize *roothairless 3* mutant, which conferred significant losses in grain yield (Hochholdinger *et al.*, 2008).

Generally, root hair bearing epidermis cells (trichoblasts) are short and contain denser cytoplasm, whereas non-root hair forming cells (atrichoblasts) are long and contain large vacuoles (Cormack, 1947). Several types of root hair patterning have been described. In dicot species such as

Arabidopsis thaliana, position-dependent root hair initiation leads to alternating longitudinal files of trichoblasts and atrichoblasts (Schiefelbein and Somerville, 1990). In contrast, some monocot species such as rice form trichoblasts via asymmetric cell divisions. Finally, random, unpredictable initiation without development of cytologically detectable distinct atrichoblasts and trichoblasts is observed in some monocot and dicot species such as maize and soybean (Clowes, 2000).

In Arabidopsis, epidermis cells overlaying two cortical cells (H cell position) develop into trichoblasts which give rise to root hairs. Cells overlaying only one cortical cell (N cell position) differentiate into hairless atrichoblasts (Schiefelbein and Somerville, 1990). In Arabidopsis, the molecular network involved in epidermis specification and thus root hair initiation is well understood (Dolan, 2001; Ishida *et al.*, 2008; Hochholdinger and Nestler, 2012). The Arabidopsis homeodomain transcription factor GLABRA2 (GL2) which is specifically expressed in atrichoblasts inhibits the expression of genes responsible for root hair formation (Masucci *et al.*, 1996). GL2 expression is activated by WEREWOLF (WER), a MYB-like transcription factor (Lee and Schiefelbein, 1999). The homologous MYB-like transcription factor CAPRICE (CPC) replaces WER in trichoblasts. CPC does not contain an activation domain and can therefore not activate *Gl2* expression (Wada *et al.*, 2002).

Root hair growth is divided into three phases: first, defined swelling to form a bulge, second, transition to tip growth and finally tip growth by oriented exocytosis (Dolan *et al.*, 1994). Recently, root hair formation from initiation to elongation in Arabidopsis was characterized via genome-wide transcriptome analyses of wild-type and 17 mutant lines leading to a complex model of transcriptional regulation (Bruex *et al.*, 2012).

Reactive oxygen species (ROS) and ROS-related proteins play an important role in root hair tip growth. Superoxide is produced by NADPH oxidases (NOXs) using oxygen as electron donor (Gapper and Dolan, 2006). Plant NADPH oxidases were identified as homologs of the mammalian gp91^{phox}, the catalytic subunit of the phagocyte NADPH oxidase, and named respiratory burst oxidase homologs (RBOHs) (Torres *et al.*, 1998). To date, the most thoroughly investigated *rboh* gene is *AtrbohC* or *ROOTHAIR DEFECTIVE 2* (*RHD2*) of Arabidopsis, which is involved in establishing hydrogen peroxide and superoxide peaks in growing root hair tips (Foreman *et al.*, 2003). The *rhd2* mutant lacks these high hydrogen peroxide and superoxide concentrations and the associated cytoplasmic Ca^{2+} influx (Wymer *et al.*, 1997). The membrane localized RHD2 protein is activated by Ca^{2+} (Takeda *et al.*, 2008). Although root hair bulges form in *rhd2* mutants they do not elongate (Schiefelbein and Somerville, 1990). Recently, it was demonstrated that *rhd2* mutants form macrotubules leading to a disturbance in mitosis in the root tip (Livanos *et al.*, 2012). In addition, the potential functions in root and root hair growth, several putative functions in shoot tissues and plant immune response have been assigned to *rboh* genes (Kwak *et al.*, 2003; Wong *et al.*, 2007; Galletti *et al.*, 2008; Lin *et al.*, 2009; Yi *et al.*, 2010; Yamauchi *et al.*, 2011; Wi *et al.*, 2012).

In maize, only a few mutants defective in root hair formation have been isolated. Two pleiotropic mutants, *disorganized aleurone layer 1* and *2* (*dil1* & *dil2*) display in addition to other developmental defects fewer root hairs and aberrant root hair morphology (Lid *et al.*, 2004). Three *roothairless* mutants (*rth1* to *rth3)* are defective at different stages of root hair formation (Wen and Schnable, 1994). Thus far only two maize genes involved in root hair elongation have been cloned. Mutants of the r*oothairless1* (*rth1*) gene condition short root hairs and a dwarf plant phenotype (Wen and Schnable, 1994) due to a defect in the encoded SEC homolog involved in polar exocytosis (Wen *et al.*, 2005). *Roothairless3* (*rth3*) encodes a monocot-specific COBRA-like protein that participates in cell-wall expansion and biosynthesis (Hochholdinger *et al.*, 2008); mutants in this gene result in very short root hairs defective in bulge formation (Wen and Schnable, 1994).

Here we describe the identification, cloning and characterization of the maize *roothairless5* gene controlling root hair initiation and outgrowth which encodes a monocot-specific NADPH oxidase involved in superoxide production.

RESULTS

The *roothairless5* gene controls both root hair length and density

An EMS mutagenesis screen yielded a recessive mutant (Schnable Laboratory Accession # 1350) that affects root hair development but has no other obvious effects on plant growth and development. Genetic crosses demonstrated that this mutant is not allelic to the previously described mutants *rth1*, *rth2* and *rth3* (Wen and Schnable, 1994). The affected gene and reference allele were designated *rth5* and *rth5-1*, respectively. Compared with wild-type (Figure 1a,c), the *rth5* mutant displays significantly shorter root hairs (Figure 1b,d). Root hair length and density of wild-type and *rth5* primary roots were analyzed via eSEM (environmental scanning electron microscopy). The length of root hairs on primary roots of 4-day-old (4 day) *rth5* mutants was decreased to 4% of wild-type length (Figure 1e). Moreover, root hair density of mutants was reduced to 64% of wild-type density (Figure 1f). In 10-day-old seedlings it is possible to observe root hairs on seminal and shoot-borne roots and the length and number of root hairs on these roots are also reduced in the mutant (Figure S1).

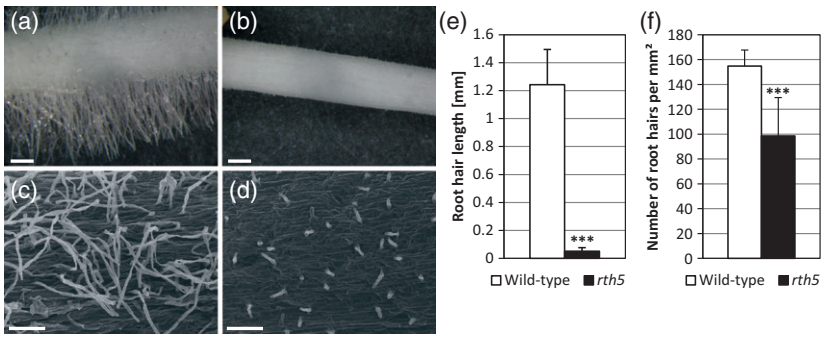

Figure 1. Phenotype of *roothairless5.*
(a–d) Binocular and eSEM pictures of 4-day-old wild-type (a, c) and *rth5* mutant (b, d) primary roots.
(e) Root hair length (n = 12).
(f) Root hair density per mm^2 (n = 3).
P-values were obtained via Student's *t*-test, *P*-value ***: ≤0.001. Error bars indicate standard deviation. Scale bars represent (a, b) 500 μm, (c, d) 100 μm.

Map-based cloning of *rth5* identified an NADPH oxidase

Using Sequenom-based bulked segregant analysis (BSA) (Liu *et al.*, 2010) the *rth5* locus was mapped to the long arm of chromosome 3. By analyzing F_2 and F_1BC populations (Experimental Procedures) the *rth5* gene was mapped to the interval 180.1–180.4 Mb of chromosome 3 (assembly version: ZmB73_AGPv1 release 4a53; http://ftp.maizesequence.org/release-4a.53/), flanked by insertion deletion polymorphism (IDP) markers IDP4064 and C3.184743 (Figure 2a). This interval harbors only five gene models, including GRMZM2G426953, encoding an NADPH oxidase (NOX), which contains a G-to-A transition at position 2462, resulting in a cysteine (C) to tyrosine (Y) conversion at amino acid position 821 close to the C-terminus of the protein (Figure 2b). This amino acid exchange is the result of a G-to-A transition relative to the B73 allele, which is characteristic of EMS-induced mutations (Greene *et al.*, 2003).

The affected cysteine residue is also conserved among divergent NADPH oxidases ranging from plants to yeast and human (Figure 2c). Hence, an alteration of this conserved residue in the predicted NAD substrate binding region might lead to a functional deficiency in the *rth5* mutant.

Confirmation of *rth5* identity via the generation of independent alleles

To confirm that the mutation in candidate gene GRMZM2G426953 indeed confers the phenotype of the *rth5* mutant, several independent putative *Mutator* alleles were isolated from Pioneer's trait utility system of corn (TUSC, Bensen *et al.*, 1995). Among these putative *Mu* insertion alleles, three displayed the roothairless phenotype and each of these alleles contained a *Mutator* insertion in the candidate gene as demonstrated by sequencing. Allele *rth5-3* contains a *Mu1* insertion in exon 5 (Figure 2b) while *rth5-2* and *rth5-4* contain *MuDR* insertions in exons 3 and 5, respectively (Figure 2b).

The gene structure

The B73 allele of the *rth5* gene consists of 14 exons and 13 introns (Figure 2b) encoding a 3792 bp mRNA (including the 5′ and 3′ UTR). This mRNA encodes an 852

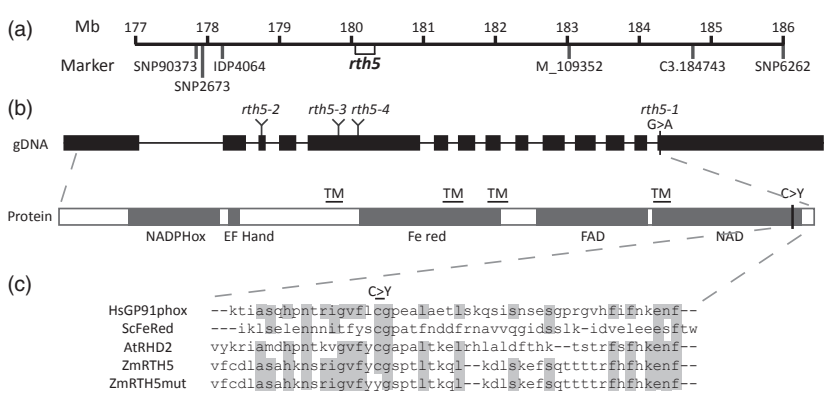

Figure 2. Mapping of *rth5.*
(a) Map-based cloning experiments positioned the *rth5* gene within a 280-kb interval of chromosome 3.
(b) A G-to-A (G→A) substitution in the last exon of the candidate gene GRMZM2G426953 results in a cysteine by tyrosine (C→Y) substitution in the protein sequence of the *rth5-1* allele at position 821. The positions of *Mu* transposon insertions in three additional alleles, *rth5-2*, *rth5-3*, and *rth5-4* are indicated.
(c) Alignment of the C-terminal 50 amino acids of human, yeast and Arabidopsis NADPH oxidases and wild-type and mutant RTH5.
At, *Arabidopsis thaliana*; EF hand, Ca^{2+} binding site; Fe red, Ferric reduction domain; FAD/NAD, FAD/NAD binding site; Hs, *Homo sapiens*; NADPHox, NADPH oxidase domain; Sc, *Saccharomyces cerevisiae*; TM, transmembrane; Zm, *Zea mays.*

amino acid protein with a predicted molecular weight of ~96 kDa.

The RTH5 protein is predicted to contain four transmembrane (TM) domains, two EF hand motifs, FAD and NAD co-factor binding sites, a ferric reductase domain, and the NADPH oxidase domain characteristic for NADPH oxidases (Figure 2b).

The NADPH oxidase family in maize

Based on sequence similarity to the RTH5 protein, 17 respiratory burst oxidase homologs (*rboh*) genes were identified in the maize filtered gene set (FGS, release: 4a53; http://ftp.maizesequence.org/release-4a.53/filtered-set/), a set of high confidence gene models. Phylogenetic reconstructions based on the full-length protein sequences of all known members of the maize, Arabidopsis, soybean and rice NADPH oxidase gene families (Figure 3) were performed. Two major groups of NADPH oxidases were observed. All NADPH oxidases exhibit diverse sequences in their N-terminus. The smaller group II is characterized by less conservation or the absence of the NADPH oxidase domain and a different conserved sequence in the NAD co-factor binding region (Figure S2). Interestingly, no soybean and only one rice and maize protein were included in group II, whereas eight Arabidopsis RBOH proteins belong to that subgroup. Along with two other maize and two rice RBOHs RTH5 is a member of a monocot-specific sub-clade of group I (Figure 3). No homolog of RTH5 resulting from an ancient genome duplication was found in the maize genome. *In silico* searches for syntenic homologs (genomeevolution.org/CoGe) identified the rice gene Os01g61880, which was also the closest homolog of RTH5 in the phylogenetic tree (Figure 3). RTH5 and its two closest maize homologs RBOH11 and RBOH13 are located in a subgroup of the phylogenetic tree characterized by the presence of a few additional amino acids at the end of the FAD co-factor binding site (Figure S2).

The rth5 gene is preferentially expressed in root hairs

Tissue-specific expression of *rth5* and all 17 *rboh* family members was examined via qRT-PCR in six different seedling tissues including the cap of the primary root, elongation zone, differentiation zone without root hairs, isolated root hairs, the coleoptilar node and the first leaf. *Rth5* transcripts accumulated in all tested tissues, but the significantly highest level was detected in root hairs (Figure 4b). Expression data of the remaining *rboh* family members in these tissues are summarized in Figure S3. Moreover, the qTeller tool (www.qteller.com) was used to compare the accumulation of *rth5* transcripts in RNA-Seq data from a wide range of tissues and organs. The highest expression values were observed in seedling roots (Figure S4). The relative expression levels of all 17 *rboh* genes in root hairs were determined via quantitative real-time PCR (qRT-PCR).

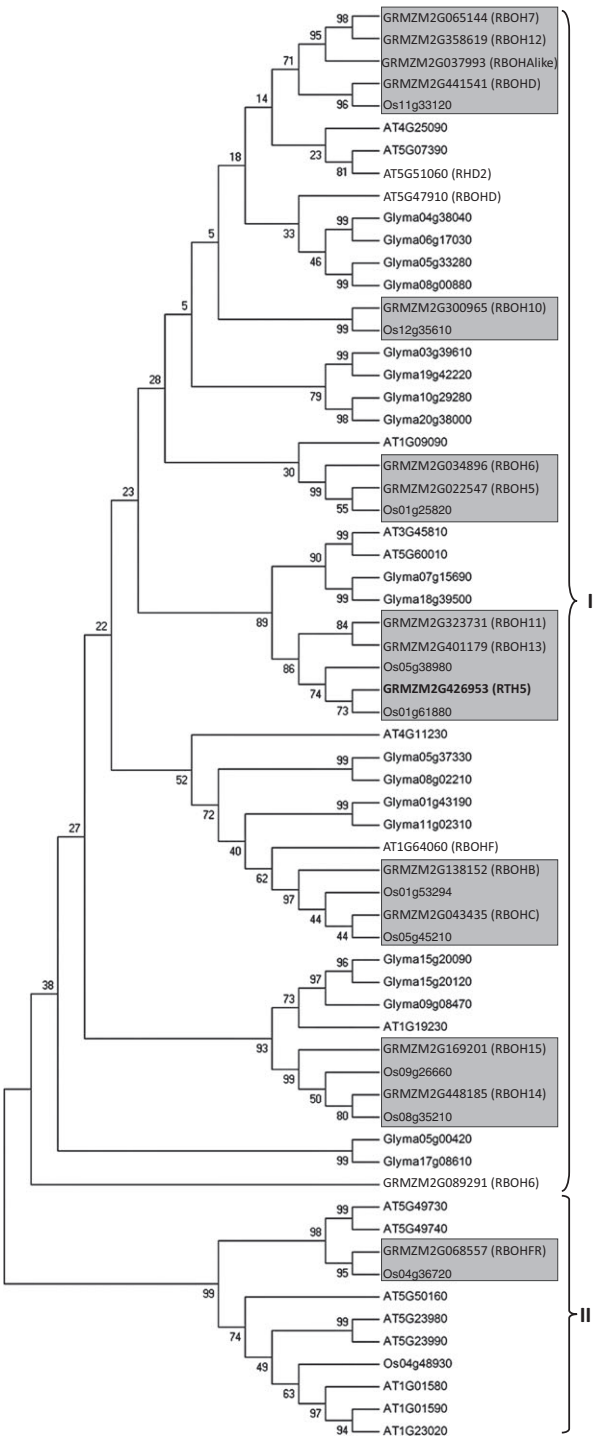

Figure 3. Phylogenetic tree of NADPH oxidases of maize, rice, Arabidopsis and soybean.
Two subfamilies can be distinguished by the presence or absence of several conserved domains in groups I and II (Figure S2). Monocot-specific clades/subclades are highlighted with gray boxes.

The *rth5* gene displayed the highest expression, accounting for 52% of the total *rboh* expression in maize root hairs (Figure 4a). The genes *rboh11* and *rboh13*, the closest

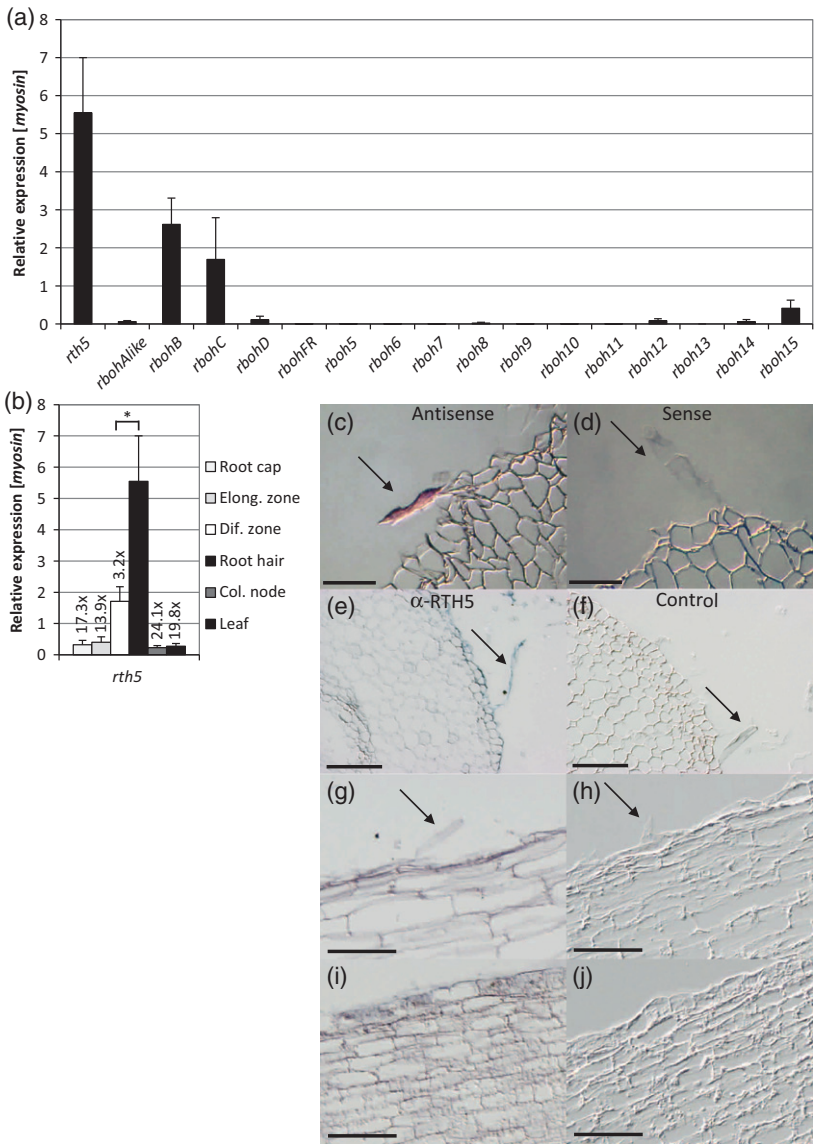

Figure 4. Tissue-specific expression of *rth5* transcripts and accumulation of RTH5 proteins.
(a) Expression of the *rth5* gene family members in root hairs by qPCR.
(b) *rth5* expression in different maize tissues by qPCR. Error bars indicate standard deviations; fold changes are indicated relative to root hairs. *P*-value obtained via Student's *t*-test, *P*-value *0.05, $n = 3$.
(c, d) RNA *in situ* hybridization of *rth5* antisense (c) and sense (d) probes on primary root cross-sections.
(e–j) Immunohistochemistry was performed on cross-sections (e, f) and longitudinal sections of the differentiation (g, h) or elongation zone (i, j). (e, g, i) Sections incubated with anti-RTH5 antibody. (f, h, j) Control sections incubated with buffer. Arrows indicate root hairs. Scale bars in (c, d) 50 μm, (e–j) 100 μm. For all experiments in (c–j), 3-day-old B73 seedling were used.

homologs of *rth5* were only very weakly expressed in root hairs.

RNA *in situ* hybridization experiments were used to study root tissue-specific expression patterns. Cross-sections hybridized with an *in vitro* transcribed *rth5* RNA antisense probe resulted in a signal in root hairs whereas the sense probe yielded no signal (Figure 4c,d).

The RTH5 protein is localized in epidermal cells of the primary root

Immunohistochemical experiments were performed to localize the RTH5 protein *in situ* (Figure 4e–j). In cross-sections of the differentiation zone, RTH5 antibody signals were detected in all epidermis cells and in root hairs (Figure 4e), whereas the control sections displayed no signal (Figure 4f).

In longitudinal sections an RTH5 signal was detected in root hairs and all epidermis cells of the root hair zone (Figure 4g). Interestingly, an RTH5 signal was also detected in longitudinal sections of the elongation zone, which does not form root hairs (Figure 4i). The control sections, showed no detectable color reaction (Figure 4h,j).

Both cross-sections and longitudinal sections displayed no difference in signal intensity or tissue organization between *rth5* and wild-type apart from the root hair defect (Figure S5).

The *rth5* mutant accumulates less ROS during root hair tip growth

Superoxide is a very short-lived radical that is rapidly converted into hydrogen peroxide (Figure 5a). Staining for both molecules was performed in wild-type and *rth5*

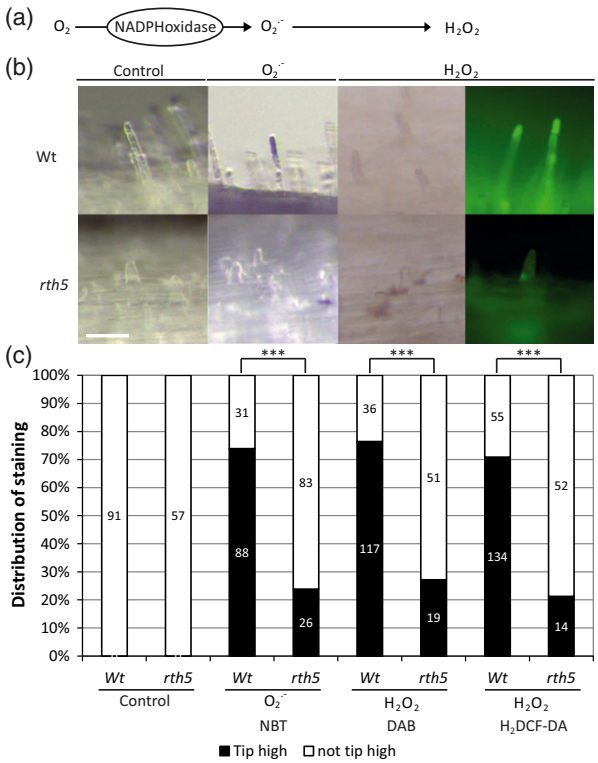

Figure 5. Reactive oxygen species (ROS) deficiency in *rth5* mutants.
(a) NADPH oxidases catalyze the formation of superoxide which is permutated into hydrogen peroxide.
(b) ROS staining of wild-type (*Wt*, upper lane) and *rth5* (lower lane) root hairs (from left to right): water control, NBT for superoxide, DAB, and H_2DCF-DA for hydrogen peroxide detection. A GFP filter was used for H_2DCF-DA detection. Scale bar 50 μm.
(c) Ratio of tip-high signals after staining in growing root hairs. Number of analyzed root hairs is indicated in the bars. *P*-values were obtained via Fisher's exact test, *P*-value ***: ≤0.001.

mutant seedlings (Figure 5b) by NBT for superoxide and DAB and H_2DCF-DA for hydrogen peroxide. NBT and DAB exclusively stained root hair cells, while H2DCF-DA not only exhibited the highest intensity of fluorescence in root hairs but also slightly stained other epidermis cells upon longer incubation. Subsequently, presence or absence of high ROS signals in root hair tips were quantified (Figure 5c). While 74% of wild-type root hairs displayed the superoxide (NBT) signal at the tip, the frequency of a tip-high signal was significantly reduced to 24% in *rth5* root hairs. Similarly, 77 and 71% of wild-type root hairs exhibited a high hydrogen peroxide signal in the tips of root hairs as detected by DAB or H_2DCF-DA, respectively, while only 27 and 21% of *rth5* root hairs displayed this signal specifically in root tips (Figure 5c). Fisher's exact test showed that these differences were significant at a *P*-value ≤0.001.

RNA-Seq of *rth5* mutants versus wild-type

To understand the influence of *rth5* on global patterns of transcription, RNA-Seq was performed on 6-day-old roots from the *rth5* mutant and wild-type seedlings (Experimental Procedures). Two biological replicates were analyzed for each genotype. Approximately 98% of raw reads from each sample passed the quality check and trimming procedure. In total, 3.7–4.7 million reads per replicate (~88% of the post-trimmed reads) were uniquely and confidently mapped to the B73 reference genome (Table S2) (Experimental Procedures). Among 21 007 genes that have a total of ≥20 RNA-Seq reads summed across all four biological replicates, 893 differentially expressed genes were identified at a false discovery rate (FDR) of 5%. Among those, 444 genes were preferentially expressed in wild-type primary roots, including 100 genes displaying a fold change (Fc) ≥2. Moreover, 449 genes were preferentially expressed in *rth5* primary roots including 195 genes displaying a Fc ≥2 (Table S3 and Figure S6). A gene ontology (GO) enrichment analysis of the 893 differentially expressed genes revealed statistically significant over-representation of three GO terms related to 'oxidation/reduction' and six GO terms related to 'carbohydrate metabolism' (Table S4). The 'oxidation/reduction' category contained four differentially expressed *rboh* genes, including *rth5.* While the *rth5* gene (GRMZM2G426953) was preferentially expressed in wild-type versus mutant primary roots (Fc: 1.4), the genes *rboh7* (GRMZM2G065144), *rboh6* (GRMZM2G034896) and *rbohAlike* (GRMZM2G037993) were preferentially expressed in mutant *rth5* roots. The relation of the 'oxidation/reduction' and 'carbohydrate metabolism' subgroups are summarized in Tables S5 and S6, respectively. Within these two classes, the subgroups 'response to oxidative stress' and 'cellulose biosynthesis' were most prominently represented. Overall, these RNA-Seq results are consistent with the view that *rth5/rbohA* functions in producing ROS ('response to oxidative stress') required for elongation ('cellulose biosynthesis') of root hairs.

DISCUSSION

The maize *rth5* mutant displays significantly shortened root hairs and reduced root hair density while aboveground development remains unaffected. Hence, *rth5* specifically controls both the elongation of root hairs and the specification of epidermis cells or the initiation of root hairs. More specifically, root hair elongation in the mutant *rth5* is affected during the transition from bulge to tip growth. In contrast to *rth5*, root hairs of the *rth3* mutant are characterized by disrupted bulges while *rth1* and *rth2* root hairs fail to elongate after transition to tip growth (Wen and Schnable, 1994). The *rth5* mutant resembles the phenotype of *rth2* and *rth3* (Wen and Schnable, 1994), which are specifically affected in root hair formation. In contrast, the maize mutants *rth1* (Wen and Schnable, 1994), *dil1* and *dil2* (Lid *et al.*, 2004) which are defective in root hair formation also display pleiotropic effects during development. The *rth1* mutant displays general growth abnormalities during development (Wen and Schnable,

1994) while *dil1* and *dil2* control cell division orientation in the aleurone, leaf and root epidermis. The maize mutant *rth5* forms fewer root hairs as compared to wild-type primary roots suggesting that the *rth5* gene is involved not only in root hair elongation but also in epidermis specification and/or root hair initiation.

In *rth5*, all root types including primary, seminal, lateral, and shoot-borne roots display defects in root hair formation, suggesting that the RTH5 protein is critical for root hair formation in all root types. A general defect in root hair formation affecting all root types has also been observed for the *rth1*, *rth2* and *rth3* mutants (Wen and Schnable, 1994; Hochholdinger *et al.*, 2008). In contrast to this general mechanism of root hair formation in all root types, the control of lateral root formation is root type-specific. For instance *rum1* (Woll *et al.*, 2005) and *lrt1* (Hochholdinger and Feix, 1998) display root type-specific defects which are confined to embryonic roots. In these mutants post-embryonic shoot-borne roots do not display any lateral root defects.

The RTH5 protein is characterized by several functional domains (Figure 2b) including four trans-membrane domains, responsible for the membrane embedding and two EF hand motifs, which are required for Ca^{2+} regulation. Moreover, the FAD and NAD domains confer binding to the co-factor FAD and the substrate NADPH, respectively. Regulation of the RBOHA/RTH5 NADPH oxidase by a mitogen-activated protein kinase cascade controlled by abscisic acid signalling was demonstrated in maize leaves (Lin *et al.*, 2009).

Based on the identification of the co-factor binding site and the functional assignment, the *rth5* gene is predicted to encode a NADPH oxidase (NOX). The NOX protein family is present in most eukaryotic species (Bedard *et al.*, 2007). The *rth5* mutant was induced by EMS, which resulted in a guanine to adenine substitution in the genomic sequence changing the cysteine (C) residue in amino acid position 821 to tyrosine (Y) (Figure 2b). Cysteine residues often form intra- or inter protein disulfide bridges to enable secondary structures. As this cysteine residue is highly conserved among a very diverse set of species (Figure 2c) it is likely that the C821Y mutation is disabling a functionally important protein domain. A point mutation of this conserved C in the human NADPH oxidase 2 (*NOX2*) gene results in the genetic disorder chronic granulomatous disease (Kawahara *et al.*, 2007). This disease affects cells of the immune system which have difficulties in forming reactive oxygen compounds, including superoxide used to kill certain ingested pathogens.

Phylogenetic reconstruction of RBOH (RESPIRATORY BURST OXIDASE HOMOLOG) proteins of rice, maize, soybean, and Arabidopsis revealed the existence of two subgroups. These subgroups are distinguished based on differences in the sequences of the NAD binding region, which are conserved within each group (Figure S2). Ten Arabidopsis RBOH proteins are classified to group I, which is consistent with previous annotations of superoxide producing NOX proteins (Kawahara *et al.*, 2007). Interestingly, all soybean proteins are assigned to group I, while eight of 18 members of the Arabidopsis RBOH protein family are classified in group II. According to their annotation, group II proteins are often connected to iron deficiency and might function as ferric reductases which are NOX proteins that do not produce superoxide (Ivanov *et al.*, 2012).

The nine members of the group I subclade that includes RTH5 are characterized by nine additional amino acids in the FAD co-factor domain as compared to the remaining group I proteins. The five monocot-specific proteins in this subclade lack 15 amino acids in their NADPH binding region. Both, the additional amino acids in the FAD, and the missing amino acids in the NAD region might confer altered binding to cofactors or substrates leading to specialized functions.

While several types of NOX proteins are found in mammals ranging from ancestral types to peroxidase containing DUOXs, in plants only homologs of EF-hand-containing NOX5-types have been identified (Bedard *et al.*, 2007). Human NOX proteins consist of two trans-membrane heterodimers ($gp91^{phox}$ and $p22^{phox}$) and four regulatory subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac2) in the cytoplasm (Lam *et al.*, 2010). Thus far, no homologs for the regulatory subunits $p47^{phox}$, $p67^{phox}$, or $p22^{phox++}$ have been found in plants (Bedard *et al.*, 2007).

The human NOX2 has been shown to play a role in the production of superoxide as a first defense response to microorganisms invading neutrophils and macrophages (Nauseef, 2008; Lam *et al.*, 2010). Plant NOX proteins have been shown to function in plant immunity and several developmental processes. Pepper and tobacco NOX proteins were demonstrated to function in plant immunity (Yi *et al.*, 2010; Wi *et al.*, 2012). Aerchenyma formation after waterlogging was shown to be conferred by the maize NOX protein GRMZM2G300965 (Yamauchi *et al.*, 2011), which showed specific expression in the root cap and the elongation zone (Figure S4; RBOH10). Finally, Arabidopsis RBOHD and RBOHF are involved in ABA-dependent stomata closure (Kwak *et al.*, 2003) and lead to an oxidative burst following infection (Galletti *et al.*, 2008).

The Arabidopsis RBOH protein RBOHC (RHD2), which is distantly related to RTH5, also controls root hair growth. Arabidopsis plants defective in *RHD2* gene function form very short root hairs that do initiate bulges which do not elongate (Schiefelbein and Somerville, 1990). Moreover, such plants display stunted root growth (Foreman *et al.*, 2003), an effect that was not observed for *rth5* mutant roots. *RHD2* is expressed in root epidermal cells, but also in the cortical cells of the elongation- and differentiation zone (Foreman *et al.*, 2003).

Consistent with its mutation in the C-terminus of the protein, the expression of the *rth5-1* mutant reference

allele was only slightly reduced as compared with wild-type primary roots (Figure S5). Similarly, protein levels detected by immunohistochemistry and western blot analyses did not show any obvious change between wild-type and *rth5-1* mutants (see Figures 4 and S5). Although expression and protein abundance are not dramatically affected, this mutation significantly affects the function of the protein as illustrated by the mutant phenotype.

Expression of *rth5* was detected in several shoot and root tissues at low levels with expression maxima in root hairs (Figures 4 and S4). Similarly, the rice *rboh* genes are also expressed in multiple plant organs and tissues (Wong *et al.*, 2007). In maize root hairs *the rth5* gene accounts for 52% of the expression of all 17 *rboh* genes and hence the majority of NADPH oxidase transcripts in root hairs. The RTH5 protein was detected preferentially in root hairs and epidermis cells but weaker signals were also detected in root cortex cells (Figure 4). In two studies mainly focusing on the influence of hormones on NADPH oxidase activity in maize leaves it was demonstrated that expression of *rth5* (*rbohA*) is induced after abscisic acid and brassinosteroid treatment as well as hydrogen peroxide application (Lin *et al.*,2009; Zhang *et al.*, 2010). These experiments suggest that RTH5 may play a role in hormone-mediated oxidative defense in leaves. Further analyses will be required to determine whether RTH5 plays a similar role in roots, in addition to its known roles in root hair initiation and elongation.

Arabidopsis root hairs display high Ca^{2+} and superoxide concentrations in their growing tips (Foreman *et al.*, 2003). The tips of root hairs of the mutant *rth5* displayed significantly less superoxide staining and accumulate less hydrogen peroxide than wild-type plants (Figure 5). These results suggest that as a consequence of a functional defect of the RTH5 protein the *rth5* mutant is unable to establish high levels of ROS in the tips of root hairs. Not all growing tips of wild-type root hairs displayed staining for superoxide and hydrogen peroxide. Some root hairs may have died during the staining procedure; some may not have taken up the indicator chemicals. Consistent with expression data the presence of RTH5 homologous RBOH proteins may account for the residual ROS content detected in the mutant (Figure S3).

A model for the function of RTH5 in root hair elongation is summarized in Figure 6(a). Superoxide molecules in the root hair tips produced by RTH5 are transformed rapidly into hydrogen peroxide, which in turn is converted to hydroxyl radicals by apoplastic peroxidases (Liszkay *et al.*, 2003). These hydroxyl radicals are cleaving polysaccharides (Fry, 1998) and therefore cause cell-wall loosening, thereby enabling polarized cell growth (Bibikova *et al.*, 1998; Liszkay *et al.*, 2004).

In Arabidopsis the molecular interactions resulting in position-dependent root hair initiation are well characterized (reviewed in: Hochholdinger and Nestler, 2012). In contrast, the molecular network underlying random root hair patterning in maize remains enigmatic (Clowes, 2000). The reduced root hair density observed in mutant seedlings suggests that RTH5 is involved not only in root hair tip growth, but also in root hair initiation or epidermis specification. The presence of RTH5 in all epidermal cells is consistent with the hypothesis that superoxide is only produced upon activation of RTH5, which thus acts as a signal converter, similar to the human NOX2 (Lam *et al.*, 2010), which subsequently promotes root hair initiation. This is supported by the finding of selective root hair cell superoxide staining in the maize differentiation zone. In contrast, all epidermis cells display superoxide staining in the elongation zone (Liszkay *et al.*, 2004). To act in root hair initiation, we hypothesize that a yet unknown root hair initiation signal molecule activates RTH5 mediated superoxide production, leading to both the establishing of root hairs and their subsequent elongation. Residual root hair initiation in the *rth5* mutant might be controlled by RTH5-independent pathways. Due to the lack of functional RTH5 proteins these root hairs fail the transition to tip growth.

The human small GTPase RAC2 is necessary for the assembly of the functional NOX2 protein (Lam *et al.*, 2010). In Arabidopsis the ROP (RHO of plants) GTPase ROP2 is required for ROS formation in root hairs (Jones *et al.*, 2007). Another possible activation/regulation pathway involves Ca^{2+}. In Arabidopsis, it was demonstrated that NADPH oxidase activity leads to higher amounts of cytoplasmic Ca^{2+}, which in turn has a positive effect on their activity mediated through EF hands (Kimura *et al.*, 2012). Similarly, a trichoblast-specific signal might activate the RTH5 protein through a small GTPase or calcium-dependent protein kinase (CDPK) to facilitate trichoblast differentiation (Figure 6b). It is likely that RTH5 is also regulated by and/or participates in feedback loops involving these factors.

As a first step towards the elucidation of the RTH5-dependent molecular networks in young maize roots, a comparative RNA-Seq analysis was performed on 6-day-old wild-type versus *rth5* primary roots. Consistent with the suggested function of RTH5 only two major biological functions were overrepresented among the differentially expressed genes in a GO enrichment analysis. First, the GO term 'oxidation/reduction' was enriched. Genes in this class included four members of the *rboh* gene family, including *rth5* as discussed above. Moreover, this class also contains many genes related to oxidative stress including several peroxidases. As discussed above these peroxidases act downstream of RTH5 and facilitate the loosening of the elongating root hairs. This function connects the 'oxidation/reduction' GO class with the GO subgroup 'cellulose biosynthesis.' Cellulose is the most abundant plant polysaccharide (Carpita and McCann, 2000) and biopolymer *per se.* Cellulose biosynthesis is a crucial step in root hair elongation that follows peroxidase

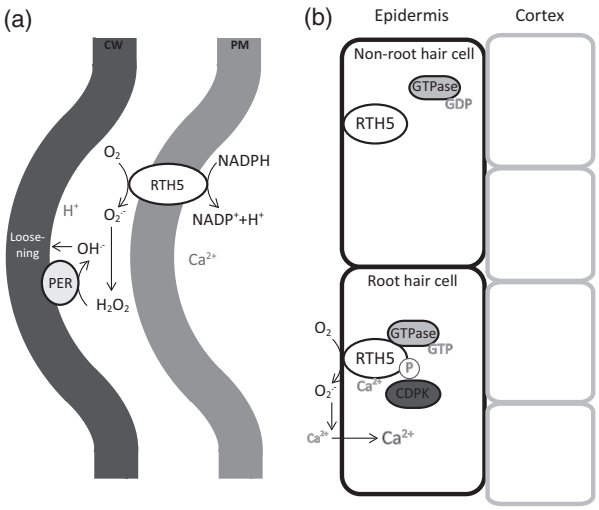

Figure 6. Models for the predicted function of RTH5 in root hair initiation and growth.
(a) Illustration of the putative role of RTH5 in tip growth by producing apoplastic superoxide, which is rapidly converted into hydrogen peroxide. Apoplastic peroxidases (PER) generate hydroxyl radicals which are cleaving celluloses and hemicelluloses leading to cell-wall loosening. The acidic cell wall (CW) allows oriented growth at the softened site. PM, plasma membrane.
(b) Suggested function of RTH5 in root hair initiation. A trichoblast-specific signal might activate the RTH5 protein through a small GTPase or calcium-dependent protein kinase (CDPK) to facilitate trichoblast differentiation.

mediated cell-wall loosening (Figure 6a). In Arabidopsis it has been demonstrated that mutations in cellulose biosynthesis genes can result in defective tip growth and aberrant elongation of root hairs (Favery *et al.*, 2001; Wang *et al.*, 2001; Park *et al.*, 2011). In summary, both overrepresented GO terms 'oxidation/reduction' and 'carbohydrate biosynthesis' are directly connected to maize root hair formation. Future reverse genetic analyses of candidate genes identified in this RNA-Seq analysis may therefore help to better define the genetic control of maize root hair formation.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The mutation was initially induced by ethyl methanesulfonate (EMS) mutagenesis of the Pioneer inbred line 114748/AD. Mutants used in the present study were progeny of plants backcrossed 10 times to the inbred line B73 (Schnable laboratory stock 00g-1542-1).

Kernels were surface sterilized as described previously (Nestler *et al.*, 2011) and germinated in moist paper rolls at a 16 h light, 8 h dark cycle at 26°C in distilled water.

Binocular imaging and environmental scanning electron microscopy

A Zeiss Stemi SV8 Binocular (www.zeiss.com) coupled with a Powershot G2 camera (Canon) (www.canon.de) was used to document root hairs at a ×20 magnification. Root hair length was determined via WinRhizo Software (Regent Instruments Inc.) (www.regent.qc.ca). A Philips XL30 FEI environmental scanning electron microscope (eSEM; www.philips.de) was used for surface illustration of fresh 4-day-old primary roots without fixation or tissue drying.

Bulked segregant analysis

Sequenom-based BSA (Liu *et al.*, 2010) was used to map the *rth5* gene. In total, 89 individuals from an F_2-family were phenotypically divided into two bulks: mutant and non-mutant. Equal quantities of root tissue were pooled from each individual in each bulk. DNA was extracted from the two tissue bulks and then subjected to Sequenom-based SNP typing using ~1000 SNP markers.

Fine-scale mapping of *rth5*

Multiple markers located on the long arm of chromosome 3 were used to genotype 80 *rth5* mutant individuals from an F_2-population (Table S1). An *rth5* mutant individual in a B73 genetic background was crossed with the Mo17 inbred line and the resulting F_1-seeds were backcrossed to Mo17 to create an F_1BC population. Two single nucleotide polymorphism (SNP) markers, SNP2673 and SNP6262, were used to genotype 512 F_1BC individuals. In total, 65 recombinants were identified between SNP2673 and SNP6262, each of which was self-pollinated. The root hair phenotypes of self-pollinated progeny were scored to infer whether or not specific F_1BC_1 recombinants carried the *rth5* mutant allele. In parallel, the recombinants were genotyped with a series of molecular markers (Figure 2a). Based on an analysis of the resulting genotyping and phenotyping data, the *rth5* mutant was mapped to the 179.9–182.3 Mb interval of chromosome 3 in AGPv1. To further narrow down the *rth5* interval, a larger F_1BC population (n = ~5000) was genotyped with SNP markers SNP90373 and M_109352. In total, 440 recombinants between these two marker loci were identified and genotyped for *rth5* as described above. After genotyping all available recombinants (65 + 440) with 35 SNP markers (15 of which are co-dominant in this population) the *rth5* gene was mapped to the interval 180.1–180.4 Mb.

Identification of independent *rth5* mutant alleles

Three independent alleles, *rth5-2*, *rth5-3*, *and rth5-4*, were identified by a reverse genetic screen of *Mutator* stocks at Pioneer Hi-Bred. Confirmation of the candidate seedlings displaying a roothairless phenotype was performed by PCR mapping of the *Mutator* insertion with a general *Mutator* (*Mu*) oligonucleotide primer (Dietrich *et al.*, 2002) and an *rth5*-specific oligonucleotide primer (5′-GCACATCTCCCGGATAAATTG-3′). The amplification product contained 39 bp beyond the Mu TIR sequence sufficient to identify the *Mutator* element (Dietrich *et al.*, 2002). *Mu* insertions were found in positions 1300, 1931, and 1968 bp of the genomic *rth5* sequence counting from the ATG start codon in the alleles *rth5-2*, *rth5-3*, and *rth5-4*, respectively.

RTH5 protein domain prediction

Several bioinformatic tools were used to predict protein domains: InterProScan (EMBL-EBI) (www.ebi.ac.uk/Tools/pfa/iprscan), MyHits (SIB) (myhits.isb-sib.ch), SMART (University of Heidelberg) (smart.embl-heidelberg.de) and TMHMM (CBS of DTU, Technical University of Denmark) (www.cbs.dtu.dk/services/TMHMM). Domains predicted by at least two search tools are summarized in the protein domain structure (Figure 2b).

Phylogenetic analyses

The protein sequences of RTH5 homologs were obtained by blasting the RTH5 sequence against species-specific databases at

maizesequence.org, rice.plantbiology.msu.edu, soybean.org, and TAIR.org. The DNA Baser software (Heracle BioSoft) (dnabaser.com) was used to combine all sequences into a Fasta file which was used for alignment and calculation of a phylogenetic tree by MEGA4.0 (Tamura *et al.*, 2007) using the Neighbor joining algorithm (Bootstrap, 1000 replications).

RNA *in situ* hybridization

Primary roots of 3-day-old seedlings were fixed and embedded in paraffin as described previously (Hochholdinger *et al.*, 2008). The RNA probe for hybridization was generated by amplification of 282 bp from the 3′-end of the *rth5* gene with the oligonucleotide primers forward 5′-GTGTACCCGAAGATCCGATG-3′, and reverse 5′-GACAGCTCGGGCAGAAAGAC-3′. The amplicon was cloned into the pGEM T-easy vector (Promega, www.promega.com) in sense and antisense directions. *In vitro* transcription of the probes and digoxigenin-labeling was performed using the SP6 polymerase (NEB, www.neb.com/nebecomm/default.asp) according to the manufacturer's protocol. RNA *in situ* hybridization was performed according to (Jackson, 1992). A Zeiss Axioplan 2 microscope (www.zeiss.com) in combination with a Photometrics Cool Snapcamera (Roper Scientific GmbH, www.roperscientific.com) was used for image acquisition.

Quantitative RT-PCR

For qRT-PCR, RNA was extracted from approximately 100 mg of different maize tissues with the RNeasy Plant Mini Kit (Qiagen, www.qiagen.com) according to the manufacturer's protocol, including on-column DNase I digestion (Fermentas, www.fermentas.de). Isolation and harvesting of root hairs was performed as described previously (Nestler *et al.*, 2011). cDNA was prepared from 500 ng total RNA by using the qScript cDNA Super Mix (Quanta BioSciences, www.quantabio.com). qRT-PCR experiments were performed using MESA Green or Blue qPCR Mastermix Plus for SYBR Assay no ROX kit (Eurogentec, www.eurogentec.com). All experiments were conducted in three biological replicates and three technical replicates per biological replicate in a CFX384 Real-Time PCR Detection System (Bio-Rad, www.bio-rad.com). The efficiency for every oligonucleotide primer pair (summarized in Table S7) was determined by a dilution series. Expression was calculated relative to the reference gene *myosin* (GenBank AC486090G09.x1), which has previously been used as expression standard in maize root assays (Dembinsky *et al.*, 2007).

Antibody preparation and immune histochemistry

A polyclonal antibody was produced by incubating rabbits with a specific RTH5 peptide with the amino acid sequence VAGMRPGRMTRMQSSAQM.

Sample preparation was slightly modified from the RNA *in situ* hybridization protocol. Fixation was performed using 4% formamide solution. After sectioning, the samples were deparaffined using RotiClear (Roth, www.carl-roth.de) and rehydrated using decreasing ethanol concentrations (90, 50, and 25%) in Microtubules stabilizing buffer (MtSB) (Albertini *et al.*, 1984). Unspecific binding was blocked by 3% bovine serum albumin (BSA) in 1 × MtSB, followed by incubation with the RTH5 antibody (1:100 in 1 × MtSB) overnight at 4°C. After six washing steps in 1 × MtSB the samples were incubated for 2 h with the secondary antibody, ZytoChem Plus AP Polymer anti-rabbit (ZytoMed, www.zytomed-systems.de) according to the manufacturer's protocol. After washing with a buffer containing 100 mM Tris buffer, 100 mM NaCl, and 50 mM $MgCl_2$, the signal was detected using the Roche NBT/BCIP stock solution (www.roche.de) by incubation in the dark for 5–10 min. The reaction was stopped by three water incubations. Images were obtained as described for RNA *in situ* hybridization experiments.

Detection of superoxide and hydrogen peroxide

Superoxide was detected by incubating root samples in 0.5 mM Nitro blue tetrazolium chloride (NBT) in 0.1 M KCl/0.1 M NaCl solution which forms an insoluble blue formazan precipitate upon reaction with superoxide (Bielski *et al.*, 1980). To detect hydrogen peroxide two dyes were used: DAB (3,3-diaminobenzidine) and 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA). DAB forms a brown precipitate when oxidized by peroxidase activity (Thordal-Christensen *et al.*, 1997). Seedlings were incubated overnight in 1 mg ml^{-1} DAB dissolved in water. H_2DCF-DA emits a green fluorescence that corresponds to cytoplasmic H_2O_2 levels (Keston and Brandt, 1965). The stock solution was prepared by dissolving 1 mg H_2DCF-DA in 1 ml DMSO, and mixing with 1 ml H_2O. To obtain the working solution a dilution in 200 ml H_2O was prepared.

Three-day-old seedlings were incubated for 45 min in the dark in Petri dishes, each of which contained a detection solution. The signal was detected by mounting primary roots in water on a microscopic slide covered by a 60-mm cover slip. Excitation of H_2DCF-DA was performed at 488 nm. Emission was detected at 525 nm using a Zeiss Axioplan 2 microscope (www.zeiss.com).

RNA-Seq experiment

Seeds from an F_2-family segregating for *rth5* after nine generations of backcrossing to the inbred B73 were grown as described above. For this step, 3-cm root tips were collected from 6-day-old *rth5* mutants and wild-type siblings. Two mutant and two wild-type pools were collected. Each pool consisted of root tissues from six individuals. From each pool RNA was extracted using RNeasy Mini Kits (Qiagen) with DNase I treatment following the manufacturer's protocol. RNA quality was analyzed using a Bioanalyzer 2100 RNA Nanochip. RNA-Seq libraries were constructed using an Illumina RNA-Seq sample preparation kit following the manufacturer's protocol. The four libraries were sequenced on an Illumina HiSeq2000 at the Iowa State University DNA facility, generating 99-bp single-end reads and deposited in the GenBank database (accession no. SRP020528).

Trimming and mapping of RNA-Seq reads

Raw reads were subjected to quality checking and trimming to remove low quality bases using a custom trimming pipeline (Liu *et al.*, 2012). Trimmed reads were aligned to the B73 reference genome (ZmB73_RefGen_v2) using GSNAP (Wu and Nacu, 2010), allowing ≤2 mismatches every 50 and 2 bp tails per 50 bp. Only uniquely mapped reads were used for subsequent analyses. The read depth of each gene in the filtered gene set (ZmB73_FGSv2; http://ftp.maizesequence.org/current/filtered-set/, Release 5b.60) was computed based on the coordinates of mapped reads and the annotated locations of genes in the B73 reference genome.

Differential expression analysis of RNA-Seq

Genes with ≥20 RNA-Seq reads across all four biological replicates were tested for differential expression between the *rth5* mutant and wild-type using the R statistical package *QuasiSeq* (Iowa State University) (http://cran.r-project.org/web/packages/QuasiSeq). The

negative binomial *QLSpline* method implemented in the *QuasiSeq* package was used to compute a *P*-value for each gene. The 0.75 quantile of reads from each sample was used as the normalization factor (Bullard *et al.*, 2010). An approach for controlling for multiple testing (Benjamini and Hochberg, 1995) was used to convert *P*-values to *Q*-values. To approximately control the false discovery rate (FDR) at 5%, only genes with *Q*-values <0.05 were declared to be differentially expressed.

Gene ontology enrichment analysis of significantly differentially expressed genes

The GO of specific genes and the over-representation of GO terms were conducted using the single enrichment analysis of the AgriGo platform (http://bioinfo.cau.edu.cn/agriGO/analysis.php).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phenotype of *roothairless 5* in different root types.

Figure S2. Alignment of RTH5 and its homologs from maize, soybean, rice and Arabidopsis.

Figure S3. Expression analysis of maize *rboh* genes.

Figure S4. Expression of *rth5* in various tissues.

Figure S5. *Rth5* transcript and RTH5 protein abundance in wild-type and *rth5* mutants.

Figure S6. RNA-Seq analysis of *rth5* versus wild-type primary roots.

Table S1. Summary of markers used in the fine-mapping experiment.

Table S2. Summary of RNA-Seq mapping.

Table S3. Summary of reads per gene and replicate and statistical analysis of differential expression between *rth5* mutants and wild-type roots.

Table S4. Gene ontology terms related to biological functions that are overrepresented among the differentially expressed genes.

Table S5. Relation of genes included in the GO term oxidation/reduction and its subgroups.

Table S6. Relation of genes included in the GO term carbohydrate metabolic process and its subgroups.

Table S7. Oligonucleotide primers used for qRT-PCR experiments.

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