Reconstruction of Signaling Networks Regulating Fungal Morphogenesis by Transcriptomics

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Coordinated control of hyphal elongation and branching is essential for sustaining mycelial growth of filamentous fungi. In order to study the molecular machinery ensuring polarity control in the industrial fungus Aspergillus niger, we took advantage of the temperature-sensitive (ts) apical-branching ramosa-1 mutant. We show here that this strain serves as an excellent model system to study critical steps of polar growth control during mycelial development and report for the first time a transcriptomic fingerprint of apical branching for a filamentous fungus. This fingerprint indicates that several signal transduction pathways, including TORC2, phospholipid, calcium, and cell wall integrity signaling, concertedly act to control apical branching. We furthermore identified the genetic locus affected in the ramosa-1 mutant by complementation of the ts phenotype. Sequence analyses demonstrated that a single amino acid exchange in the RmsA protein is responsible for induced apical branching of the ramosa-1 mutant. Deletion experiments showed that the corresponding rmsA gene is essential for the growth of A. niger, and complementation analyses with Saccharomyces cerevisiae evidenced that RmsA serves as a functional equivalent of the TORC2 component Avolp. TORC2 signaling is required for actin polarization and cell wall integrity in S. cerevisiae. Congruently, our microscopic investigations showed that polarized actin organization and chitin deposition are disturbed in the ramosa-1 mutant. The integration of the transcriptomic, genetic, and phenotypic data obtained in this study allowed us to reconstruct a model for cellular events involved in apical branching.

The formation of complex structures of multicellular organisms is a pivotal question in biology. Breaking cell symmetry has been recognized as the first step in patterning an organism. This step from apolar to polar growth involves the perception and transmission of environmental and/or internal signals and results in the formation of a cellular axis. Establishing and maintaining cell polarity are thus fundamental prerequisites for the morphogenesis of organisms. Examples for a polarized mode of cell growth can be found in yeast (budding), filamentous fungi (hyphal tip growth), algae (rhizoids), plants (root hairs and pollen tubes), and animals (neurons). As tip-growing hyphal cells provide examples of highly polarized growth, filamentous fungi are attractive eukaryotic model systems to study the mechanisms underlying this process.

In addition, filamentous fungi such as Aspergillus niger are also used as cell factories for the production of chemicals, pharmaceuticals, and proteins. During the last years, however, it has become clear that the morphology of filamentous fungi seriously limits the product yields obtained (37, 64, 72). Previous studies suggested a link between protein production and the abundance of actively growing hyphal tips (36, 107); however, only contradictory results have been reported so far. An increase in the number of hyphal tips has been reported to increase protein production and secretion in some cases (14, 106), whereas no correlation has been found in others (14). Thus, no generally accepted model exists so far that can be used as basis for rationally optimizing the morphology of filamentous fungi with respect to protein secretion and their rheological behavior in a bioreactor. In order to improve the morphological features of filamentous fungi in industrial processes, much more basic knowledge is required to obtain a deeper insight into the molecular networks regulating fungal morphology.

The formation of highly polarized hyphae is a defining attribute of filamentous growth. Various protein complexes (e.g., the polarisome, exocyst, and Arp2/3 complex), cytoskeletal elements, the Spitzenkörper, lipid rafts, and signaling molecules (GTPases, calcium, and cyclic AMP) are essential for establishing and maintaining polarized growth (for reviews, see references 8, 43, 44, 92, and 102). Basically, it is thought that secretory vesicles delivering proteins and cell wall material to the hyphal apex are transported along microtubules to the Spitzenkörper (8). The Spitzenkörper is a vesicle-rich region present in the apexes of fungal hyphae and defines the center
and direction of growth (34, 35, 39, 108). The Spitzenkörper is thought to be newly formed at sites of spore germination and branch formation and is visible only in rapidly growing hyphal tips. The Spitzenkörper consists of vesicles (chitosomes, calcium-containing vesicles, and other vesicles of unknown content), proteins (F-actin, tubulin, formins, and calmodulin), and ribosomes and is viewed as a switching station from microtubule-based to actin microfilament-based vesicle transport (8–10, 20, 39, 45, 80, 92, 97, 103). Actin microfilaments focus in the center of the Spitzenkörper and organize vesicle transport to the plasma membrane (8, 19). The polarisome is a multi-protein complex adjacent to the Spitzenkörper and is thought to play a key role in the nucleation of actin microfilaments and in governing maximal polar growth rate (44, 55, 66, 102, 111).

In vivo studies with strains of Neurospora crassa showed that three different chitin synthases concentrate in the center of the Spitzenkörper (80). Besides these known fungal polarity determinants, a kinase complex which is conserved throughout eukaryotic evolution mediates spatial control of cell growth by regulating the actin cytoskeleton. This complex, named TORC2, has been shown to be essential for the determination of cell polarity in Saccharomyces cerevisiae, Dicyostelium discoideum, and mammalian cells (47). However, such a role of TORC2 has not yet been established in any filamentous fungus.

Hyphal branching leads to mycelial development. Usually, branches arise from basal regions (lateral branching); however, new branches can also be formed by tip splitting (apical branching). The physiological details of the process of apical branching have been studied in vegetative hyphae of A. niger, using the temperature-sensitive hyperbranching ramosa-1 mutant (77, 79). Four short-term events were identified: (i) cytoplasmic contraction thought to be triggered by a transient alteration of the cytoskeleton, (ii) retraction of the Spitzenkörper, (iii) disappearance of the Spitzenkörper accompanied by a sharp reduction in the hyphal elongation rate, and (iv) de novo formation of two Spitzenkörper giving rise to two apical branches. Similar events were also observed in other fungi, including wild-type A. niger, Neurospora crassa, and Trichoderma atroviride (78). It is believed that apical branching results from abnormal accumulation of vesicles at the tip and/or from increased tip-directed transport of vesicles, which exceeds the capacity of the leading tip. To accommodate the abnormal accumulation of the vesicles, the tip divides into two new branches (42).

Only little is known about the molecular basis of apical branching in filamentous fungi. In N. crassa, the act’ mutant, in which actin is positioned subapically instead of apically, displays increased tip splitting (101). Furthermore, the spray mutant (with altered intracellular calcium distribution) and the frost mutant (with disturbed manganese homeostasis) showed excessive apical-branching phenotypes (15, 90). In a large-scale genetic screen for morphological mutants of N. crassa, more proteins whose mutations led to hyphal tip splitting were identified (87), e.g., Ypk1, an ortholog of the yeast and nematode TORC2 effector proteins Ypk1p and SGK-1, and Cdc24, an activator protein of the GTPase Cdc42 (49, 50, 87). A requirement for Cdc42 in tip splitting was also supported by observations of the filamentous yeast Ashbya gossypii (85). Other proteins reported to be specifically required for apical branching in A. gossypii are the protein kinase AgCIAp4, the paxillin-like protein AgPxl1p, and the polarisome components AgSpa2p and AgBni1p (5, 55, 56, 85). Congruently, mutations in the AgBni1p ortholog SepA evoked increased apical branching in Aspergillus nidulans (89).

In order to understand the molecular basis for apical branching in A. niger, we have identified and characterized the genetic locus affected in the ramosa-1 mutant. Here, we report that a single point mutation in the rmsA gene (An02g04280) responsible for the mutant phenotype of the ramosa-1 mutant. The RmsA protein is homologous to the Avolp/Sin1 protein, which is conserved from yeast to humans and, as a component of the TORC2 complex, is involved in regulating actin cytoskeleton polarity (104, 110). We show that RmsA serves as a functional equivalent of Avolp and has a pivotal role in polarity maintenance in A. niger. We furthermore present for the first time a transcriptomic fingerprint of apical branching, which enabled us to obtain valuable mechanistic insights into the signaling machinery controlling morphogenesis of A. niger.

### MATERIALS AND METHODS

#### Strains, culture conditions, and molecular techniques.

The A. niger and S. cerevisiae strains used in this study are listed in Table 1. Escherichia coli strain XL1-Blue served as the host for all plasmid work. General cloning procedures in E. coli were done as described by Sambrook and Russell (82). A. niger strains were cultivated in minimal medium (13) containing 1% glucose as a carbon source or in complete medium, consisting of minimal medium supplemented with 1% yeast extract and 0.5% Casamino Acids; 10 mM uridine was added when required. Fermentation medium (FM) is composed of 0.75% glucose, 0.45% NH₄Cl, 0.15% KH₂PO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.1% salt solution (13), and 0.0035% yeast extract. The pH of FM was adjusted to 3. S. cerevisiae strains were cultivated in yeast-extract-peptone (YP) medium containing either 1% glucose or 1% galactose as a carbon source. Transformation of A. niger strains was conducted as described earlier (75). S. cerevisiae genetic methods as described by Guthrie and Fink (40) were used.

#### Bioreactor cultivation.

Freshly harvested conidia (5 × 10⁸) from strain T312 and the ramosa-1 mutant were used to inoculate 5 liters of FM. Cultivations were performed in a BioFlo3000 bioreactor (New Brunswick Scientific), where the

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**TABLE 1. Strains used in this study**

| Strain | Description | Source or reference |
|--------|-------------|---------------------|
| A. niger |             |                     |
| N402 | Wild type | Lab collection |
| AB4.1 | pyrG | 98 |
| MA70.15 | ΔkasA pyrG⁻ | 65 |
| T312 | Wild type | 77 |
| ramosa-1 | Mutant of T312 obtained after | 77 |
| mutant | UV mutagenesis | |
| 14, 17 | Heterokaryotic strain, ΔmsA | This work |

| S. cerevisiae |                      | Research Genetics |
|---------------|----------------------|-------------------|
| 26474 | Mata/a his3Δ1/Δ1 leu2Δ0Δ1 met15Δ0/MET15 lys2A/Δ LYS2 ura3Δ0 YOL078w/Yol078w::Kan' |                     |
| ARY1 | 26474 containing pGal-RmsA | This work |
| ARY2 | 26474 containing pGal-Avo1 | This work |
| ARY1.1B | Segregant from ARY1, ΔYol078w, pGal-RmsA | This work |
| ARY2.4B | Segregant from ARY2, ΔYol078w, pGal-Avo1 | This work |
| ARY2.4C | Segregant from ARY2, pGal-Avo1 | This work |
temperature, pH (set to 3), and agitation speed were controlled online using the program NBS Biocommand. The cultivation program followed four consecutive phases: (i) 24°C, agitation speed of 250 rpm, and headspace aeration for 13 h; (ii) 37°C, agitation speed of 750 rpm, and sparger aeration for 4 h; (iii) 25°C, agitation speed of 750 rpm, and sparger aeration for 3 h; and (iv) 37°C, agitation speed of 750 rpm, and sparger aeration for 1 h. Mycelial samples were taken after certain time points for microarray and microscopic analyses (see below).

Identification and cloning of mrsA. The mrsA gene was identified by complementation of the temperature-sensitive phenotype of the ramosa-1 mutant using the cosm id library pAOpyrGecoARP containing genomic DNA of wild-type A. niger (kindly provided by F. Schuren and P. Punt, TNO Nutrition). The complementing plasmid isolated was named pRamosa-13. Restriction analysis revealed that pRamosa-13 contained a 9-kb NcoI fragment as well as a 3.4-kb ClaI/NcoI fragment that were each fully capable of complementing the ramosa-1 mutant phenotype. The 9-kb NcoI fragment and the 3.4-kb ClaI/NcoI fragment were cloned into pUC21 (100), giving pRamosa-18 and pRamosa-19, respectively. Double-stranded DNA sequencing of pRamosa-18 revealed that the 9-kb insert harbored a 2,683-bp-long open reading frame (ORF) that was designated mrsA.

Deletion of mrsA. To construct an mrsA deletion plasmid, pRamosa-18 was digested with NotI and Kpnl to obtain a 6-kb fragment containing the 5′ region of the mrsA gene. A 0.7-kb fragment containing the 3′ flanking region of the mrsA gene was obtained using a Xhol/NotI double restriction of pRamosa-19. A 2.5-kb Kpnl/Sall fragment containing the Aspergillus oryzae pyrG gene was obtained from pAO4-13 (29). Three-way ligation of this fragment resulted in the disruption plasmid pΔmrsA. Before transformation into A. niger strain AB4.1 or MA70.15, pΔmrsA was linearized using BglIII. Disruption of the mrsA gene in A. niger was analyzed by Southern blot analysis. Genomic DNAs of putative ΔmrsA strains (i.e., a wild-type parent strain (T312) with the deletion plasmid digested with ClaI, and a transformant with a 718-bp Xhol/NcoI mrsA fragment that was labeled by the random-primer method using [α-32P]dATP was used. Hybridizations were carried out at 65°C. The heterokaryon rescue technique (71) was used to show that mrsA encodes an essential protein. Conidiaospores from primary mrsA transformants in the MA70.15 (ΔmrsA) background were analyzed for growth on selective medium (lacking uridine). Conidiospores from transformants that did not grow on selective medium (lacking uridine) and digested with ClaI, and digested with pRamosa-19, were used as transformants that produced viable conidiospores. For the up- and downregulated sets of genes, 500,000 bootstraps were interpreted as the background distribution. The probabilities of equal or greater extent compared to the requested set was interpreted as the P value.

Microarray data accession number. The microarray data have been deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE17641.

RESULTS

The apical-branching phenotype of the ramosa-1 mutant is reversible. Previous studies have characterized the phenotype of the ramosa-1 mutant and its respective wild-type strain T312 using mature vegetative hyphae. Hyphae of the ramosa-1 mutant developed apical branches when subjected to a restrictive temperature (34 to 40°C), whereas at a permissive temperature (23°C), the ramosa-1 phenotype was similar to that of the wild-type strain (79). In this study, we questioned whether this observation is also valid for young germlings of the ramosa-1 mutant. In order to ensure controlled and equal growth conditions, we cultivated spores of both strains in a bioreactor using a defined temperature program (see below). As followed by the dissolved oxygen tension, equal growth behavior was observed for the ramosa-1 mutant and T312 for the first 17 h of cultivation (data not shown).

First, spores of both strains were inoculated at 24°C and allowed to germinate for 13 h. After initial swelling, the majority of the spores (>90%) of both strains formed unbranched germ tubes and displayed indistinguishable phenotypes (Fig. 1).
After this phase, the temperature was set to the restrictive temperature of 37°C for a period of 4 h. Within the first hour, already 75% of ramosa-1 germlings had started to develop apical branches, whereas germlings of T312 continued elongating with no branching (Fig. 1A, panels c and d). During further cultivation at 37°C, the majority of T312 germlings grew in an apical fashion, and lateral branches were only rarely observed (Fig. 1A, panels f, h, and j). In contrast, all germlings of the ramosa-1 mutant displayed apical branches, and newly formed tips branched again (usually one of the two apical tips branched apically), suggesting that the ability of ramosa-1 germlings to maintain stable polarity axes is limited to a short period, after which new polarity axes become established via apical branching. Cultivation at the restrictive temperature also produced increased septation and subapical branches next to the septa (Fig. 1A, panels e, g, and i). However, when the temperature was shifted back to the permissive temperature (24°C) and kept there for the next 3 h of cultivation, ramosa-1 germlings regained their ability to elongate without producing apical branches; i.e., they maintained stable polarity axes (Fig. 1B, panel a). This observation indicated that the morphogenetic program of the ramosa-1 mutant can be manipulated by simply altering the ambient growth temperature. Indeed, after an additional upshift to 37°C for 1 h, new polarity axes became established (Fig. 1B, panel b). Thus, establishment and (re)maintenance of new polarity axes can be induced or repressed in ramosa-1 germlings.

RmsA shows homology to Avo1p/Sin1 proteins. To identify the genetic locus responsible for the mutant phenotype of the ramosa-1 mutant, a complementation approach was followed. When cultivated on solid medium at 37°C, the ramosa-1 mutant has a drastically reduced radial colony growth rate and forms small, very compact colonies lacking conidiophores and conidia (79). A cosmid library was transformed to the ramosa-1 mutant, and transformants were selected based on their ability to grow and conidiate at 37°C (data not shown). The isolation and subsequent sequencing of the complementing plasmid pRamosa-18 resulted in the identification of a single ORF, designated rmsA.

The 2,683-bp-long ORF is interrupted by two introns, the positions of which were confirmed by sequencing of the corresponding cDNA. As depicted in Fig. S1 in the supplemental
material, the deduced 838-amino-acid sequence of RmsA shows a high degree of homology to the Avo1p/Sin1 protein family. In *Saccharomyces cerevisiae*, Avo1p (adhères voraciously to TOR2) has been shown to be an interactor with the TOR (target of rapamycin) protein complex (TORC2), which is necessary for the polarization of the actin cytoskeleton (61). The *Schizosaccharomyces pombe* rmsA ortholog Sin1 was originally reported to interact with the mitogen-activated protein (MAP) kinase Sty1 (105); however, most recently, it has also been described as an essential component of TORC2 (46).

Avo1p/Sin1 proteins, in general, are highly conserved in metazoan species and fungi (104). Five regions with considerable identity (SCDs I to V) have been identified in Avo1p/Sin1 orthologous proteins (104) and are also present in the RmsA protein (SCD I, amino acids [aa] 1 to 39; SCD II, aa 104 to 180; SCD III, aa 369 to 517; SCD IV, aa 702 to 767; SCD V, aa 768 to 816) (Fig. 2A). Schroder et al. have also additionally identified two putative domains within most Avo1p/Sin1 orthologs, namely, a Raf-like Ras binding domain and a pleckstrin homology domain (86), which were also identified in RmsA (Ras binding domain, aa 594 to 677; pleckstrin homology domain, aa 699 to 789) (Fig. 2A). Sequence comparison of the *rmsA* genes in T312 and the *ramosa-1* mutant revealed a single point mutation, resulting in an exchange of a highly conserved tyrosine to asparagine at aa 447 (Fig. 2B). Remarkably, this mutation is necessary for the polarization of the actin cytoskeleton (61). In order to get a first insight into the function of RmsA, a heterozygous *AVO1::kanMX4/AVO1* strain was transformed with a plasmid that contained the *rmsA* wild-type gene under the control of the *S. cerevisiae* GAL1 promoter (plasmid pGal-RmsA). As controls, the recipient strain was transformed with the *S. cerevisiae* AVO1 gene under the control of the same promoter (plasmid pGal-Avo1) and with the empty plasmid (pYES2). Transformed diploid cells were allowed to sporulate, and haploid spores were dissected on galactose plates. The analysis confirmed that *AVO1* is an essential gene, as only two viable spores per tetrad were obtained after dissecting *avo1/AVO1* heterozygote strains containing the empty pYES2 plasmid. Dissection of the *avo1/AVO1* diploid strain containing pGal-Avo1 resulted in four viable spores, and subsequent analysis showed that expression of...
**AVOI from the GAL1 promoter could rescue the avo1 deletion on both galactose and glucose media (Fig. 3).** The rmsA gene was also fully competent in rescuing the loss of AVOI function, indicating that RmsA is a functional homolog of Avo1p. However, complementation was obtained only on galactose medium, indicating that high levels of rmsA are required for full complementation. S. cerevisiae wild-type strains derived from dissecting the avo1/AVOI heterozygote diploid strain containing the plasmid pGal-RmsA or pGal-Avo1, respectively, did not result in any altered phenotype compared to the wild-type situation (data not shown), suggesting that overexpression of Avo1p or RmsA is not detrimental to S. cerevisiae.

**Mutation of RmsA results in actin and chitin depolarization.** Avo1p has been described to be essential for the maintenance of TORC2 integrity in S. cerevisiae (109). TORC2 is a protein complex that is required for actin polarization, thereby mediating spatial control of cell growth, as well as for positive regulation of the cell wall integrity (CWI) pathway via activation of Rom2p, a GDP/GTP exchange factor for Rho1p (28, 84). We assumed that if a similar complex existed in A. niger and had the same function, actin localization and cell wall organization would be disturbed in the ramosa-1 mutant when grown at 37°C due to a nonfunctional (or partially functional) RmsAV4477p protein.

We thus visualized actin via immunofluorescence and chitin via CFW staining in ramosa-1 and T312 germings shifted for 4 h to the restrictive temperature (similar to those in panels i and j in Fig. 1A). As depicted in Fig. 4, actin localization differed in ramosa-1 germings compared to the wild-type situation. Actin patches were highly clustered at the apex, i.e., at the extreme tip, in 42 out of 49 hyphal tips analyzed from wild-type germings (85.7%) (Fig. 4c). In seven hyphal tips (14.3%), we could observe a faint actin spot at the apex together with actin patches behind the apex (Fig. 4d). These patches presumably form a cortical actin ring corresponding to the actin collar most recently observed in mature hypha of A. nidulans by time-lapse microscopy (94). In comparison, only 21 out of 68 hyphal tips (30.4%) from the ramosa-1 mutant displayed a considerable congregation of actin patches at the hyphal apex (similar to that shown in Fig. 4e), and in only three of these could we detect a basal actin collar (similar to that in Fig. 4d). In the majority of ramosa-1 tips analyzed (69.6%), however, actin patches were scattered randomly along the sub-apex (Fig. 4a and b), resembling the actin delocalization effect of the actin-depolymerizing agent cyclochalasin A in A. nidulans (94) and the localization pattern of actin in the act1' mutant of N. crassa (101).

Actin polarization at the hyphal tip has been shown to be required for polarized chitin synthesis in A. nidulans (95). This observation might also hold true for A. niger, as chitin was found to be accumulated at the hyphal apex in wild-type germings (Fig. 4f). This cap-like distribution, however, was not present in ramosa-1 germings (Fig. 4e), suggesting that loss of actin polarization in the ramosa-1 mutant distorts chitin synthesis at the hyphal tip. Remarkably, the overall CFW fluorescence was found to be much stronger for ramosa-1 germings than for T312 germings (Fig. 4g and h), possibly hinting at a higher chitin abundance in the cell walls of the ramosa-1 mutant, which is in accordance with the previous finding that disappearance of the Spitzenkörper during apical branching is accompanied by considerable cell wall thickening (77).

No differences between ramosa-1 and wild-type germings were observed when they were subjected to DAPI staining. The nuclear distributions, i.e., a regular spacing between nuclei, were similar in both strains (data not shown). Likewise, staining for ROS, described to be required for polarized growth of plant organs (22) and fungi (88), did not reveal any obvious discrepancies between the two strains. A tip-localized accumulation of ROS was found for the ramosa-1 mutant and T312 (Fig. 4i and j).

**Gene expression profiling in the ramosa-1 mutant induced for apical branching.** Microarray analyses were performed using RNA samples obtained from each three bioreactor runs (biological triplicate) of the ramosa-1 mutant and T312 (negative control). To analyze early events in apical branching, germings (corresponding to panels c and d in Fig. 1A) were harvested 1 h after the temperature upshift to 37°C. Microarray data were processed as described in Materials and Methods, and genes showing at least 1.5-fold-higher or -lower expression in the ramosa-1 mutant were evaluated as being differentially expressed. Expression of 156 genes out of 14,165 A. niger genes was modulated, and 109 thereof displayed increased expression levels in the ramosa-1 strain. A comprehensive list of all differentially expressed genes is depicted in Table S2 in the supplemental material. Gene orthologs which have been shown to be important for polar growth in N. crassa (52, 87) are indicated there. In order to validate the changes in gene expression, Northern analyses for 13 selected genes were performed using the same RNA samples as utilized for the microarray analyses. As shown in Fig. 5, the Northern data are in good agreement with the microarray data; i.e., genes showing high or low levels of differential expression in the ramosa-1 mutant displayed comparable signals of intense or modest upregulation (or downregulation) in the Northern experiment.

Functional classification of all responsive genes of the ramosa-1 mutant into FunCat categories (81) revealed that the category with the largest number of differentially expressed genes is that of genes involved in metabolism (Table 2). Apical branching of the ramosa-1 mutant has been reported to be accompanied by substantial reduction of the elongation rate of the parent hypha prior to appearance of apical branches (77). A possible explanation for this observation might be diminished ATP production as a result of reduced glucose uptake caused by decreased expression of An09g04810 (predicted glucose transporter) and a switch to anaerobic energy generation.
reflected by increased transcription of genes encoding a pyruvate decarboxylase (An02g06820) and a alcohol dehydrogenase (An02g02060) (Table 3).

Within the category of metabolism, genes coding for proteins involved in glutamate metabolism (An11g07960 coding for glutaminase and An02g14590 coding for a NADPH-dependent glutamate dehydrogenase) were upregulated (Table 3 and Fig. 6). Most interestingly, deletion of the NADPH-dependent glutamate dehydrogenase results in reduced branching frequencies in *A. nidulans* and *Penicillium chrysogenum* (96). Glutamate is one of the main cellular precursors used for the synthesis of other amino acids, suggesting that amino acid starvation might be sensed in the *ramosa-1* mutant. Alternatively, glutamate might have been used to fuel the citrate cycle.

FIG. 4. Phenotypic analysis of *ramosa-1* and T312 germlings. Samples were taken after cultivation for 13 h at 24°C, followed by cultivation for 4 h at 37°C. Microscopic pictures were taken for at least 50 germlings from each strain, and representative pictures are shown. (a to d) Actin immunostaining of the *ramosa-1* mutant (a and b) and T312 (c and d). Polarized actin localization is indicated by arrows, an actin collar by arrowheads, and depolarized actin patches by stars. (e to h) CFW staining of the *ramosa-1* mutant (e and g) and T312 (f and h). Pictures for panels e and f were taken using an automatic exposure time, and pictures for panels g and h were taken using a 100-ms exposure time. Polarized chitin localization as visible in panel f is indicated by arrows. The increase in CFW fluorescence intensity in panel g suggests enhanced chitin levels at *ramosa-1* cell walls. (i and j) Microscopic images of the *ramosa-1* mutant (i) and T312 (j) stained with NBT. The presence of ROS is reflected by blue hyphal tips. Bars, 5 μm for panels a to d and 10 μm for panels e to j.
or as a precursor of γ-aminobutyrate, whose further metabolism is important for counteracting oxidative stress in *S. cerevisiae* and *Arabidopsis thaliana* (25, 31). The key enzyme of the γ-aminobutyrate shunt, γ-aminobutyrate transaminase, showed enhanced expression in the *ramosa*-1 mutant (An17g00910 in Fig. 6), which could probably point toward increased ROS production in the *ramosa*-1 mutant. Supportive of this assumption is upregulation of other *A. niger* genes which show considerable sequence homology to oxidative stress-responsive and ROS-scavenging proteins from other eukaryotes, e.g., d-arabinose dehydrogenase (An01g06970), platelet-activating factor acetylhydrolase (An09g01050), and manganese superoxide dismutase (An01g12530) (1, 58, 99).

Finally, 13 genes in the category related to metabolism with predicted function in cell wall biosynthesis and integrity were significantly upregulated in the *ramosa*-1 mutant, whereas two genes were downregulated (Table 3 and Fig. 7), indicating that apical branching is accompanied by considerable cell wall reorganization. Congruently, the *mkkA* gene (An18g03740), encoding a MAP kinase kinase predicted to function in the CWI signaling pathway of *A. niger*, showed increased expression. To the group of upregulated cell wall genes belonged a chitin synthase gene (An07g05570), whose increased expression might explain the apparent enhanced chitin level in the *ramosa*-1 mutant as shown in Fig. 4g.

Six genes predicted to function in the synthesis of (phospho)lipid signaling molecules such as phosphatidate (PA) (An02g08050 and An15g07040; reactions 3 and 4 in Fig. 7), phosphatidylinositol-4'-monophosphate (PIP) (An18g06410, reaction 5 in Fig. 7), diacylglycerol (DAG) (An04g03870 and An11g05330, reactions 1 and 2 in Fig. 7), and inositolpyrophosphates (IP) (An16g05020, reaction 6 in Fig. 7) were upregulated in the *ramosa*-1 mutant (Table 3 and Fig. 7). These molecules play important roles in the regulation of actin polarization, CWI, and calcium signaling in lower and higher eukaryotes (see Discussion and Fig. 7), thus probably reflecting an important influence of phospholipid signaling in the process of apical branching.

Furthermore, genes encoding putative effector proteins of the calcium signaling machinery were upregulated, such as two Ca²⁺/calmodulin-dependent protein kinases (An02g05490 and An16g03050) and three vacuolar Ca²⁺ pumps (An02g06350, An01g03100, and An05g00170), hinting at the possibility that the mechanisms underlying apical branching might, beside CWI and phospholipid signaling, also involve the calcium signaling machinery (Fig. 7). In support of this notion is the most recent observation that a strong calcium spike accompanies apical branching in *Fusarium oxysporum* hyphae (54). Finally, 12 genes putatively encoding transport proteins for ions (P⁴⁺, Na⁺, K⁺, Fe²⁺, and Zn²⁺) and small molecules (phospholipids, amino acids, peptides, and glucose) displayed differential transcription in the *ramosa*-1 mutant (Table 3), suggesting that (i) apical branching might in general require ion homeostatic and metabolic control systems and/or (ii) the RmsA protein has a function not only in actin polarization but additionally in ion homeostasis and energy metabolism.

**Promoter analysis of differentially expressed genes.** In order to unravel potential transcription factors involved in up- or downregulation of the 136 differentially expressed genes, we screened the 1,000-bp upstream regions of all genes for the presence of binding sites established for 25 transcription factors from different *Aspergillus* and *Trichoderma* species (using the in-house-developed TFBSF). We also used MEME (6) to screen for the presence of common but new DNA binding motifs. We determined the frequency of occurrence of these motifs in the genomic region of each gene. On average, each probe was scored with all 25 transcription factors with a probability of occurrence (PO). A list of the top 10 transcription factors can be found in Table S2 in the supplemental material. The most frequently occurring motifs were selected and used to refine the putative transcription factors involved in the regulation of apical branching.

**TABLE 2. Functional categories of genes up- or downregulated in the *ramosa*-1 mutant compared to wild-type strain T312**

| Functional category                  | Upregulated | Downregulated |
|--------------------------------------|-------------|---------------|
| Metabolism                           | 32          | 11            |
| Amino acid metabolism                | 4           | 1             |
| Nitrogen and sulfur metabolism       | 2           | 1             |
| Nucleotide metabolism                | 1           | 1             |
| Phosphate metabolism                 | 1           | 1             |
| C compound and carbohydrate metabolism | 18         | 7             |
| Lipid, fatty acid, and isoprenoid metabolism | 6         | 1             |
| Energy                               | 2           | 1             |
| Cell cycle and DNA processing        | 2           | 1             |
| Transcription                        | 2           | 1             |
| Protein synthesis                    | 7           | 2             |
| Protein fate                         | 7           | 2             |
| Cellular transport and transport mechanism | 5         | 2             |
| Cellular communication               | 1           | 1             |
| Cell rescue, defense, and virulence  | 3           | 1             |
| Regulation of interaction with cellular environment | 2         | 1             |
| Subcellular localization             | 2           | 1             |
| Transport facilitation               | 1           | 1             |
| Unclassified proteins                | 50          | 10            |

*An annotated list of all responsive genes, including the fold change, P value, and classification, can be found in Table S2 in the supplemental material. Statistically enriched FunCat subcategories, determined by using the gene set enrichment analysis tool (93) (false discovery rate, <0.05), are also depicted in Table S2 in the supplemental material."
TABLE 3. Selected responsive genes in the *ramosa*-1 mutant, ordered into different processes and functions

| Category and ORF | Gene\(^a\) | Fold change\(^b\) | \(P\) value | Predicted protein function\(^c\) | Closest \(S.\) cerevisiae homolog |
|-----------------|-------------|----------------|-----------|---------------------------------|-----------------------------|
| Energy generation | An02g06820 | 2.62 | 0.024 | Pyruvate decarboxylase | Pdc6 |
| | An02g02060 | 2.15 | 0.039 | Alcohol dehydrogenase | |
| Amino acid metabolism | An11g07960 | (9.74) | 0.025 | Glutaminase | |
| | An17g00910 | 6.03 | 0.004 | \(\gamma\)-Aminobutyrate transaminase | |
| | An02g15980 | 3.15 | 0.029 | NAD\(^+\)-dependent glutamate dehydrogenase | |
| Oxidative stress-responsive proteins | An06g10500 | (22.64) | 0.038 | PAFAH, removal of oxidized membrane phospholipids | |
| | An15g07670 | (10.66) | 0.055 | Tyrosinase involved in melanin synthesis | |
| | An02g00610 | (3.97) | 0.029 | Heat shock protein | |
| | An01g01840 | 2.49 | 0.038 | Secoisolariciresinol dehydrogenase | |
| | An01g12530 | 2.39 | 0.055 | Manganese superoxide dismutase | |
| Cell wall synthesis and remodeling | An16g06120 | gelF \(^d\) | (78.40) | 0.004 | Glycophosphatidylinositol-anchored \(\beta\)-1,3-glucanosyltransferase |
| | An04g05830 | 18.18 | 0.006 | Glycophosphatidylinositol-anchored cell wall protein | |
| | An13g02510 | 7.49 | 0.003 | Glycophosphatidylinositol-anchored chitin transglycosylase | |
| | An11g06540 | 6.506 | 0.005 | D-Arabinose dehydrogenase | |
| | An06g01610 | (3.97) | 0.029 | Heat shock protein | |
| | An18g03740 | 2.26 | 0.038 | Secoisolariciresinol dehydrogenase | |
| | An01g06400 | 0.14 | 0.013 | Glycophosphatidylinositol-anchored chitinase, similar to ChiA of *A. nidulans* | |
| Phospholipid signaling | An02g08050 \(^d\) | (12.36) | 0.056 | Phosphatidyl synthase, synthesis of phosphatidyl alcohols | |
| | An01g07000 | 10.75 | 0.051 | \(C_{14}\) sterol reductase, ergosterol synthesis | Erg24 |
| | An16g05020 \(^d\) | 7.34 | 0.002 | Inositol hexaki-/heptakiphosphate kinase, synthesis of IP6/IP7 | Vip1 |
| | An02g04160 \(^d\) | 4.31 | 0.011 | Plasma membrane protein promoting PHF synthesis | Sfl1 |
| | An15g07040 \(^d\) | 3.77 | 0.026 | Phospholipase D, synthesis of PA | Mkk12 |
| | An04g03870 \(^d\) | (3.24) | 0.018 | PA phosphatase, synthesis of DAG | |
| Calcium signaling and homeostasis | An02g05490 | 2.97 | 0.028 | \(Ca^{2+}\)/calmodulin-dependent protein kinase | |
| | An16g03050 | 2.39 | 0.033 | \(Ca^{2+}\)/calmodulin-dependent protein kinase | |
| | An02g06350 | (5.48) | 0.025 | Vacular \(Ca^{2+}\)/\(H^+\) exchanger | Pmc1 |
| | An01g03100 | 4.49 | 0.009 | Vacular \(Ca^{2+}\)/\(H^+\) exchanger | Vcx1 |
| | An09g00170 | 2.15 | 0.054 | Vacular \(Ca^{2+}\)/\(H^+\) exchanger | Vcx1 |
| Other signaling processes | An15g01560 | (3.21) | 0.029 | GTPase-activating protein involved in protein trafficking | Gyp7 |
| | An04g01500 | 2.98 | 0.044 | Putative C2H2 zinc finger transcription factor | |
| | An09g07090 | 0.06 | 0.054 | SUN family protein involved in replication | Sim1 |
| | An16g07890 | 0.04 | 0.037 | Similar to *A. nidulans* transcription factor RosA | Ume6 |
| | An12g06710 | 0.29 | 0.031 | Negative regulator of Cdc42 | Vtc1 |
| Transporters | An02g41460 | 4.85 | 0.010 | Mitochondrial phosphate translocator | Mir1 |
| | An12g02320 | (4.70) | 0.027 | Vascular glutathione S-conjugate ABC transporter | Yci1 |
| | An09g09303 | 3.44 | 0.055 | Na\(^+\)/K\(^+\)-exchanging ATPase alpha-1 chain | |
| | An02g04180 | 3.78 | 0.021 | MFS multidrug transporter | |
| | An16g06300 | 3.18 | 0.013 | Low-affinity Fe(III) transporter | |
| | An04g06840 | 2.39 | 0.052 | \(Ca^{2+}\)/phospholipid-transporting ATPase | |
| | An14g18600 | 0.41 | 0.051 | Mitochondrial carrier protein | |
| | An12g05510 | 0.38 | 0.024 | Siderophore-iron transporter | |
| | An05g09300 | 0.32 | 0.049 | Vacular zinc transporter | Zrc1 |
| | An18g01220 | 0.29 | 0.059 | Allantoic permease | Dal5 |
| | An15g07460 | 0.26 | 0.023 | Oligopeptide transporter | |
| | An09g68410 | 0.06 | 0.011 | High-affinity glucose transporter | |

\(^a\) Proposed protein abbreviations according to reference 73.

\(^b\) Parentheses indicate that the gene has an “Absent” flag in the control experiment.

\(^c\) PAFAH, platelet-activating factor acetylhydrolase; MFS, major facilitator superfamily.

\(^d\) Superscript numbers refer to reactions indicated in Fig. 7.
motifs in the group of 109 upregulated and 27 downregulated genes and evaluated whether these motifs are statistically significant over- or underrepresented compared to groups of the same size comprising randomly selected genes of *A. niger* (500,000 bootstrap samples, \( P / H_{11021} < 0.05 \)).

Using these in silico approaches, we were able to identify four motifs with the TFBSF tool and 27 motifs with the MEME tool as being enriched or underrepresented within the upsteam regions of genes upregulated in the *ramosa-1* mutant. As shown in detail in Table S3 in the supplemental material, the four enriched *Aspergillus* motifs identified with the TFBSF tool were also among the sites identified by MEME and are binding motifs of the transcription factors AbaA (asesexual development and dimorphism) (2, 17, 57), CrzA (calcium signaling, cell wall formation, and polar growth) (33, 91), CreA (carbon catabolite repression and polar growth) (59, 112), and AmdR (amino acid utilization) (27), respectively. Among the set of MEME sites, we could additionally identify one site as a BrlA site (asesexual development) (23) and three sites as cyclic AMP-responsive elements involved in protein kinase A signaling (metabolism, polar growth, and dimorphism) (11, 16, 38). Within the set of genes downregulated in the *ramosa-1* mutant, three overrepresented sites were identified with the TFBSF tool which were also present among the 10 identified MEME sites (see Table S3 in the supplemental material): Seb1 (osmotic stress response) (74), AnCP/AnCF (CCAA binding factor involved in, e.g., respiration) (21, 53), and BrlA (asesexual development) (23). These data are in good agreement with the microarray and phenotypic data obtained which suggested a complex reorganization in the *ramosa-1* mutant involving polar growth control, carbon and nitrogen metabolism, respiration, and calcium homeostasis.

For the majority of the known *Aspergillus/Trichoderma* transcription factor binding sites analyzed (15), we found a similar frequency of occurrence in genes differentially expressed in the *ramosa-1* mutant compared to randomly selected genes (see Table S3 in the supplemental material). Although this does not necessarily preclude these trans factors from any role in the *ramosa-1* mutant transcriptional response, it can be speculated that their involvement in regulating early events during apical branching is less important than the involvement of transcription factors showing enriched or underrepresented motifs. To our surprise, however, binding sites for the effector regulator of the CWI pathway RlmA are not significantly overrepresented in genes upregulated in the *ramosa-1* mutant, suggesting that (i) RlmA does not have the same prominent function as its *S. cerevisiae* ortholog Rlm1p (i.e., not all *A. niger* cell wall-related genes are regulated by RlmA), (ii) CWI activates not only RlmA but also another as-yet-unknown transcription factor(s), or (iii) CWI signaling is not the only pathway responsible for the expression of cell wall-related genes.

**DISCUSSION**

The *ramosa-1* mutant as a model system to study polarity control. This study shows that the *ramosa-1* strain can be considered an excellent model system to study the morphogenetic program of *A. niger*. All critical steps of hyphal morphogenesis (establishment, maintenance, and loss of maintenance of polar axes) can be altered within a single system by changing the cultivation temperature. Hence, different aspects of fungal polarity can be simultaneously studied in this strain. In addition, this model system can be used to address the question of whether an increase in the number of branches (per total cell mass) will indeed improve protein secretion in *A. niger*. Using the approach described in this study, the frequency of branching as well as the length of hyphal compartments can easily be adjusted by running defined temperature programs. Determin-
niger to asparagine has profound consequences on the growth of the ramosa-1 mutant when it is cultivated at 25°C. The lethality of AVO1 deletion in S. cerevisiae can be complemented by rmsA, demonstrating that RmsA is a functional equivalent of Avo1p, a component of the S. cerevisiae TORC2 complex (61). Avo1p is essential for the maintenance of TORC2 integrity (109), a complex that is composed of six proteins, namely Avo1p, Avo2p, Avo3p/Tsc11p, Lst8p, Bit61p, and Tor2p (30, 61, 76). With the exception of Avo2p and Bit61p, sequences with obvious similarity to all S. cerevisiae TORC2 components can be found in the genome of A. niger (see Table S4 in the supplemental material). Respective sequences (except for Avo2p and Bit61p, which appear to be unique to S. cerevisiae) are also present in other eukaryotes ranging from S. pombe over Drosophila to mammals and have at least partially been shown to associate in a TORC2 complex (109). This high degree of evolutionary conservation makes it reasonable to predict that a TORC2-like complex exists in A. niger and that RmsA is a component of it.

TORC2 has an essential function for actin polarization and hence determination of cell polarity in S. cerevisiae, Dictyostelium discoideum, and mammalian cells but not in the fission yeast S. pombe (63, 109). In this study, we have observed a depolarized actin localization at the hyphal tip when the ramosa-1 mutant is cultivated at the restrictive temperature (Fig. 4), strongly suggesting that RmsA, i.e., the proposed A. niger TORC2, carries out a function in cytoskeletal organization. Most interestingly, the disturbed actin localization observed is reminiscent of the subapical localization of actin in the act1 mutant of N. crassa, a mutant that displays increased apical branching (101). Loss of actin polarization in the ramosa-1 mutant (hypothetically due to reduced TORC2 integrity as a consequence of a less functional RmsA$^{Y447N}$) may thus be considered as a key event of apical branching.

Transcriptomic insights into the process of apical branching. What, then, are the cellular events involved in apical branching? The transcriptomic data obtained in this work are most probably a reflection of two events: (i) the consequences of a defective RmsA/TORC2 function(s) (resulting in loss of polarity and disturbance of other TORC2-controlled processes) and (ii) establishment of two new polarity axes. The function of TORC2 in polar growth control via regulation of actin polarization and/or protein kinase C (PKC) activities is conserved from yeast to mammals (reviewed in reference 49). Additionally, TORC2 has also been reported to be an upstream regulator of another TOR-containing complex (TORC1) in mammals (49). TORC1 is also conserved from yeast to mammals and regulates a myriad of cell growth-related processes (e.g., transcription, translation, and protein turnover) by integrating signals from nutrients, energy status, and stressors. Thus, TORC1 regulates temporal growth, whereas TORC2 regulates spatial growth (49). However, the discovery that mammalian TORC2 acts not only in parallel to but also upstream of TORC1 brings challenge in understanding TORC2 signaling mechanisms and in answering the question posed above. The transcriptomic response of the ramosa-1 mutant includes changes in carbon, nitrogen, (phospho)lipid, temperature but disturbed at higher temperature, or (iii) RmsA is crucial for the survival of A. niger at high but not at low temperature. Our data seem to exclude the third possibility, as deletion of the rmsA gene is already lethal for A. niger when it is cultivated at 25°C.

A proposed role for RmsA in polarity control. Only a single point mutation within the rmsA gene is responsible for the mutant phenotype of the ramosa-1 mutant. The Y447N mutation of RmsA seems to have no apparent consequence for the growth of the ramosa-1 mutant when it is cultivated at low temperature (24°C), suggesting that RmsA$^{Y447N}$ can still fulfill its cellular function. However, at higher temperature, Y447 seems to be essential for the function of RmsA, and its change to asparagine has profound consequences on the growth of A. niger, such as loss of polarity maintenance, slow growth, and defective asexual development (this work and reference 77). Sev eral scenarios are imaginable to explain this temperature-dependent phenotype: (i) RmsA$^{Y447N}$ is stable but lowered in its stability at 37°C and becomes readily degraded, (ii) an interaction of RmsA$^{Y447N}$ with other proteins is possible at low temperature but disturbed at higher temperature, or (iii) RmsA is crucial for the survival of A. niger at high but not at low temperature. Our data seem to exclude the third possibility, as deletion of the rmsA gene is already lethal for A. niger when it is cultivated at 25°C.

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and energy metabolism as well as in the stress response and ion homeostasis. As mentioned above, probably not all of these responses are related to the process of apical branching. Below, we propose and discuss a possible involvement of three cellular processes in the process of apical branching—(phospho)lipid signaling, calcium signaling, and CWI signaling—and their suspected connections to TORC2 functions.

The synthesis of important (phospho)lipid signaling molecules (PA, DAG, and PIP) and IP seems to be increased during apical branching, as genes encoding the corresponding synthetic enzymes showed enhanced expression (Table 3 and Fig. 7). Although the function of PA has not been studied in detail for filamentous fungi, a recent report demonstrated that reduced production of PA causes polarity defects in A. nidulans (62), which supports our data. Importantly, PA serves as precursor for DAG in S. cerevisiae, which in turn functions as activator of PKC (Pck1p), a component of the CWI pathway which is localized upstream of the MAP kinase kinase Mkk1/2p (MkkA) (Fig. 7) (for a review, see reference 103). PA, however, has multiple regulatory roles in eukaryotes, such as promotion of actin polymerization and activation of TOR and PI(4,5)2 kinases to increase conversion from PIP to phosphatidylinositol-4,5-bisphosphate (PIP2) (103). PIP2 in turn can stimulate actin polymerization via interaction with actin-interacting proteins (reviewed in reference 24) and promotes actin remodeling by recruiting TORC2-interacting proteins (Sln1p and Sln2p) and different GTPases (e.g., Rho1p and its activator Rom2p) to membrane compartments (3, 4, 83). The GTPase Cdc42 has also been reported to activate mammalian PI(4,5)2 kinases, thereby increasing local PIP2 levels (reviewed in reference 83). Interestingly, we found a downregulation of An12g04710, which displays similarity to the yeast Vtc1p and Nrf1p proteins, which are negative regulators of Cdc42 (70). Reduced expression of An12g04710 could thus also hint at increased PIP2 synthesis during apical branching. Finally, An16g05020, a gene with homology to the genes encoding the S. cerevisiae Vip1p and S. pombe Asp1p proteins (inositol hexaki-/heptaki-phosphate kinase; reaction 6 in Fig. 7), showed increased expression. Asp1p has been reported to be necessary for the integrity of cortical actin patch organization (32).

Remarkably, PIP2 serves as precursor for phosphatidylinositol-1,4,5-triphosphate (PIP3), which has been described as inducing calcium release from intracellular stores in mammalian cells (reviewed in reference 7). An increase in cytosolic calcium levels has been shown to activate the calcium/calmodulin/calcineurin/Crz1p signaling pathway in S. cerevisiae, which induces calcineurin- or Crz1p-dependent transcription of genes whose protein products are involved in ion homeostasis, cell wall synthesis, and signaling (reviewed in reference 26). All components of this pathway are conserved in A. niger (see Table S5 in the supplemental material). CrzA, the A. nidulans homolog of Crz1p, has also been demonstrated to be positively involved in transcriptional regulation of a chitin synthase gene (chsB) and the vcaA gene, encoding a vacuolar Ca2+ pump (91). Among the genes upregulated in the ramosa-1 mutant, at least five genes whose protein products can be predicted to function in calcium signaling and homeostasis were identified, i.e., two Ca2+/calmodulin-dependent protein kinases and three vacuolar Ca2+ pumps (two of these encode VCX1/vcaA-homologous genes). However, other A. niger genes also might be effectors of a calcium response, as CrzA binding motifs were significantly overrepresented in genes upregulated in the ramosa-1 mutant (see Table S3 in the supplemental material). Furthermore, a recent finding in S. cerevisiae demonstrated that calcineurin negatively controls TORC2 and that TORC2 negatively regulates calcineurin, a mutual antagonism that is also reflected by the observation that about 50% of the genes which are upregulated during TORC2 inhibition overlap with calcineurin/Crz1p-dependent genes (69). Among this set of overlapping S. cerevisiae genes are also genes showing increased expression in the ramosa-1 mutant (An15g01560/GTPase Gyp7p, An02g06350/vacuolar Ca2+/H+ exchanger Pmc1p, An02g05490 and An16g03050/protein kinase Cmk2, and An00g07168/aspartic protease) (Table 3; see Table S2 in the supplemental material). Most importantly, this report has evidenced that calcineurin-mediated events cause depolarization of the actin cytoskeleton, whereas TORC2 counteracts this process—opposing control mechanisms that have also been observed in mammalian cells (see reference 69 and references therein). The ramosa-1 transcriptome data hint at the possibility that a similar antagonism might occur in A. niger, i.e., actin depolarization in the ramosa-1 mutant might be a consequence of increased calcium signaling which originates from defective TORC2 signaling.

The actin organization defect in an S. cerevisiae conditional tor2 loss-of-function mutant (tor2Δ) can be suppressed by overexpressing CWI pathway components (for a review, see reference 60). Our data demonstrated enhanced transcription of the predicted CWI component mkkA1, point toward activation of PkcA by DAG, showed increased expression of cell wall biosynthesis and remodeling genes, and suggested increased chitin deposition in the ramosa-1 mutant (Fig. 4 and Table 3). These responses resemble the connection between TORC2 and CWI signaling in S. cerevisiae, suggesting that reinforced CWI signaling in the ramosa-1 mutant could be an adaptive response to counteract defective RmsaX477N (i.e., TORC2) function.

Finally, the transcriptomic data also point toward increased expression of oxidative stress response genes in the ramosa-1 mutant (Table 3). However, the fact that we could not detect any differences in tip-localized ROS staining in ramosa-1 and wild-type germlings (Fig. 4) suggests that increased ROS production can be sufficiently counteracted by enhanced expression of ROS-scavenging genes in the ramosa-1 mutant. An appealing explanation for the observed induction of oxidative stress genes might be given by a recent report on S. cerevisiae, where it has been demonstrated that TORC2 inhibits the response to oxidative stress (69). In other words, defective TORC2 signaling in the ramosa-1 mutant could mimic the presence of oxidative stress within the cell and might further reflect a similar interconnection between TORC2, oxidative stress, CWI, and calcium signaling pathways in A. niger as reported for S. cerevisiae. Taken together, the transcriptomic fingerprint of the ramosa-1 mutant suggests that at least four signaling cascades might be involved in the process of apical branching: (phospho)lipid, calcium, TORC2, and CWI signaling. In our previous work, we used a pharmacological approach to induce (sub)apical branch formation in A. niger germlings. The transcriptomic profiles obtained likewise suggested a role...
for (phospho)lipid, TORC2, and CWI signaling during polarity control (67).

A hypothetical model for cellular events involved in apical branching. We previously proposed “that the apical branching phenotype in ramosa-1 is triggered by a molecular event that induces a transient alteration in cytoskeleton organization” (77, 78). Our present transcriptomic analysis shows a number of changes that either singly or in combination would bring about the physiological changes that contract the actin cytoskeleton, dislocate the Spitzenkörper, interrupt hyphal elongation, and set the stage for the subsequent formation of two new centers of polarized growth. Based upon transcriptomic, genetic, and phenotypic data obtained in this study and in our previous work (77, 78), we propose the following working model for the process of apical branching in A. niger (Fig. 8). We hypothesize that the actin polarization defect in the ramosa-1 mutant is evoked by a nonfunctional or partially functional RmsA<sup>Y447N</sup> protein, whereas in wild-type cells, the primary trigger for a momentary disruption of actin integrity has yet to be identified. As a consequence of actin depolarization, the Spitzenkörper detaches from the apex and disintegrates, and polarity maintenance becomes lost in the leading hypha. This event is counteracted by increased (phospho)lipid and CWI signaling aiming at actin repolarization and increased cell wall biosynthesis, whereupon two new Spitzenkörper and thereby two new polarity axes become established. The stability of these polarity axes, however, cannot be maintained in the

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