

Identification of Three Urease Accessory Proteins That Are Required for Urease Activation in Arabidopsis¹

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Urease is a nickel-containing urea hydrolase involved in nitrogen recycling from ureide, purine, and arginine catabolism in plants. The process of urease activation by incorporation of nickel into the active site is a prime example of chaperone-mediated metal transfer to an enzyme. Four urease accessory proteins are required for activation in *Klebsiella aerogenes*. In plants urease accessory proteins have so far been only partially defined. Using reverse genetic tools we identified four genes that are necessary for urease activity in Arabidopsis (*Arabidopsis thaliana*; ecotypes Columbia and Nössen). Plants bearing T-DNA or Ds element insertions in either the structural gene for urease or in any of the three putative urease accessory genes *AtureD*, *AtureF*, and *AtureG* lacked the corresponding mRNAs and were defective in urease activity. In contrast to wild-type plants, the mutant lines were not able to support growth with urea as the sole nitrogen source. To investigate whether the identified accessory proteins would be sufficient to support eukaryotic urease activation, the corresponding cDNAs were introduced into urease-negative *Escherichia coli*. In these bacteria, urease activity was observed only when all three plant accessory genes were coexpressed together with the plant urease gene. Remarkably, plant urease activation occurred as well in cell-free *E. coli* extracts, but only in extracts from cells that had expressed all three accessory proteins. The future molecular dissection of the plant urease activation process may therefore be performed in vitro, providing a powerful tool to further our understanding of the biochemistry of chaperone-mediated metal transfer processes in plants.

The first step of the hydrolysis of urea to ammonium and carbonic acid is catalyzed by the nickel metalloenzyme urease, which is found in many microbes (Mulrooney and Hausinger, 2003) and plants (Frankenberger and Tabatabai, 1982; Witte and Medina-Escobar, 2001). In plants, urea originates from the breakdown of Arg, especially important during germination (Zonia et al., 1995; Goldraij and Polacco, 1999), and from purines or ureides. Because ureides serve as nitrogen-transport compounds in nitrogen-fixing legumes their degradation has received special attention (Stebbins and Polacco, 1995; Todd and Polacco, 2004). Externally applied urea as fertilizer is also made accessible to plants by urease (Witte et al., 2002a). Since urea is one of the most used nitrogen fertilizers worldwide (<http://faostat.fao.org>), its enzymatic hydrolysis is a process of great agricultural importance.

Activation of urease is best studied in *Klebsiella aerogenes*. It requires the carbamoylation of a Lys

residue and the incorporation of two nickel ions per active site (Kuchar and Hausinger, 2004). For in vivo urease activation, three urease accessory proteins, UreD, UreF, and UreG, are absolutely necessary and a fourth accessory protein, UreE, facilitates this process. The precise role of the accessory proteins is not yet clearly understood. Apo-urease and UreD, UreF, and UreG form a complex that is competent to incorporate CO₂ and nickel upon GTP hydrolysis carried out by UreG (Soriano and Hausinger, 1999). Nickel is delivered by UreE, a nickel-binding metallochaperone (Soriano et al., 2000). After activation the complex dissociates.

Prokaryotic ureases are mostly encoded by three genes that are collinearly fused to one gene in eukaryotes (Mulrooney and Hausinger, 2003). Plant urease genes have first been characterized from soybean (*Glycine max*). The soybean genome contains an embryo-specific urease encoded by the gene *Eu1* (Meyer-Bothling and Polacco, 1987) and a ubiquitous urease encoded by *Eu4* (Torisky et al., 1994). In contrast, potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), several other solanaceous species, and also Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia [Col]) possess only a single urease gene (Witte et al., 2005). Using microbial accessory protein sequences, putative plant orthologs for UreD, UreF, and UreG were identified in the databases and the corresponding cDNAs cloned (Freyermuth et al., 2000; Witte et al., 2001; Bacanamwo et al., 2002). Sequence identities determined from pair-wise alignments of *K. aerogenes*

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UreD and UreF with Arabidopsis UreD and UreF candidates are about 20% in both cases, providing only a weak indication that these proteins may be orthologous. A plant gene with similarity to bacterial *ureE* was not found, at least in the genome of Arabidopsis. It was hypothesized that the function of the bacterial metallochaperone UreE may have been taken over in plants by the UreG protein. In comparison to bacterial UreG proteins, plant UreGs contain an extended N terminus rich in His and Asp residues and were shown to bind nickel (Freyermuth et al., 2000; Witte et al., 2001).

A function for the putative plant urease accessory proteins in plant urease activation has so far been demonstrated only for an UreG ortholog from soybean. A mutation in the corresponding gene *Eu3* eliminated both urease activities of this plant (Freyermuth et al., 2000). Functional complementation of defective microbial urease activation complexes indicated a possible role of other putative plant accessory protein orthologs in urease activation. A *K. aerogenes* urease operon carrying a mutation in *ureG* could be partially complemented by a potato *ureG* ortholog (Witte et al., 2001) and a *Schizosaccharomyces pombe ureF* mutant was partially complemented by a soybean protein with similarity to microbial UreF (Bacanamwo et al., 2002). However, a role in plant urease activation still needs to be established for possible plant UreF orthologs. Attempts to complement *ureD* mutants of *K. aerogenes* or *S. pombe* with putative plant UreD orthologs have failed so far (Witte, 2001; Bacanamwo et al., 2002). A further mutant lacking all urease activities in soybean is encoded by the *Eu2* gene (Meyer-Bothling et al., 1987). However, the *Eu2* mutant could not be assigned to any of soybean accessory protein candidate genes so far (Bacanamwo et al., 2002).

Here we report that Arabidopsis null mutants of the genes encoding At2g35035, At1g21840, and At2g34470 lack urease activity *in vivo*, identifying these proteins as genuine urease accessory proteins *AtUreD*, *AtUreF*, and *AtUreG*. Simultaneous coexpression of *Aturease*, *AtUreD*, *AtUreF*, and *AtUreG* in *Escherichia coli* led to activation of plant urease in bacterial cells and in cell-free extracts, showing that these proteins are the core components for plant urease activation.

RESULTS

Isolation of Mutants

Using the insertion mutant collections of the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003), the German Plant Genomics Research Program (GABI-Kat lines; Rosso et al., 2003), and the Plant Functional Genomics Research Group at the RIKEN Genomic Science Centre (Kuromori et al., 2004), we isolated a homozygous mutant in the *urease* gene, two homozygous mutants in the putative *ureF* and *ureD* genes, respectively, and three homozygous mutants in the *ureG* gene of Arabidopsis (Fig. 1; Table I). *Urease*,

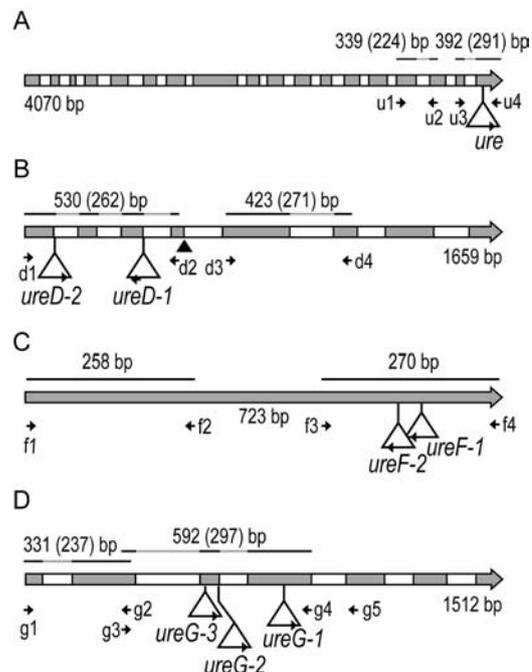


Figure 1. Schematic overview of Arabidopsis *urease* (A), *ureD* (B), *ureF* (C), and *ureG* (D) unspliced coding sequences. Exons are shown in gray, introns are shown in white. Primer positions are indicated by arrows. The positions of insertions in the mutant alleles are marked by the triangles. The arrow on the triangle is drawn near the left border for T-DNAs and on the G-side for Ds transposons indicating the orientation of the insertions. PCR product sizes for primer pairs used in RT-PCR experiments are given above the respective drawings. Product sizes without introns are given in parenthesis. The black triangle in B indicates the position of the stop codon in alternatively spliced *ureD**.

ureG1 and 2, and both *ureD* mutants were obtained from T-DNA insertion collections of the Salk Institute and GABI-Kat in ecotype Col. Both *ureF* mutants and mutant *ureG-3* were obtained from the Ds transposon insertion collection of RIKEN in ecotype Nössen (Table I). As recommended by RIKEN, the Ds donor line DS5-392-12 (Kuromori et al., 2004) instead of Nössen wild type was used as control in all following experiments.

All four genes are single copy in Col; the copy numbers in Nössen are unknown. The positions of the insertions were confirmed by sequencing the PCR products amplified with a primer binding to the left border of the T-DNA or the G-side of the Ds element in combination with a gene-specific primer, respectively (see "Materials and Methods"). Except for mutant lines *ureD-2* and *ureG-2*, the insertions had occurred in exons, likely preventing the generation of an intact mRNA. The insertion in *ureD-2* was found in intron 1 8 bp downstream of the intron-exon boundary, possibly compromising correct splicing. In *ureG-2* the T-DNA inserted exactly at the boundary of exon 3 to intron 4, eliminating the 5' splice site of intron 4. The *ureF* gene encodes a protein of 240 amino acids and does not contain introns. In both *ureF* mutants the reading frame is truncated and altered at the 3' end. *UreF-1* encodes a protein of 217 amino acids, the

Table 1. Mutant lines

Gene	Gene Code	Insertion Type	Mutant Collection	Ecotype	Line ID	Mutant Name	Insertion Position ^a
<i>urease</i>	At1g67550	T-DNA	Salk	Col	N538002	<i>ure-1</i>	5' of base 3,909 (exon 18)
<i>ureD</i>	At2g35035	T-DNA	Salk	Col	N605718	<i>ureD-1</i>	3' of base 407 (exon 3)
<i>ureD</i>		T-DNA	GABI-Kat	Col	399D03	<i>ureD-2</i>	5' of base 108 (intron 1)
<i>ureF</i>	At1g21840	Ds	RIKEN	Nössen	15-1020-1	<i>ureF-1</i>	3' of base 600 ^b
<i>ureF</i>		Ds	RIKEN	Nössen	15-4563-1	<i>ureF-2</i>	3' of base 564 ^b
<i>ureG</i>	At2g34470	T-DNA	GABI-Kat	Col	294B06	<i>ureG-1</i>	5' of base 821 (exon 4)
<i>ureG</i>		T-DNA	GABI-Kat	Col	386A08	<i>ureG-2</i>	5' of base 615 (intron 3)
<i>ureG</i>		Ds	RIKEN	Nössen	16-2845-1	<i>ureG-3</i>	5' of base 572 (exon 3)

^aInsertion position in the unspliced coding sequence of the respective gene, 5' or 3' of the last unaltered base. ^b*ureF* does not contain introns.

C-terminal 17 amino acids being altered, and *ureF-2* encodes a 190-amino acid protein with two changed residues at the C terminus.

None of the mutants displayed a visible phenotype under standard growth conditions used for Arabidopsis (see "Materials and Methods").

Reverse Transcription-PCR Analysis of Mutants

Insertion mutants were characterized by reverse transcription (RT)-PCR experiments testing for the presence and integrity of mRNA transcribed from the genes affected by the insertions. For each mutant two primer pairs were used, one spanning the insertion and one in an unaltered region of the gene but always spanning an intron where possible (Fig. 1). To test the RT reactions, control PCRs with primers for *actin 2* (At3g18780) were carried out (Fig. 2D). Control reactions without RT to test for the presence of genomic DNA were negative in all cases (Fig. 2B, lanes 7–12; data not shown). Reactions were repeated at least twice.

These experiments demonstrated that all mutants lacked an intact wild-type mRNA for the respective mutant gene. In detail, for none of the mutants except *ureG-1* an RT-PCR product was obtained with primers spanning the insertion (lanes marked "s" in Fig. 2), while the corresponding wild-type controls were positive yielding products of the expected size (for expected product sizes see Fig. 1). With RNA from *ureG-1* plants, a product of reduced length was obtained, possibly because the corresponding template in this mutant is subject to aberrant splicing due to the insertion (Fig. 2A, lane 8). With the primer pairs binding in unaltered regions of the respective genes, RT-PCR products were obtained in all cases albeit in strongly reduced amounts for *ureF-1*, *ureF-2*, and *ureG-3* (Fig. 2C, lanes 3, 5, and 9). For *ureG-1* the obtained product was of increased size (Fig. 2A, lane 7) again arguing for altered splicing of the template in this mutant. Also, for *ureG-2* splicing may be partially affected (lane 9). *UreD* transcript was previously described to be alternatively spliced in part leading to the incorporation of a stop codon exactly 3' of exon 4 (Fig. 1B, black triangle; accession no. AJ312203; Witte, 2001). RT-PCR experiments presented here indicated that *ureD* transcript is subject to further alternative or inefficient splicing because several higher M_r products were

consistently observed (Fig. 2B). These products were *ureD* specific because they were absent in reactions performed with RNA from the *ureD* insertion mutants (lanes 4 and 6). Control RT-PCR reactions without RT were negative (lanes 7–12), demonstrating that the products amplified from an RNA template.

Urease Activity Assays

All mutant plants were unable to sustain growth on urea as sole nitrogen source (Fig. 3). In contrast, both wild-type accessions grew well either with nitrate or with urea. This experiment was repeated three times with identical results.

For urease activity quantification, protein extracts were prepared from pooled leaves of four to five plants for each line, respectively. The plants were grown on soil supplemented with NiCl_2 to allow efficient urease activation. For the wild-type plants, leaf urease activities of $13.5 \pm 0.7 \text{ mU mg}^{-1}$ (Nössen Ds donor line DS5-392-12) and $17.9 \pm 1.6 \text{ mU mg}^{-1}$ (Col 0) were determined (errors are confidence intervals for $P = 95\%$). Comparable activities were reported from leaves of other plants (Witte and Medina-Escobar, 2001). For none of the mutants could urease activity be detected. Thus, urease activity in all mutants was absent or at least strongly reduced below detection limit of the assay.

These findings demonstrate that the Arabidopsis urease accessory proteins *AtUreD*, *AtUreF*, and *AtUreG* are required in vivo to generate a functional urease enzyme.

Reconstitution of Plant Urease Activation in *E. coli*

To investigate whether *AtUreD*, *AtUreF*, and *AtUreG* are not only necessary but also sufficient for plant urease activation at least in a heterologous system, we coexpressed these accessory proteins together with *Aturease* in *E. coli* and measured urease activity in bacterial extracts. While some Enterobacteria like *K. aerogenes* contain a urease operon and are urease positive, *E. coli* generally does not possess ureolytic activity (Mobley et al., 1995).

When *Aturease* and all three Arabidopsis accessory proteins were simultaneously expressed, ureolytic activity was observed in *E. coli* extracts. Lack of *AtureD*, *AtureF*, or *AtureG* eliminated urease activation

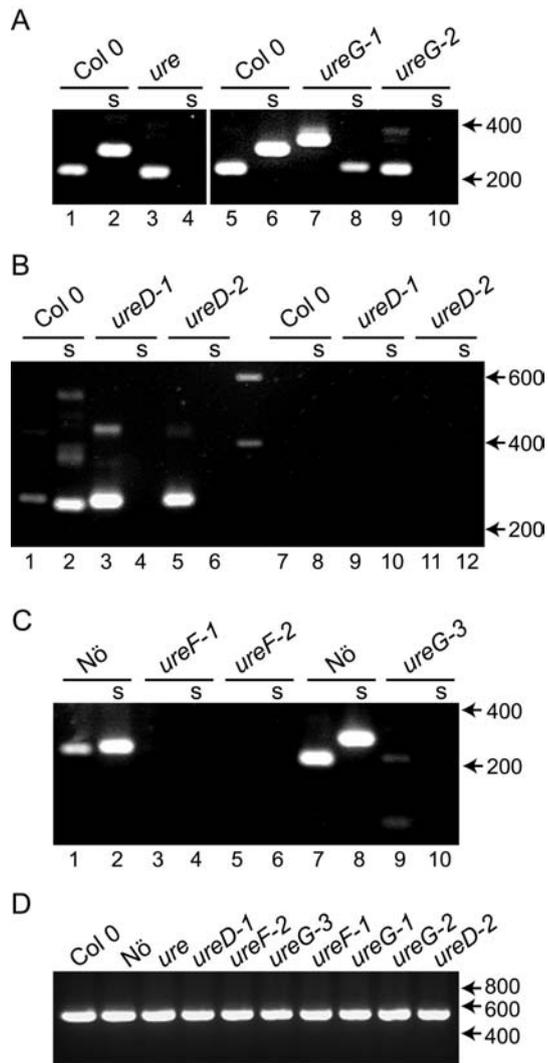


Figure 2. Characterization of mutants by RT-PCR. Lanes labeled with “S” show results obtained with primers spanning the T-DNA or Ds transposon insertion region. Unmarked lanes show results with primers that generate products either upstream or downstream of the insertion region (for primer positions and expected product sizes see Figure 1). A, Results for Col 0 wild type, the *urease* mutant, and mutants *ureG-1* and *ureG-2*. B, Results for Col 0 and the mutants *ureD-1* and *ureD-2*. Lanes 1 to 6, with RT; lanes 7 to 12, without RT. C, Results for the Ds element donor line DS5-392-12 (Nö) and *ureF-1*, *ureF-2*, and *ureG-3*. D, Control reactions with *actin 2* primers.

in this system (Fig. 4A). Presence of the corresponding mRNAs was shown by RT-PCR (Fig. 4B). Thus, the three accessory proteins are not only necessary but also comprise the core components required for the activation of plant urease. Replacing *ureD* with the alternative splice variant *ureD** (stop codon directly 3' of exon 4; Fig. 1) also led to urease activation but again only in the presence of the other accessory proteins.

If the activation occurred only inside the *E. coli* cells but not after extraction, a constant activity would be measured in the extract. This appeared to be the case in the presence of truncated *ureD** where a linear increase of ammonium concentration was observed in

the assay (Fig. 4A). Alternatively, if the activation occurred in the cells but continued after extraction in vitro, the activity would be expected to increase during the enzyme assay. This was observed in the presence of the full-length *ureD* gene. To confirm that the nonlinear increase of ammonium production was in fact due to in vitro urease activation, an experiment

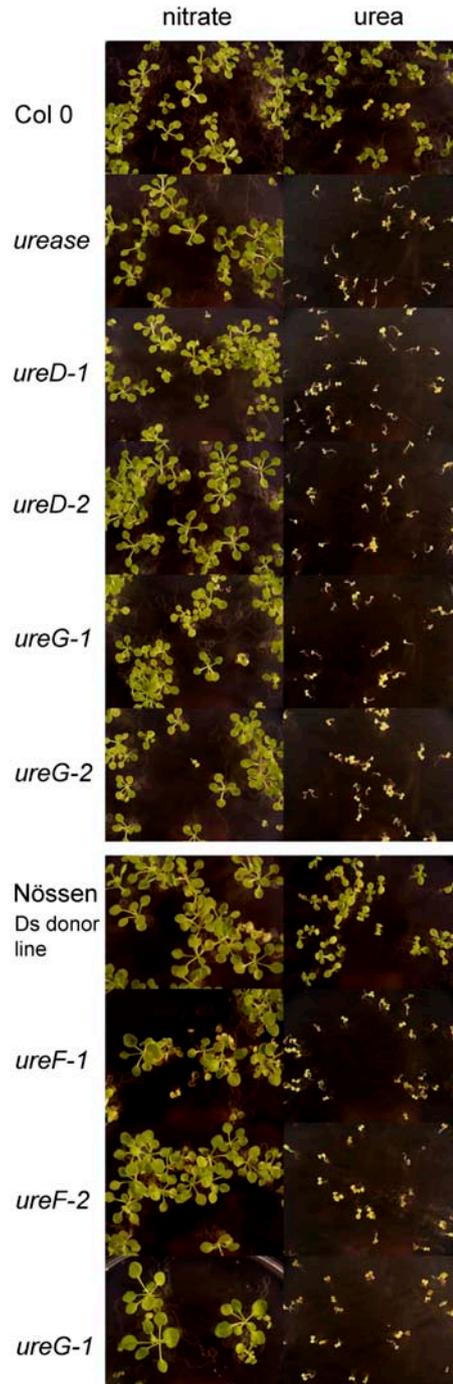


Figure 3. Growth test on agar plates with nitrate or urea as nitrogen source. Left column, plates supplemented with nitrate; right column, plates supplemented with urea. Photographs were taken 4 weeks after germination.

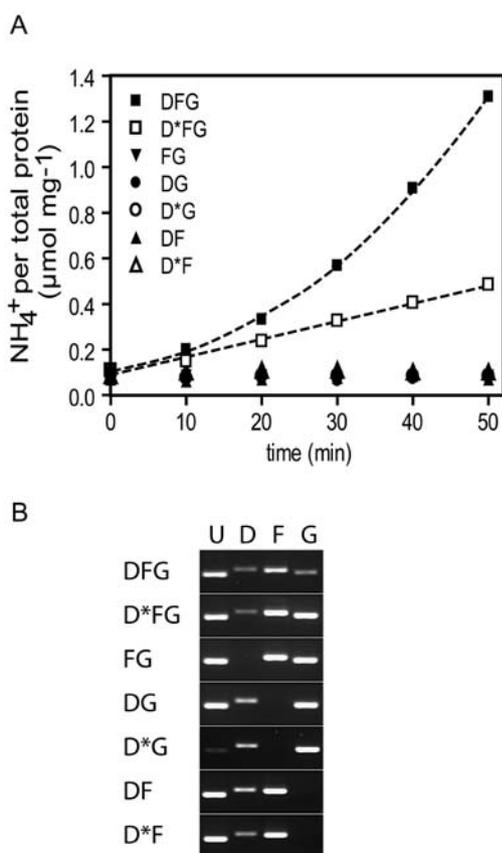


Figure 4. Activation of *Aturease* by Arabidopsis urease accessory proteins coexpressed in *E. coli*. A, Ammonium generation from urea per milligram total protein in extracts of *E. coli* cultures that had expressed urease and different sets of urease accessory proteins: UreD (D); UreF (F); UreG (G), and UreD* (D*: truncated UreD from alternatively spliced mRNA). B, RT-PCR results from RNA extracted 2 h after gene induction with IPTG. The accessory proteins expressed in the respective cultures are indicated on the left. The mRNAs amplified are indicated above: *Urease* (U), *ureD* (D), *ureF* (F), and *ureG* (G).

combining different *E. coli* extracts was performed. Extracts of cells that had expressed urease in combination with UreF and UreG (1), or UreD and UreG (2), or UreD and UreF (3) did not display activity, whereas the combined extracts of (1) and (3) or (2) and (3) allowed urease activation *in vitro* (Fig. 5). These experiments also provided a control showing that yellow fluorescent protein (YFP), which was always present when one of the accessory proteins was missing (see "Materials and Methods"), did not interfere with urease activation. Interestingly, combining extracts (1) and (2) did not lead to *in vitro* urease activation (Fig. 5). If either UreF or UreD had been lacking in the cell, extracts were not competent for *in vitro* urease activation even if extracts from cells that had expressed the missing protein were added.

DISCUSSION

Urease allows plants to recycle urea-nitrogen originating from Arg breakdown and purine or uride

catabolism. Since nitrogen availability is generally growth limiting for plants (Bray, 1983), efficient recycling is likely to give plants an ecological advantage. We demonstrate in this study that Arabidopsis requires three accessory proteins to generate urease activity. Soybean plants with defects in urease activity were previously shown to contain mutations in the *urease* and *ureG* genes (Meyer-Bothling and Polacco, 1987; Torisky et al., 1994; Freyermuth et al., 2000). Transgenic *ureG*-antisense potato plants also displayed reduced urease activity (C.P. Witte, M.A. Taylor, and H.V. Davies, unpublished data). These data could be corroborated by our results for *urease* and *ureG* mutants in Arabidopsis.

Comparing accessory protein sequences from Arabidopsis and *K. aerogenes* shows that UreG is conserved best (42.8% identity) while UreD and UreF are only 21.8% and 19.4% identical, respectively. However, structural conservation of urease accessory proteins UreF from soybean and UreG from potato is sufficient to allow partial complementation of defective *S. pombe* and *K. aerogenes* urease activation complexes, respectively (Witte, 2001; Bacanamwo et al., 2002). In contrast, *ureD* mutants of *S. pombe* or *K. aerogenes* were not able to activate urease in the presence of UreD from soybean or Arabidopsis. Also, Arabidopsis UreF did not complement *K. aerogenes* urease activation in the absence of bacterial UreF (Witte, 2001; Bacanamwo et al., 2002). Our results show that the proteins used in these complementation studies, in particular plant UreF and UreD, are genuine urease accessory proteins. Thus, the structural conservation of the urease activation complex across kingdoms is only partial. Especially UreD but also UreF are not compatible with any type of urease although the primary sequence and the metalcentre of urease enzymes were strongly conserved during evolution (Mulrooney and Hausinger, 2003).

In *K. aerogenes* UreD and UreF form a stable complex with apo-urease, to which UreG associates more

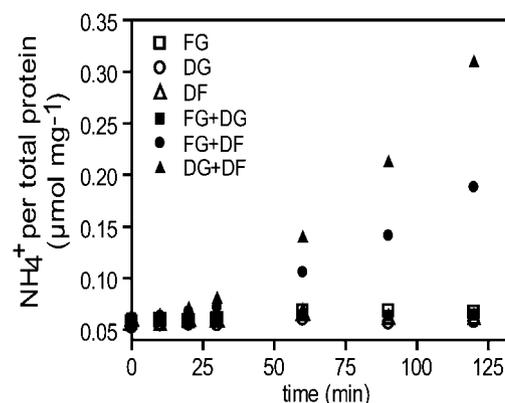


Figure 5. Arabidopsis urease activation *in vitro*. Ammonium generation from urea per unit total protein in single (white symbols) or combined (black symbols) extracts from *E. coli* cultures that had expressed urease and different combinations of urease accessory proteins. Legend as in Figure 4.

weakly and UreE binds only transiently (Soriano and Hausinger, 1999). Binding of UreD is necessary for association of UreF to the complex (Park and Hausinger, 1995). UreD may be very specific for its respective urease because it forms a tight complex with urease that can be chemically cross-linked at several residues (Chang et al., 2004). UreF in *K. aerogenes* was proposed to prevent nickel binding before carbamoylation (Moncrief and Hausinger, 1996) and to induce conformational change of the complex (Park and Hausinger, 1995; Chang et al., 2004). UreF masks UreD epitopes (Moncrief and Hausinger, 1996) and prevents chemical modification of UreD (Chang et al., 2004) upon binding to the complex, indicating that both proteins are closely associated and may act in a collaborative fashion. Interestingly, combining two *E. coli* extracts from cells that had expressed *Aturease* and an incomplete but complementary set of Arabidopsis accessory proteins led to in vitro plant urease activation. However, in vitro activation was observed only if one of the extracts was obtained from cells that had expressed *AtUreD* and *AtUreF* together (Fig. 5). A possible interpretation is that *AtUreD* and *AtUreF* form a complex with apo-urease in the *E. coli* cells but not in the cell-free extracts, while *AtUreG* can join the complex in vivo as well as in vitro. Similar to activation complex formation in *K. aerogenes* there may be an order of plant accessory protein association with urease, or plant UreD and UreF binding may only be possible during apo-urease protein synthesis in vivo. While improved tools, like, for example, antibodies for the individual accessory proteins, are needed to experimentally test these interpretations, the findings highlight the future potential of analyzing the plant urease activation process in vitro.

An alternatively spliced variant of *AtureD* mRNA incorporating a premature stop codon directly 3' of exon 4 has been described (accession no. AJ312203; Witte, 2001). RT-PCR results in this study indicate that *AtureD* transcript is subject to further differential splicing (Fig. 2B). Differential or ineffective splicing may be a mechanism for reducing the intact mRNA level of plant *ureD*. In *K. aerogenes*, *ureD* possesses an ineffective GTG start codon, and UreD overexpression hinders bacterial urease activation in vivo (Park et al., 1994). However, *AtUreD** generated from differentially spliced *ureD** mRNA and comprising only the first four exons is still sufficient to promote plant urease activation in *E. coli* (Fig. 4). It needs to be tested whether *AtUreD** is also active in planta.

Arabidopsis UreD, UreF, and UreG are necessary and sufficient to activate *Aturease* in *E. coli*. In planta further proteins may be required. The soybean mutant *Eu2* eliminates both urease activities of this plant (Meyer-Bothling et al., 1987) but could not be assigned to any of the accessory protein candidates and was not affected in nickel uptake and movement (Holland and Polacco, 1992). *Eu2* may represent a UreE ortholog or a urease activation component specific to eukaryotes.

The possibility to assemble a functional plant urease in a heterologous system and in vitro will allow a detailed biochemical analysis of the plant (eukaryotic) urease activation process. Great advances in understanding this process using the urease operon of *K. aerogenes* expressed in *E. coli* have been made, but the precise function of the urease accessory proteins remains in the dark. The comparison of the prokaryotic to the eukaryotic urease activation system may further our understanding of protein-mediated metal transfer processes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Mutants of Arabidopsis (*Arabidopsis thaliana*) from the Salk Institute collection (Alonso et al., 2003) and the Ds donor line DS5-392-12 (Kuromori et al., 2004) were ordered from the European Arabidopsis Stock Centre. GABI-Kat T-DNA mutants (Rosso et al., 2003) were received from the GABI-Kat mutant collection at the Max-Planck-Institute for Plant Breeding Research. Ds transposon mutants in ecotype Nössen were obtained from RIKEN (Kuromori et al., 2004). See Table I for details of the mutants. For genetic characterization and to isolate homozygous mutants, plants were grown on turf-based compost (Mini Tray; Balster Einheitserdewerk) in a controlled growth chamber (16 h light of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$, 22°C day, 18°C night, 60% relative humidity).

Agar plates were prepared with half-strength Murashige and Skoog nutrients without vitamins, sugars, or amino acids. Ammonium nitrate was omitted and cobalt chloride was replaced by $0.5 \mu\text{M}$ nickel chloride (Witte et al., 2002b). Plates contained either 9.35 mM KNO_3 or 4.68 mM urea as sole nitrogen source. To urea plates 9.35 mM KCl was added to maintain the same molarity of potassium ions as on the nitrate plates. Plates were incubated for 4 weeks in a controlled growth room in short-day conditions (see below) inside a transparent plastic bag to avoid water loss.

Mutant Characterization

Mutants from the Salk Institute and the GABI-Kat collections were screened with a primer binding at the left border of the T-DNA insert (TGGACCGC-TTGCTGCAAC for the Salk Institute collection and ATATTGACCATCA-TACTCATTGC for GABI-Kat). For transposon mutants from RIKEN a primer binding at the G-side of the Ds element was used: TACCTCGGGTTCGA-AATC. The following gene-specific primers were used, respectively: for *urease*, primer u5: ATCCTCTAGTCTAACAAACATTG; for *ureD-1*, primer d1: ATGGC-GACAGGGAAAG; for *ureD-2*, primer d2: TTGATCCTATTGCCTTGTACAC; for both *ureF* mutants, primer f1: ATGGAAGAAGACGAAAG; and for all *ureG* mutants, primer g5: CTAGTCTCTACTGAAATTAGCAG (Fig. 1). The PCR products from the mutants were cloned and sequenced to confirm the position of the insertions (Table I).

RT-PCR

RNA from plants was prepared using TRI reagent (Sigma) and treated with DNaseI (Sigma) following the manufacturer's instructions. RT using $1.0 \mu\text{g}$ total RNA was performed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and a poly-T primer. PCR reactions employed the following primers (see Fig. 1): for *urease*: u1 + u2 (GACAGCTGACAAGATG-AAG; CTCCTTTGATTATCATTCTG) and u3 + u4 (GTGATATCAAGACC-TATGTTTG; AAAGAGGAAATAGTCCG); for *ureD*: d1 + d2 (see above) and d3 + d4 (CTCTTTTGGTTGTGATACCAG; TGATAGTCTTGCATCCGTTG); for *ureF*: f1 + f2 (above; TGTCTCTATATCAGGAGATTG) and f3 + f4 (CTT-GTATGTGGTTTACTCG; AGAGCAAAAACAGTCTAGAAAACAAG); for *ureG*: g1 + g2 (ATGGCATCACAGACCAC; GCCAATACCGACGGTAA-AAG) and g3 + g4 (CTTTTACCCTCGGTATTGGC; CACCGGATTCACAAA-GAAGC); and for *actin 2* (At3g18780): a1 + a2 (GTGAACGATTCCTGGAC-CTGCCTC; GAGAGGTTACATGTTACCACAAC), spanning an intron.

For *urease*, *ureF*, and *ureG*, a PCR of 35 cycles (50°C annealing temperature) was performed, while for *actin 2* only 30 cycles were used. For *ureD* a reaction with 40 cycles (55°C annealing temperature) was carried out.

RNA from *Escherichia coli* was extracted with TRI reagent from a cell pellet of 1 mL culture (optical density = 0.8) two hours after iso-propyl-thiogalactoside (IPTG) induction. RNA was treated with DNaseI following the manufacturer's instructions. Four-tenths of a microgram of RNA was used in the RT reaction with Moloney murine leukemia virus reverse transcriptase employing the gene-specific primers u2, d2, f2, and g2, respectively. The RT reaction was diluted 20 times in the PCR reactions that contained the primer pairs u1 + u2, d1 + d2, f1 + f2, and g1 + g2 (24 cycles; 50°C annealing temperature). Control RT-PCR reactions were carried out without adding reverse transcriptase.

Urease Assay

Homozygous mutant plants and controls were grown on turf-based compost (Mini Tray; Balster Einheitserdewerk) in a controlled-climate chamber (8 h light of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 22°C day, 18°C night, 60% relative humidity). The compost was supplemented twice during growth with 5 mL 1 mM NiCl₂ per plant. The second supplement was given 1 week before the experiment to ensure optimal activation of urease.

Four-tenths of a gram of leaf material from 6-week-old plants was pooled from leaves of four to five plants of each line, and proteins were extracted with 2.5 mL sodium phosphate buffer (50 mM, pH 7.5) containing 50 mM NaCl, 1 mM EDTA, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 mM dithiothreitol. Extracts were centrifuged (20,000 g; 15 min; 4°C), and 1.5 mL of the supernatants were passed through a 5-mL HiTrap G25 desalting gel filtration column (GE Biosciences) equilibrated with half-strength extraction buffer without 4-(2-aminoethyl)benzenesulfonyl fluoride and dithiothreitol. Desalted extracts were subjected to urease assay as described previously (Witte and Medina-Escobar, 2001). Ammonium accumulation from urea hydrolysis was determined with the phenol hypochloride reaction in 10-min intervals during a total of 50 min (generating six data points per assay). Linear regression analysis and a statistical test to analyze whether the slope of the regression line was significantly divergent from zero were performed with the GraphPad Prism software package (www.graphpad.com). One unit was defined as the production of 1 μmol ammonium per minute. Protein concentrations were determined with a commercial Bradford assay (Bio-Rad).

Cloning in Bacterial Expression Vectors and Expression in *E. coli*

Urease from *Arabidopsis* was amplified by RT-PCR from leaf RNA introducing an *Nde*I site at the start codon and a *Sma*I site directly 3' of the stop codon. The urease was cloned into a derivative of the pET30 expression vector (Novagen, Merck) denominated pET30-CTH (Glinski et al., 2003) via the *Nde*I and *Eco*RV restriction sites of the vector. *UreD* and the alternative splice variant of *ureD* (accession no. AJ312203) were amplified from clones obtained previously (Witte, 2001; Bacanamwo et al., 2002) introducing an *Nco*I site at the start codon and a *Hind*III site directly 3' of the stop codon (the second stop codon in case of the alternative splice variant). *YFP* was amplified from pMON999-YFP (Shah et al., 2001), introducing an *Nco*I site at the start codon and a *Bgl*II site directly 3' of the stop codon. *UreD* and the alternative splice variant of *ureD* were cloned into the *Nco*I and *Hind*III sites of the vector pAlter-Ex2 (Promega). *YFP* was cloned into the *Nco*I and *Bam*HI sites of this vector. These cloning steps generated the expression vectors pAlter-ureD (1), pAlter-ureD* (2), and pAlter-YFP (3). *UreG* was amplified from pUni51 (clone 61276; Yamada et al., 2003) introducing an *Nco*I site at the start codon and a *Bgl*II site directly 3' of the stop codon. *UreF* was excised with *Nde*I from a clone obtained previously (Witte, 2001) containing a *Nde*I site at the start codon and a second *Nde*I site 3' of the stop codon. *YFP* was amplified again from pMON999-YFP, introducing an *Nde*I site at the start codon and a *Bgl*II site directly 3' of the stop codon. These genes were cloned into the expression vector pCDF-Duet-1 (Novagen, Merck) containing two multiple cloning sites (mcs) generating the constructs pCDF-ureG-ureF (4), pCDF-YFP-ureF (5), and pCDF-ureG-YFP (6). *UreG* was cloned into the *Nco*I and *Bam*HI sites of mcs1 and *ureF* was cloned into the *Nde*I site of mcs2. *YFP* was cloned into mcs1 via *Nco*I and *Bam*HI for construct pCDF-YFP-ureF or into mcs2 via *Nde*I and *Bgl*II for construct pCDF-ureG-YFP. All constructs were confirmed by sequencing when PCR steps were involved. The vectors pET30-CTH, pAlter-Ex2, and pCDF-Duet1 possess the compatible origins of replication ColEI, P15A, and CDE, respectively, allowing the simultaneous presence of all vectors in the same cell. The *E. coli* expression strain BL21(DE3) was transformed with the pET30-CTH-urease construct. The resulting cells were transformed sequentially with constructs (1) + (4), (1) + (5), (1) + (6), (2) + (4), (2) + (5), (2) + (6),

and (3) + (4), allowing the coexpression of urease with all three accessory proteins or coexpressing urease with any two of the accessory proteins and YFP. All transformants were resistant to kanamycin (50 $\mu\text{g mL}^{-1}$), streptomycin (50 $\mu\text{g mL}^{-1}$), and tetracycline (5 $\mu\text{g mL}^{-1}$) on plate. In liquid culture antibiotic concentrations were reduced by 40% each.

For expression, 20 mL *E. coli* cultures (Luria-Bertani medium) supplemented with 100 μM NiCl₂ were grown at 30°C to an optical density of 0.6 and induced with 1 mM IPTG for two hours. Cells were washed twice with 20 mL 25 mM phosphate buffer (pH 7.5) containing 25 mM NaCl and 0.5 mM EDTA and finally resuspended in 1.5 mL of this buffer. Cells were ruptured by sonification and urease assays were performed with the clarified extracts at 50°C in the presence of 50 mM urea (Witte and Medina-Escobar, 2001). For extract combination experiments equal volumes of extracts were mixed and subjected to the urease assay. Ammonium production was referred to total protein content of the extract determined by a commercial Bradford assay (Bio-Rad).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_105422 for the *urease* gene, NM_179908 for the *ureD* gene, NM_102032 for the *ureF* gene, and NM_128999 for the *ureG* gene.

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