Evidence for a Ustilago maydis Steroid 5α-Reductase by Functional Expression in Arabidopsis det2-1 Mutants

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We have identified a gene (udh1) in the basidiomycete Ustilago maydis that is induced during the parasitic interaction with its host plant maize (Zea mays). udh1 encodes a protein with high similarity to mammalian and plant 5α-steroid reductases. Udh1 differs from those of known 5α-steroid reductases by six additional domains, partially predicted to be membrane-spanning. A fusion protein of Udh1 and the green fluorescent protein provided evidence for endoplasmic reticulum localization in U. maydis. The function of the Udh1 protein was demonstrated by complementing Arabidopsis det2-1 mutants, which display a dwarf phenotype due to a mutation in the 5α-steroid reductase encoding DET2 gene. det2-1 mutant plants expressing either the udh1 or the DET2 gene controlled by the cauliflower mosaic virus 35S promoter differed from wild-type Columbia plants by accelerated stem growth, flower and seed development and a reduction in size and number of rosette leaves. The accelerated growth phenotype of udh1 transgenic plants was stably inherited and was favored under reduced light conditions. Truncation of the N-terminal 70 amino acids of the Udh1 protein abolished the ability to restore growth in det2-1 plants. Our results demonstrate the existence of a 5α-steroid reductase encoding gene in fungi and suggest a common ancestor between fungal, plant, and mammalian proteins.

The basidiomycete Ustilago maydis is a facultative biotrophic fungus that grows in a yeast form in culture. The pathogenic life cycle is initiated when compatible, haploid sporidia fuse and form a filamentous dikaryon, which is able to penetrate into host tissue (Snetselaar and Mims, 1993; Mills and Kotzé, 1981; for review, see Banuett, 1995). Prerequisites for compatibility are different a and b loci (for review, see Kahmann et al., 2000). The a locus encodes a pheromone-based recognition system required for mating ( Bölker et al., 1992; Spellig et al., 1994). The b locus encodes the homeodomain proteins Be and Bw that trigger pathogenic development of the dikaryotic hyphae (Kronstad and Leong, 1990; Schulz et al., 1990; Gillissen et al., 1992).

The pathogenic phase is characterized by extensive hyphal growth within the infected plant tissue followed by karyogamy and differentiation of the diploid teliospores (Banuett and Herskovitz, 1996). The plant reacts to the infection by tumor formation, which is associated with cell enlargement and proliferation (Snetselaar and Mims, 1994; I. Potrykus, personal communication). For more than four decades, it has been speculated that phytohormones, like auxins, cytokinins, and gibberellins released by U. maydis, may trigger the morphological alterations in the host (Wolf, 1952; Turian and Hamilton, 1960; Mills and van Staden, 1978; Sokolovskaya and Kuznetsov, 1984; Basse et al., 1996). However, up to now there is no convincing evidence, to our knowledge, that these compounds are responsible for tumor induction. Another class of hormones that have a wide distribution throughout the plant kingdom and that control plant growth and development are brassinosteroids (Man-dava, 1988; for review, see Altmann, 1998; Clouse and Sasse, 1998). In Arabidopsis, det2-1 mutants defective in brassinolide biosynthesis have been isolated (Chory et al., 1991). They are characterized by dramatic dwarfism, dark green leaves as consequence of an increased number of chloroplasts in a reduced cell volume, reduced male fertility and apical dominance, and delayed senescence and flowering (Chory et al., 1991; Li et al., 1996; for review, see Altmann, 1998; Clouse and Sasse, 1998). det2-1 plants are mutated in the DET2 gene, which encodes a steroid 5α-reductase with high similarities to mammalian steroid 5α-reductases. To date, DET2 represents the only cloned plant steroid 5α-reductase. In mammals, this class of enzyme catalyzes the NADPH-dependent conversion of testosterone to dihydrotestosterone, the potent androgen in male sex differentiation (for review, see Russell and Wilson, 1994). Detailed metabolic investigations have shown that the Arabidopsis DET2 protein catalyzes the 5α-
reduction of (24R)-24-methylcholest-4-en-3-one to (24R)-24-methyl-5α-cholestan-3-one, the third step in a four-step process leading to the formation of campestanol from campesterol (Li et al., 1996; Fujioka et al., 1997; Noguchi et al., 1999). In microorganisms, the only gene with similarity to steroid 5α-reductases resides in the genome of Schizosaccharomyces pombe (GenBank accession no. T39193), however, evidence for a function as steroid 5α-reductase is lacking.

Here, we describe the U. maydis udh1 gene, which encodes a protein with high similarity to known steroid 5α-reductases from mammals and Arabidopsis and provide evidence for its function by complementation of Arabidopsis mutants homozygous for det2-1.

RESULTS
Isolation of udh1

A number of U. maydis genes that are strongly expressed during the tumor stage of infected maize (Zea mays) plants have been identified by applying the method of differential display (Basse et al., 2000, 2002). One of these U. maydis genes is udh1 (for U. maydis DET2 homolog). A fragment comprising the udh1 3’ portion was used to identify genomic clones from a U. maydis cosmid library. Sequence analysis of a genomic AgeI-HindIII fragment revealed an open reading frame (ORF) of 1,164 bp encoding a predicted protein of 388 amino acids with an estimated molecular mass of 42.3 kD (Fig. 1). The absence of introns in the udh1 ORF sequence and the presence of a poly(A) site were demonstrated by the isolation of cDNA clones and reverse transcription (RT)-PCR analysis (Fig. 1; see "Materials and Methods"). The deduced amino acid sequence of the udh1 gene is most similar to those of type 1 steroid-5α-reductases from mammals (Andersson and Russell, 1990; Lopez-Solache et al., 1996), the Arabidopsis DET2 protein (Li et al., 1996), and a putative steroid reductase from S. pombe (GenBank accession no. T39193), with 30% sequence identity to the mammalian and plant enzymes and 35% sequence identity to the predicted S. pombe protein (Fig. 2A). For comparison, the Arabidopsis DET2 and rat type 1 steroid-5α-reductase (rS5R1) proteins share 41% sequence identity. In a number of positions, the Udh1 sequence matched either the mammalian or the Arabidopsis sequence (Fig. 2B), implying a common ancestor protein. In support of a function as steroid-5α-reductase, the Glu-311 residue of the Udh1 protein aligned with an invariant Glu residue of mammalian enzymes that is absolutely required for activity. This residue is also conserved in DET2 but is replaced by Lys-204 in the mutant det2-1 protein (Fig. 2A; Li et al., 1996). Furthermore, the Gly-52 residue of the Udh1 sequence matched the conserved Gly-34 residue of human type 2 steroid-5α-reductase (hS5R2) implicated in testosterone binding (Thigpen and Russell, 1992). In addition, Udh1 contains five (Arg-266, Pro-295, Gly-297, Asn-307, and Arg-380) of six conserved amino acids that are part of a cofactor binding domain typical for mammalian 5α-reductases (for review, see Russell and Wilson, 1994). The Udh1 amino acid sequence remarkably differed in length from known 5α-reductases by about an additional 130 amino acids (Fig. 2A). The additional amino acids are inserted in six stretches (I–VI) and comprise positions 1 to 25 (I), 80 to 102 (II), 174 to 213 (III), 222 to 234 (IV), 271 to 289 (V), and 330 to 348 (VI). Two of these stretches (II and V) also partially exist in the S. pombe ORF sequence (Fig. 2A) and emphasize the closer relationship between the fungal proteins. The U. maydis amino acid sequence contained eight potential transmembrane-spanning domains according to the

Figure 1. Nucleotide sequence and derived amino acid sequence of udh1. Shown is the DNA sequence of the 1,722-bp genomic Agel-HindIII fragment (GenBank accession no. AF502086) containing the udh1 gene. The translation start ATG codon is underlined and the stop codon of udh1 is indicated by an asterisk. Primers used for generating udh1-eGFP fusions are indicated by arrows. The deletion in udh1 strains comprised the region from Phe-78 to Ala-358 (boxed). The poly(A) site is indicated at position 1,453.
program TMPRED, of which four aligned with the transmembrane-spanning domains of rS5R1 predicted by the same program (Fig. 2A). Two of the additional transmembrane domains in the *U. maydis* ORF sequence overlapped with stretches II and III. Thus, the *U. maydis* Udh1 seems to form a distinct class among 5α-steroid reductases.

**Expression of udh1**

As judged by northern analysis the *U. maydis udh1* gene was expressed at similar levels during budding growth of haploid sporidia and during filamentous growth of either dikaryotic hyphae resulting from a cross of haploid strains FB1 and FB2 or the solopatho-
genic, diploid strain FBD11 (Fig. 3A). During biotrophic growth, *udh1* transcripts were detected 3 d after inoculation, which coincides with an increase of fungal mass and the onset of visible tumor development (Fig. 3B). *udh1* transcript levels were markedly increased 7 and 8 d after inoculation compared with the barely detectable transcript levels of the constitutively expressed *ppi* gene during biotrophic growth (Fig. 3B). This reflects the small amount of fungal biomass within infected leaves and emphasizes up-regulation of the *udh1* gene during tumor formation, thus, explaining the identification of the *udh1* gene by differential display analysis at this stage. Standardized in comparison with the *ppi* signal strength, *udh1* transcript levels were 26-fold increased 7 d post-inoculation compared with those in strain FB2 during axenic growth.

**Localization of Udh1**

Previous studies have indicated that human and rat 5α-steroid reductase isozymes reside in either the membranes of the endoplasmic reticulum (ER) or the nucleus depending on the tissue source of the enzyme as demonstrated by immunological detection and sedimentation analysis of enzyme activity (for review, see Russell and Wilson, 1994). To localize the *U. maydis* Udh1 protein, the green fluorescent protein encoding eGFP gene was translationally fused to the 3′ end of *udh1*. Two independent *U. maydis* strains CL13/pugh1#8 and CL13/pugh1#10 harboring ectopic insertions of the *udh1:eGFP* fusion construct under control of *udh1* promoter sequences were analyzed by fluorescence microscopy. EGFP fluorescence mainly resided in the periphery of nuclei, which were localized by 4,6-diamidino-2-phenylindole (DAPI) staining (Fig. 4). This is indicative for localization of the Udh1 protein in membranes of either the nucleus or the ER, which is continuous with the nuclear membrane. In addition, fluorescent patches were visible at the cell periphery, which are indicative for the typical ER network structure in close vicinity to the plasma membrane (Fig. 4; Pichler et al., 2001; Wedlich-Söldner et al., 2002).

**Complementation of Arabidopsis det2-1 Mutants**

Previous investigations have demonstrated that the Arabidopsis *DET2* gene was functional in cultured human cells and, conversely, human 5α-steroid type
1 and type 2 reductases could complement Arabidopsis det2-1 mutants (Li et al., 1997). To investigate whether udh1 encodes a functional 5α-steroid reductase, the udh1 gene was introduced into the binary vector pBAR-35S in such a way that expression was driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985). Homozygous Arabidopsis det2-1 and wild-type Columbia (Col-0) plants carrying the resulting plasmid pBUB1 were generated by Agrobacterium tumefaciens-mediated transformation and selection for Basta resistance. Transgenic lines from two independent transformation experiments were investigated in four series (Table I). All transgenic lines were tested for the insertion of the udh1 gene into their genomes by PCR using primers derived from the CaMV 35S promoter and a primer located in the 3′-untranslated udh1 sequence and by a subsequent sequence analysis of the PCR products (Table I). This analysis confirmed the transgene in most plants analyzed but also revealed its absence in a few homozygous det2-1 plants, implying that Basta selection was not completely tight. As expected, these plants showed a dwarf phenotype (Table I). To confirm the genetic background of all transgenic lines, cleaved amplified polymorphic sequence (CAPS) analysis based on the absence of the MnlI site at position 610 in the det2-1 ORF was employed (Fig. 5; Table I). Whereas PCR products amplified from Col-0 DNA contained a MnlI restriction fragment of 152 bp, a 193-bp MnlI fragment was retrieved from amplified det2-1 DNA (Fig. 5). Three of the investigated descendants from infiltrated homozygous det2-1 plants that resisted Basta selection were heterozygous (DET2/det2-1) and have probably emerged from cross-pollination (Table I). In all cases, plants that carried the transgene were homozygous with respect to the det2-1 allele.

Northern analysis was performed to determine whether transgenic plants carrying the pBUB1 construct expressed the transgene (Fig. 6A). RNA was extracted from leaves of det2-1/pBUB1#1, 2, 8, and 12 plants (see Fig. 7D) and probed with the udh1 gene. RNA preparations from Arabidopsis Col-0 and U. maydis FB1 were included as control to exclude cross-hybridization of the udh1 gene with Arabidopsis RNA and to indicate the length of the U. maydis udh1 transcript. Expression of the udh1 transcript size of pBUB1 transformants was larger than that of the endogenous udh1 gene expressed in U. maydis, implying differences in transcription start sites conferred by the different promoters.

Transformed plants were compared with homozygous det2-1 mutants and wild-type Col-0 plants at different time points. Under the experimental conditions employed, Col-0 plants rarely displayed elongated shoots or flowers 42 d after sowing but normally required an additional period of at least 10 d for further development (Fig. 7A; Table I). At the

Table I. Analysis of transgenic Arabidopsis lines

<table>
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<th>Lines</th>
<th>Analyzed Lines</th>
<th>Genotypea</th>
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<th>Flower/Seed Developmentc Phenotyped</th>
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<td>All (3) after 50 d A</td>
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<tr>
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<td>Det2/Det2</td>
<td>Yes</td>
<td>All (3) after 50 d B</td>
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<td>Det2/Det2</td>
<td>–</td>
<td>1 (0) after 50 d C</td>
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<td>det2-1/det2-1</td>
<td>–</td>
<td>None (0) after 50 d D</td>
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<td>–</td>
<td>None (0) after 43 d C</td>
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* The genotypes of all transformants, all det2-1/DET2 heterozygous and det2-1 homozygous plants, and some wild-type Col-0 plants were confirmed by CAPS (see “Materials and Methods”). ** Confirmed by PCR/sequencing (see “Materials and Methods”). c The number of seed pods developed is indicated in brackets. A. Accelerated stem elongation, flower and seed development, thin stems, and reduced size and number of rosette leaves. B. Like A, but increased stem thickness and size and number of rosette leaves. C. Wild-type Col-0 phenotype. D. det2-1 phenotype.

x/y/z, x is the number of plants that corresponds to the genotype in column 3 and carries the transgene. y is the number of heterozygous plants. z is the number of plants that is homozygous for det2-1, escaped Basta selection, and lacked the transgene.
same stage, control det2-1 plants showed a characteristic dwarf phenotype, small dark green leaves, and lacked flowers (Fig. 7B; Table I). All homozygous Arabidopsis det2-1 lines harboring the udh1 transgene remarkably were no longer dwarfs. Instead, they displayed an elongated main stem and developed flowers even earlier than wild-type Col-0 plants, indicating accelerated development due to the transgene (Fig. 7C; Table I). In addition, these plants differed from Col-0 plants by a reduced size and number of rosette leaves (Fig. 7, A and C). During further growth, transgenic plants showed accelerated seed pod development and additionally differed from wild-type Col-0 plants by excessively elongated stems, which were unable to keep the plant in an upright position (Fig. 7, D and E; Table I). The phenotype of Col-0 plants carrying pBUB1 resembled the one of det2-1/pBUB1 transformants with respect
to accelerated elongation of the main stem, flower, and seed pod formation. However, main stems were more stable and the number of rosette leaves was less severely reduced than in the det2-1 transformants (Table I; data not shown).

To test whether the phenotype of pBUB1 transformants relied on the Udh1 function or were a consequence of overexpression, transgenic det2-1 lines expressing the DET2 gene under control of the CaMV 35S promoter were generated (see “Materials

Figure 7. Development of Arabidopsis pBUB1, pBDET2, and pBUA1 transformants. A to C, Plants from series 2, 42 d after sowing. A, Wild-type Col-0. B, Mutant det2-1. C, Mutant det2-1 transgenic for pBUB1. Arrows indicate terminal flowers that developed in Col-0 and transgenic det2-1 plants. D and E, Plants from series 3, 61 d after sowing. D, Mutant det2-1 transgenic for pBUB1 (det2-1/pBUB#1, 2, 8, and 12). Seed pods are indicated by arrows. E, Wild-type Col-0 plants. Seed pods have not yet developed. F to H, Plants from series 4, 43 d after sowing. F, Transformants det2-1/pBDET2#1, 2, 3, 6, 7, and 8 are numbered from 5 to 10. det2-1 plants carrying the pBDET2 construct (5–10) display seed pod development (arrows). Homozygous det2-1 (1–3) and heterozygous DET2/det2-1 (4) plants that escaped Basta treatment but lacked the transgene. G, Control wild-type Col-0 plants. H, pBUA1 transgenic det2-1 plants (2 and 3) are phenotypically indifferent from a det2-1 plant (1) that escaped Basta selection but lacked the transgene.
and Methods”). The genomic DET2 sequence was introduced into pBAR-35S and the resulting vector pBDET2 was transformed into homozygous det2-1 plants.

Transformants det2-1/pBDET2#1, 2, 3, 7, and 8 were analyzed for DET2 expression (Fig. 6B). This indicated expression of the transgene in all cases, whereas expression of the endogenous gene was barely detectable in wild-type Col-0 or mutant det2-1 plants (Fig. 6B, lanes 6–8). The expected position of the transgenic DET2 transcript was deduced from the faint endogenous DET2 signal of Col-0 and det2-1 RNA preparations. The presence of transcripts heterogeneous in size may reflect the use of different transcription start sites in transgenic plants. Strongly elevated DET2 expression levels like in the det2-1/pBDET2#3 transformant were not reflected by additional phenotypic alterations compared with the det2-1/pBDET2#1 transformant, which expressed the transgene at a 10-fold reduced level (see below), pointing to saturation in the conversion of the DET2 reaction product.

Like det2-1/pBUB1 transformants, all det2-1/pBDET2 transformants displayed long, thin stems, few rosette leaves of reduced size, and accelerated flower and seed pod development (Fig. 7, F and G; Table I), indicating that overexpression of either the udh1 or the DET2 gene accounted for the morphological differences compared with wild-type Col-0 plants.

The Udh1 amino acid sequence contained an N-terminal extension, which was absent in known 5α-steroid reductases (see Fig. 2A). To assess whether a truncated ORF starting from the second in-frame-positioned Met-71 codon resulted in a functional enzyme, the plasmid pBUA1 was constructed, which was derived from pBAR-35S by insertion of the N-terminal 70 amino acids for Udh1 activity. From Fig. 8, D and E). Furthermore, stems from 19 of 22

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<th>Lines</th>
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\(^a\) Plants were always kept in the greenhouse with a 12/12-h photo (15,000 lux)/dark period at 22°/18°C. \(^b\) Plants were kept in the greenhouse for 17 d as described in (a) and then transferred to a phytochamber maintained with a 16/8-h photo (16,000 lux/dark period at 22°/18°C). In brackets, number of lines that were also analyzed with respect to the presence of the transgene by PCR and the genotype by CAPS (see “Materials and Methods”). These all corresponded to the genotypes indicated in column 3 and carried or lacked the transgene as indicated in column 4. \(^c\) Number of flowering plants. In brackets, number of plants with elongated stems (≥2 cm) and flower buds. \(^d\) Number of plants having seed pods developed.

Accelerated Growth in the T2 Progeny under Different Light Conditions

To further investigate whether a general stress response from repeated Basta selection promoted the observed growth difference between wild-type and transgenic plants, we analyzed the T2 progeny derived from selfing of the T1 det2-1/pBUB1#1 plant (series 1). According to Mendelian inheritance, three-fourths of the T2 progeny should carry at least one copy of the transgene. Seedlings derived in the absence of Basta selection from T1 det2-1/pBUB1 and Col-0 were transferred to individual pots and either kept in the greenhouse with a 12-h photoperiodicity or transferred to the phytochamber with a 16-h photoperiodicity. Of the investigated T2 progeny from T1det2-1/pBUB1 plants, 78% displayed rescued growth (Table II), indicative for the presence of the transgene. As expected, some T2 plants (22%) displayed a dwarf phenotype reminiscent of det2-1 mutant plants. All of these plants lacked the transgene (Table II), demonstrating a strict linkage between its presence and rescued growth in the T2 progeny. Under long-day conditions development of transgenic T2 plants was weakly favored compared with F1 Col-0 plants, as illustrated from accelerated stem and flower bud formation and from seed pod development between 31 and 35 d after sowing (Table II; Fig. 8, D and E). Furthermore, stems from 19 of 22
**Figure 8.** Development of T2det2-1/pBUB1 plants (series 5 and 6) compared with F1Col-0 plants. A to C, Plants from series 5, 33 d after sowing. D to F, Plants from series 6, 33 d after sowing. All presented T2det2-1/pBUB1 plants, four (B), and six (E) Col-0 plants, respectively, were analyzed with respect to the genotype and the presence (A and D) or absence (B, C, E, and F) of the transgene. Growth conditions were as specified in Table II. A and D, T2det2-1/pBUB1. B and E, Wild-type Col-0. C and F, T2det2-1/pBUB1 that displayed a dwarf phenotype and lacked the transgene. Each pot contains two plants. The numbers in D refer to the plants used for northern analysis (see Fig. 9).

T2det2-1/pBUB1 plants exhibited lengths between 10 and 19.5 cm compared with stems of the Col-0 variety with only 1 of 10 having elongated to a length of 10 cm 35 d after sowing (data not shown). Surprisingly, differences became more apparent upon exposure to a shorter photoperiod.

Although development of F1 Col-0 plants exposed to a 12-h photoperiod was attenuated compared with growth under long-day conditions (Table II; Fig. 8, B and E), development of det2-1/pBUB1 plants was comparable under both light conditions, as illustrated by early flowering and seed pod development between 31 and 35 d after sowing (Table II; Fig. 8, A and D). This suggests that udh1 overexpression can compensate for reduced light conditions and demonstrates the accelerated growth phenotype in the absence of Basta selection. In addition, all det2-1/pBUB1 plants developing under reduced light conditions differed from Col-0 plants by decreased numbers of rosette leaves and weakened stem stability, as already noticed in the T1 plants (Fig. 8A; data not shown). Northern analysis of transgenic T2 plants from series 6 demonstrated udh1 expression in all investigated plants. Although transgene expression levels were severely reduced in the T2det2-1/pBUB1#9 plant, they still exceeded the faintly detectable endogenous det2-1 expression levels (Fig. 9). The T2det2-1/pBUB1#11 and 12 plants displayed significantly reduced stem lengths and had not-yet-developed flowers despite udh1 transcript levels as high as in the fully developed T2det2-1/pBUB1#4 plant (Fig. 8D; Fig. 9), indicating that extensive overexpression was not sufficient to promote accelerated growth in all plants under the chosen conditions.
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Figure 9. Expression of the udh1 gene in differently developed transgenic Arabidopsis plants. RNA (1 μg) isolated from rosette leaves of T2det2-1/pBUB1 plants from series 6, 33 d after sowing and from U. maydis strain FB2 (4 μg) grown on CM charcoal for 2 d was loaded (see “Materials and Methods”). Northern blots were probed with the 32P-labeled AgeI-HindIII udh1 fragment, the DET2 EcoRV fragment, and the Arabidopsis 32P-labeled actin probe (ACT2) as loading control. The ratio of quantified signals (udh1/ACT2) was calculated (arrowhead). Staining with methylene blue reflected the amounts of total RNA loaded. The full-length udh1, endogenous U. maydis udh1, ACT2, and the fungal and maize ribosomal RNA bands are indicated (arrowheads). Bottom, Developmental stage of investigated plants 35 d after sowing: B, Flower bud; F, flower; S, seed pods. The numbers refer to stem lengths in centimeters.

Similar results were obtained when udh1 transcript levels were determined by northern analysis in T2det2-1/pBUB1 plants that all displayed accelerated growth under a 12-h photoperiodicity (see Fig. 8A). From six analyzed plants, five exhibited comparable levels of strong overexpression, whereas accumulation of udh1 transcripts was 10-fold reduced in one plant, indicating that moderate levels of overexpression are sufficient for accelerated growth under reduced light conditions (data not shown). In all cases, udh1 transcript levels significantly exceeded those of the endogenous det2-1 gene of T2det2-1/pBUB1 plants and the DET2 gene of wild-type Col-0 plants from the same series, respectively (data not shown).

Deletion Analysis of udh1 in U. maydis

To assess the function of the udh1 gene in U. maydis, the respective ORF was deleted in compatible, haploid U. maydis strains (see “Materials and Methods”). Such strains were viable, microscopically indistinguishable from wild-type cells, and not affected in growth (data not shown). Three independent Δudh1 strain combinations were assessed for pathogenicity in sweet corn (Zea mays var Early Golden Bantam). Tumors comparable in morphology and size with wild-type tumors developed in 47 of 76 plants after infection with Δudh1 combinations, which compares with tumor formation (33 of 42) by the respective wild-type strains FB1 and FB2. Furthermore, the udh1 gene was deleted in the haploid strain SG200, which is solopathogenic due to the presence of a hybrid b locus composed of the compatible bE1 and bW2 genes. Again, the frequency of tumor development was comparable in plants (sweet corn var Early Golden Bantam) inoculated with either SG200Δudh1 (72 of 79 plants) or SG200 strain (34 of 35 plants). In addition, the development of teliospores was unaffected by the udh1 deletion (data not shown). A reduction in virulence of Δudh1 strains may possibly be reflected by attenuated biotrophic growth. We, therefore, inoculated mixtures of four independent SG200Δudh1 and wild-type SG200 strains and determined their ratio in individual tumors 10 d postinoculation (sporulation stage). Intensities of strain-specific fragments amplified from Δudh1 and wild-type strains were comparable when using DNA from preparations of tumors A to C, whereas the Δudh1-specific fragment was slightly less efficiently amplified from DNA preparation of tumor D (Fig. 10A). This indicated that Δudh1 mutants were able to proliferate at the same rate as wild-type strains during infection. To assess the possibility of gene redundancy, Southern hybridization was performed under non-stringent conditions using an udh1 fragment, which spanned the deleted ORF region of the mutant allele (Fig. 10B). Although genomic fragments of the expected sizes were detected in restricted chromosomal DNA from wild-type strain SG200, distinct cross-hybridizing signals were not detectable in restricted chromosomal DNA from two independently generated SG200Δudh1 strains. Under the chosen non-stringent hybridization conditions a 2,504-bp BamHI fragment, which overlapped with only 43 bp of the udh1 probe, showed weak hybridization. This suggests that genes homologous to udh1 are either absent in U. maydis or have become highly diverged.

DISCUSSION

Our studies provide molecular evidence for the existence of a 5α-steroid reductase in U. maydis, which, based on sequence alignment (see Fig. 2, A and B), likely arose from a common ancestor between fungi, plants, and animals. However, the Udh1 amino acid sequence is unique with respect to six additional protein stretches whose significance remains to be determined. Two of them overlapped
with predicted transmembrane domains, suggesting that the Udh1 protein may be more tightly embedded in the lipid bilayer than mammalian steroid 5α-reductases, which contain four putative transmembrane domains. Membrane localization of monkey type 1 and type 2 and human and rat steroid-5α-reductases have been described (Andersson et al., 1989; for review, see Russell and Wilson, 1994). Fluorescence microscopy of single U. maydis cells expressing an Udh1-eGFP fusion protein corroborates the predicted localization of steroid-5α-reductases in membranes of the ER. Although the Udh1 sequence contains an ER retention element (HDEL) at position 258, it is unclear whether this is functional because these elements are generally positioned at the C-terminal end (for review, see Pelham, 1990). As an alternative, the Udh1 protein may be associated with a protein targeted to the ER.

To determine whether udh1 encodes a functional 5α-steroid reductase, transgenic Arabidopsis plants carrying the udh1 gene under control of the constitutive CaMV 35S promoter were generated. In all cases, transformation with pUBU1 rescued the dwarf phenotype of Arabidopsis det2-1 plants. Intriguingly, all transformants displayed accelerated stem growth and flower and seed development compared with wild-type Col-0 plants. Because both pUBU1 and pBDET2 transformants promoted the same phenotype in homozygous det2-1 plants, increased levels of 5α-steroid reductase activity, which may encounter elevated levels of brassinosteroids, are responsible for accelerated development compared with wild-type Col-0 plants. This is consistent with the low endogenous DET2 expression levels in wild-type Col-0 plants (see Fig. 6B). The reduction in size and number of rosette leaves and the formation of thin, elongated steps in plants overexpressing 5α-steroid reductase may be the consequence of an imbalanced concentration of brassinosteroids. The phenotypic differences observed in T1 transgenic plants did not result from repeated Basta selection, because the accelerated growth phenotype was stably inherited to the T2 generation in the absence of Basta treatment. Under a 16-h photoperiod, individual developmental differences of det2-1/pUBU1 plants were still maintained in the T2 generation despite similar overexpression levels, whereas under reduced light conditions, accelerated growth dominated. The observation that phenotypic differences between det2-1/pUBU1 and wild-type Col-0 plants were strongly favored under a 12-h photoperiod compared with a 16-h photoperiod of the same light intensity suggests that enhanced levels of 5α-steroid reductase activity can compensate for low-light conditions, consistent with a role of brassinosteroids in photomorphogenesis (Clouse, 1997). This conclusion agrees with the recent observation that Arabidopsis antisense lines of the CPD gene, which is implicated in brassinosteroid biosynthesis, exhibit a more delayed development compared with wild-type plants if exposed to low-light conditions (Schlüter et al., 2002). All investigated plants expressed transgene levels that strongly exceeded those of the endogenous DET2 gene. This points to saturated levels of 5α-steroid reductase activity and may explain why
differences in the strength of overexpression were not reflected by an additional growth phenotype. To verify the translational start codon of udh1, the DNA sequence encoding the region between Met-1 to Met-71 was deleted. This has indicated that the less conserved N-terminal portion of the Udh1 protein, which corresponds to the N-terminal 58 amino acids of the rat s55K protein is crucial for activity. A four-amino acid segment (VSIV) and the conserved Gly residue (matching Gly-52 of Udh1) suggested in substrate binding of the rat type 1 enzyme and human type 2 enzyme, respectively (Thigpen and Russell, 1992), are contained in this region.

Enzymes of steroid metabolism have been described in fungi. 17β-hydroxy-steroid-dehydrogenase activity was detected in Cochliobolus lunatus and S. pombe (Lanisnik et al., 1992; Dlugonski and Willman ska, 1998). 5α-Steroid reductase activity was detected in Penicillium chrysogenum and Penicillium crustosum, which in culture were able to reduce the 4,5-double bond in testosterone to give dihydrotestosterone (Cabeza et al., 1999). Which role these enzymes play in fungal growth or development remains to be shown. The finding that U. maydis 4Udh1 strains are unaffected in growth, virulence, and tumor induction capacity rules out the possibility that this enzyme is responsible for the synthesis of a plant hormone. However, an alternative mechanism for the Δ4-5 reduction cannot be excluded. In mammals, steroid 5α-reductases exist as type 1 and type 2 isoforms implicated in catabolic and anabolic functions, respectively (Normington and Russell, 1992; for review, see Russell and Wilson, 1994), and the genome of Arabidopsis reveals the presence of a steroid 5α-reductase-like protein (GenBank accession no. T51384). Because the Udhl sequence was more similar to mammalian type 1 enzymes, a possible catabolic function may be envisaged. However, screening the udh1 enzymes, a possible catabolic function may be envisaged. However, screening the udh1 and Wilson, 1994), and the genome of Arabidopsis 

**Plant Material, Strains, and Growth Conditions**

The standard wild-type genotype used was Arabidopsis Col-0. The det2-1 mutation is in the Arabidopsis ecotype Col-0 background (Chory et al., 1991). The Agrobacterium tumefaciens strain GV3101pMP90RK (Koncz and Schell, 1986), kindly provided by Dr. R. Kunze (Köln, Germany) transformed with the pBAR-b 35S derived vector constructs (see below) was used to transform Arabidopsis Col-0 and det2-1 plants by the vacuum infiltration method (Bechtold et al., 1993). Seeds were incubated in potting soil for 2 d at 8°C to induce germination. Five days after maintenance with a 16-h photoperiod (5,000 lux) at 21°C, seedlings were subjected to four cycles of Basta (0.01% [v/v] Basta [Aventis CropScience, Strasbourg, France], in 0.01% [v/v] Silwet L-77: Lele Seeds, Round Rock, TX) selection in 1-d intervals. Basta-resistant seedlings were transferred to individual soil pots and maintained with a 16-h photoperiod at 21°C. To assess the influence of the udh1 gene on development, a T2 progeny was harvested from mature siliques of self-pollinated det2-1/det2-1 plants carrying the pBUB1 transgene, and a F1Col-0 progeny line was obtained from self-pollinated Col-0 plants. Seeds were stored under identical conditions and plants were grown as specified in Table II.

Haploid Ustilago maydis strains FB1 (a1b1), FB2 (a2b2), and the diploid strains FBD11 (a1a2b1b2) have been described (Banuett, 1995). CL13 (a1bE1bW2) and SG200 (a1a2bElW2) are solopathogenic haploid strains ( Bölker et al., 1995). Cells were grown at 28°C in yeast/potato/Suc (YEPS; Tsukuda et al., 1988) or complete medium (CM; Holliday, 1974). To test for mating, strains were cospotted on charcoal containing potato dextrose plates and incubated at room temperature for 48 h. Plant infections were done as described (Basse et al., 2000) with the sweet corn var Early Golden Bantam (Olds Seed, Madison, WI). Escherichia coli K12 strain DH5α (Bethesda Research Laboratories, Bethesda, MD) and TOP10 (Invitrogen, Karlsruhe, Germany) were used as hosts for plasmid amplification.

**DNA and RNA Procedures**

U. maydis chromosomal DNA was prepared according to Hoffman and Winston (1987). Maize (Zea mays) and Arabidopsis chromosomal DNA was isolated with the DNEasy Plant Mini Kit (Qiagen, Hilden, Germany). Transformation of U. maydis followed the protocol of Schulz et al. (1990). DNA from U. maydis grown on solid medium and U. maydis-infected maize tissue was isolated according to Schmitt et al. (1990). Total RNA was extracted from Arabidopsis plants with the TRIzol reagent (Invitrogen) or the NucleoSpin RNA II Kit (CLONTECH, Heidelberg). Radioactive labeling of RNA was performed with the megaprime DNA labeling kit (Amersham-Pharmacia Biotech, Freiburg, Germany). Detection and quantification of the signals was done using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant software. Nucleotide sequences were determined by automated sequencing using an ABI Prism 377 DNA sequencer (PerkinElmer life Sciences, Boston). All PCR products were sequenced. Southern hybridization under non-stringent conditions was performed at 55°C. Stringent washing steps were omitted. All other DNA manipulations followed standard procedures as described by Sambrook et al. (1989). Nucleotide
sequences were compared using the BLAST program (Altschul et al., 1997). Prediction of transmembrane helices were made with TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html).

Plasmids and DNA Fragments

For subcloning and sequencing, plasmids pUC18, pUC19 (Amer sham-Pharmacia Biotech), and pCR2.1-TOPO (Invitrogen) were used. The U. maydis cosmid library has been described ( Bölker et al., 1995). Hygromycin (hph) and carboxin resistance (ipi: encoding succinate dehydrogenase) cassettes and the ppi fragment (Bohlmann, 1996) as selectable markers and probes, respectively, for U. maydis have been described (Basse et al., 2000). The eGFP gene (CLON-TECH) was excised as a 1,030-bp NcoI-EcoRV fragment from plasmid p123 (Aichinger, 2000). Udhi was contained in cosmids 9B4, 9B10, and 29C12. A genomic Agel-HindIII fragment of 1,722 bp that contained the udh1 gene was isolated from cosmid 9B4. Plasmid pudh1 contained the 1,722 bp fragment as described (Basse et al., 2000) using the forward primer udh1-2 containing a EcoRV restriction site (5'-AGGTCCATGGAGATTCCTTCGCCCATC-3'; positions 1,394–1,413 in Fig. 1). PCR reactions were performed in the presence of Taq precision polymerase (Stratagene, Amsterdam) using parameters as described (Basse et al., 2000). PCR products were cleaved with EcoRV and NcoI and ligated to the eGFP gene isolated as NcoI-EcoRV fragment to yield pug1. The sequence of the amplified udh1 gene showed one deviation at the wobble position of the Ala-206 codon, which, therefore, did not alter the amino acid sequence. The plasmid pug1 was generated from pug1 by ligating the hph cassette as end-filled NotI fragment into the EcoRV site. The 6,274-bp SplI-SplI fragment was isolated from pug1 and ectopically integrated into U. maydis strain CL13 (strains CL13/pugh18 and CL13/pugh110). For Southern analysis to confirm ectopic integration, genomic DNA was cleaved with BamHI and probed with the udh1 Agel-HindIII fragment. U. maydis cells grown in YEPS medium were fixed with 4% (w/v) formaldehyde and stained with 0.5 µg mL−1 DAPI (Sigma, Taufkirchen, Germany) in phosphate-buffered saline (pH 7.2) for 15 min at 60°C and subsequent washing with phosphate-buffered saline. Samples were observed with differential interference contrast optics or under fluorescence microscopy (excitation/emission for DAPI: 365 nm/397 nm; excitation/emission for eGFP: 450–490 nm/515–565 nm) using an Axioskop (Zeiss Jena, Germany).

Differential Display and Isolation of udh1 cDNA Clones

Differential display was performed as described (Basse et al., 2000). Two individual RNA preparations from leaf tumor tissue 6 d after inoculation and mock-infected control leaf tissue were reverse-transcribed using T11GG primers. For PCR amplification, the oligo(dT) primers from the first step combined with a 10-mer primer with a defined but arbitrary sequence (5'-SCCATGCACG-3') were used. The 8.8-kb EcoRI-BamHI fragment spanning the 3' portion of udh1 was used as probe to isolate two independent cDNAs from a cDNA library of FBD11 (Schauwecker et al., 1995). Both clones terminated approximately 100 bp downstream of the predicted translational initiator ATG codon. The remaining 5' end of the cDNA was obtained by RT-PCR with DNase-treated RNA isolated from leaf tumors 6 d after inoculation with strains FB1 and FB2 as described (Basse et al., 2000) using the forward primer Bsp (5'-GGACTAGTCTCATGTCTCACCTCTCGATC-3'; positions 251–272 in Fig. 1) and the reverse primer Absp (5'-TGTGATGAGGCGAAGAGC-3'; positions 566–586 in Fig. 1).

eGFP-udh1 Fusion Constructs and Microscopy

For construction of the udh1:eGFP fusion construct pugh1, pudh1 was amplified with the forward primer udh1-2 containing an EcoRV restriction site (5'-TGTCGATATCGCCTCTCCATCAATC-3'; positions 1,419–1,438 in Fig. 1) and the reverse primer udh1-1 containing a NcoI restriction site (5'-AGGTCTAGGAGAAAAAGGGATGATGCCC-3'; positions 1,394–1,413 in Fig. 1). PCR reactions were performed in the presence of Taq precision polymerase (Stratagene, Amsterdam) using parameters as described (Basse et al., 2000). PCR products were cleaved with EcoRV and NcoI and ligated to the eGFP gene isolated as NcoI-EcoRV fragment to yield pug1. The sequence of the amplified udh1 gene showed one deviation at the wobble position of the Ala-206 codon, which, therefore, did not alter the amino acid sequence. The plasmid pug1 was generated from pug1 by ligating the hph cassette as end-filled NotI fragment into the EcoRV site. The 6,274-bp SplI-SplI fragment was isolated from pug1 and ectopically integrated into U. maydis strain CL13 (strains CL13/pugh18 and CL13/pugh110). For Southern analysis to confirm ectopic integration, genomic DNA was cleaved with BamHI and probed with the udh1 Agel-HindIII fragment. U. maydis cells grown in YEPS medium were fixed with 4% (w/v) formaldehyde and stained with 0.5 µg mL−1 DAPI (Sigma, Taufkirchen, Germany) in phosphate-buffered saline (pH 7.2) for 15 min at 60°C and subsequent washing with phosphate-buffered saline. Samples were observed with differential interference contrast optics or under fluorescence microscopy (excitation/emission for DAPI: 365 nm/397 nm; excitation/emission for eGFP: 450–490 nm/515–565 nm) using an Axioskop (Zeiss Jena, Germany).

A. tumefaciens Transformation Vectors

Plasmids pBUB1 and pBUA1 were derived from Saccharomyces cerevisiae vectors p426ADH-udh-B1 and p426ADH-udh-A1, which contained the full-length udh1 gene and a 5'-truncated udh1 gene fragment, respectively (C. Basse, unpublished data). For construction of p426ADH-udh-B1 and p426ADH-udh-A1, the udh1 5' portions were amplified from puh1 using Pfu polymerase (Stratagene) and the primer combinations Spel/Asp and Apel/Asp610 (5'-AGGTCCATGGAGATTCCTTCGCCCATC-3'; positions 461–482 in Fig. 1)/Asp, respectively. Primers Asp610 and Bsp contained Spel restriction sites. PCR products were restricted with Spel/BamHI and ligated into the respective sites of p426ADH1#1041 (kindly provided by Dr. R. Kunze, Köln, Germany) to yield p426AuB1 and p426AuA1, respectively. p426ADH-udh-B1 and p426ADH-udh-A1 were obtained by ligating the BamHI fragment, which contained the 3' portion of the udh1 gene (positions 534–1,629 in Fig. 1), into the BamHI sites of p426AuB1 and p426AuA1, respectively. The end-filled HindIII-Spel fragments containing the udh1 gene were isolated from p426ADH-udh-B1 and p426ADH-udh-A1, respectively, and ligated into the Smal site of pBUB-B. The resulting vectors were termed pBUB1 and pBUA1, respectively. The DET2 gene was isolated from genomic Col-0 DNA by PCR in the presence of Pfu polymerase, the forward primer udhe5n (5'-AATCTGATACCCCGAAAAATGGAGAAA-ATCG-3'; positions from –8 to +13 in the DET2 ORF), and the reverse primer udhe3n (5'-AATCTGATACCCGGAATATTACACCCGAGACG-3'; positions 78–97 downstream of
the DET2 ORF). Both primers contained EcoRV recognition sites. Sequence analysis of the genomic DET2 clone confirmed the presence of a short intron with a length of 85 bp inserted at position 397 of the DET2 ORF (Li et al., 1996). However, two alterations to the sequence described by Li et al. (1996) were detected (a C to G transversion and a C to T transition at positions 516 and 592, respectively). Whereas the first substitution affected the wobble position of the Arg-172 codon, the latter resulted in an Arg-198 to Cys conversion. A comparison with the respective region of the Arg-172 codon, the latter resulted in an Arg-198 to Cys conversion. A comparison with the respective region of the Arg-172 codon, the latter resulted in an Arg-198 to Cys conversion. A comparison with the respective region of the Arg-172 codon, the latter resulted in an Arg-198 to Cys conversion.

Construction of udh1 Deletion Strains

For construction of the udh1 deletion plasmid, pudh1 was amplified with the forward primer udet3 (5'-AAGGCCCTCTCATAGCTACCAACGGTTCAC-3'; positions 1,326–1,346 in Fig. 1) and the reverse primer udet1 (5'-AAGGCTGATGGCAGGAATCTCC-3'; positions 464–482 in Fig. 1), which both contain a SalI restriction site. The deletion comprised the complete udh1 ORF except the initial 231 bp at the 5' end and the terminal 91 bp at the 3' end. PCR parameters were as described (Basse et al., 2000). PCR products were cleaved with SalI and ligated to the hph cassette as an end-filled BamHI/EcoRV fragment to yield pUdet1ko. To replace the resident udh1 gene with the hph cassette, a 4,434-bp SspI-SphiI fragment was isolated from pUdet1ko and transformed into U. maydis strains FB1, FB2, and SG200. For Southern analysis, genomic DNA was cleaved with either AgeI/HindIII or BamHI and probed with the udh1 containing AgeI-HindIII fragment. Because udh1 null-mutants were generated only in strains SG200 and FB2, FB1 udh1 null-mutants were obtained from the sexual offspring of a cross between FB2Δudh1 and FB1.

Detection of U. maydis DNA in Maize Tissue Infected with Mixtures of SG200 and SG200Δudh1 Strains

Maize plants (var Early Golden Bantam) were inoculated with mixtures of SG200 and SG200Δudh1 cultures adjusted to a density of 7 to 10 × 10⁷ cells mL⁻¹. Four independent SG200Δudh1 strains were used. In each case, DNA was prepared from a single leaf tumor of an infected maize plant 10 d after inoculation using the DNeasy plant kit (Qiagen). Chromosomal DNA was used as template for PCR under standard conditions. The following primer combinations were used to amplify fragments from (a) the hph gene flanked by udh1 5' ORF sequences and (b) the udh1 gene: (a) 5'-GCAAGTTTCTGTTCTGATAGC-3'/5'-CCATGCAGTCTACCATGTCG-3'; (b) 5'-CCCGTTGTCGACCTAGACGGGTTGG-3'/5'-GGCAAAGACAAGCGTGGTG-3'. Primer combinations a and b resulted in comparable amplification efficiencies using identical amounts of chromosomal DNA from either SG200Δudh1(a) or SG200(b) strains as template.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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