RESEARCH LETTER

Oxalic acid biosynthesis is encoded by an operon in *Burkholderia glumae*

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Received 9 November 2009; accepted 27 December 2009.
Final version published online February 2010.

DOI:10.1111/j.1574-6968.2010.01895.x

Editor: Geoffrey Gadd

Keywords
transposon; oxalate; polycistronic.

Abstract

Although the biosynthesis of oxalic acid is known to occur in a number of bacteria, the mechanism(s) regulating its production remains largely unknown. To date, there is no report on the identification of an oxalic acid biosynthetic pathway gene from bacteria. In an attempt to identify such a gene(s), a mutant screen was conducted using the simple oxalic acid-producing phytopathogenic bacterium, *Burkholderia glumae*. Four mutants that failed to produce oxalic acid were isolated from a transposon-mutagenized *B. glumae* library and named *Burkholderia* oxalate defective (Bod)1. DNA sequence analysis revealed that each mutant contained an insertion event at different sites in the same ORF, which we referred to as the oxalate biosynthetic component (*obc*)*A* locus. Complementation of the Bod1 mutant with the *obcA* gene, however, resulted only in a partial restoration of the oxalic acid-producing phenotype. Further complementation studies utilizing a larger DNA fragment encompassing the *obcA* locus coupled with deletion mutagenesis led to the identification of another ORF that we named the *obcB* locus, which was essential for higher levels of oxalic acid production. Transcript analysis indicated that both *obcA* and *obcB* are coexpressed and encoded on a single polycistron message.

Introduction

Oxalic acid is the simplest of the organic dicarboxylic acids. It is considered a relatively strong acid with good reductive power, making it prevalent in a variety of industrial applications (Strasser *et al.*, 1994; Rymowicz & Lenart, 2003; Meyer-Pinson *et al.*, 2004). Currently, the bulk of the acid is produced chemically, but there has been some interest in the development of fermentative processes utilizing oxalic acid-producing microorganisms (Strasser *et al.*, 1994; Rymowicz & Lenart, 2003; Meyer-Pinson *et al.*, 2004). As in the chemical industry, oxalic acid is also common in nature; its biosynthesis has long been known to occur in a variety of organisms such as bacteria, fungi, plants, and animals (Hodgkinson, 1977; Franceschi & Nakata, 2005). The functional role of oxalate in each organism can differ along with its chemical form and distribution (Hodgkinson, 1977; Dutton & Evans, 1996; Franceschi & Nakata, 2005). In microorganisms, oxalic acid has been shown to serve a number of important functions, which include roles in metal tolerance (Dutton & Evans, 1996; Sayer & Gadd, 1997; Appanna & Hamel, 1999; Green & Clausen, 2003), nutrient acquisition (Shimada *et al.*, 1994; Dutton & Evans, 1996; Gharieb, 2000; Munir *et al.*, 2001), competition (Dutton & Evans, 1996), and pathogenicity (reviewed by Dutton & Evans, 1996; Hegedus & Rimmer, 2005).

Despite the important functional roles for oxalic acid in microorganisms, the mechanisms regulating the production of this acid remain largely unknown. Thus far, there have been two reports of a biosynthetic gene identified from fungi (Pedersen *et al.*, 2000; Han *et al.*, 2007), but none from bacteria. Difficulties have been encountered in deciphering the multiple oxalic acid biosynthetic activities identified (Akamatsu *et al.*, 1991; Akamatsu & Shimada, 1994; Tokimatsu *et al.*, 1998), purifying the biosynthetic activities (Li *et al.*, 1999) and ultimately the genes that encode them. Efforts to understand this biosynthetic pathway(s) would greatly benefit from the identification and isolation of the molecular components required for its production. Thus, we adopted a molecular-genetic approach to complement the existing biochemical methodologies. *Burkholderia glumae* was chosen as the model organisms for this endeavor

because it is a simple bacterium, produces ample amounts of oxalate, is amenable to molecular-genetic techniques (Nakata, 2002), has an established biochemical assay for oxalic acid biosynthesis (Li *et al.*, 1999), a recently sequenced genome (Lim *et al.*, 2009), and is an economically important phytopathogen. *Burkholderia glumae* is the known causal agent of bacterial panicle blight and seedling rot in rice (Tsushima *et al.*, 1996; Song & Kim, 1999; Nandakumar *et al.*, 2009) as well as bacterial wilt in a number of crop plants (Jeong *et al.*, 2003; Lim *et al.*, 2009).

As a first step toward elucidating the regulatory mechanisms of oxalic acid biosynthesis, here, we report the identification and isolation of the first set of oxalic acid biosynthetic genes from bacteria. We refer to these new genes as oxalate biosynthetic component (obc)*A* and *obcB*, both of which are essential for elevated oxalic acid production in *B. glumae*. Transcript analysis showed that both genes are encoded in a single polycistronic message, forming, at least in part, an oxalic acid biosynthetic operon.

Materials and methods

Strains, media, and culture growth

Burkholderia glumae (ATCC no. 49703, Manassas, VA) as well as strains of *Escherichia coli* [DH5α, Invitrogen Life Technology, Carlsbad, CA; BLR (DE3), EMD Biosciences Inc., Madison, WI] were grown in Luria–Bertani broth (LB) (Invitrogen Life Technology) media at 30 °C. If required, 50 μg mL^{-1} of the appropriate antibiotic was added.

Transposon mutagenesis and mutant screen

A transposon-mutagenized *B. glumae* library was generated as described previously (Nakata, 2002), with the exception that the EZ::TN^{TM} ⟨KAN-2⟩ (Epicentre, Madison, WI) rather than the EZ::TN^{TM} ⟨R6K-γori/KAN-2⟩ was used to create the insertion mutants. Individual colonies were selected and used to inoculate 1 mL of LB. The cultures were grown to saturation (1–2 days) at 30 °C with shaking. Oxalic acid production was visualized using an enzymatic reaction protocol (as described below). The tubes were then visually screened for alterations in the intensity of the purple-colored reaction product.

Oxalic acid measurement

Oxalate measurements were performed using the Sigma oxalate diagnostic kit (catalog no. 591-D; St. Louis, MO), according to the manufacturer's instructions. In brief, the oxalate was oxidized by oxalate oxidase to carbon dioxide and hydrogen peroxide. The hydrogen peroxide generated was then allowed to react with 3-methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino)benzoic acid in the presence of peroxidase to yield an indamine dye that was read at 590 nm. Cells were removed by centrifugation before quantifying the oxalic acid levels in the media. Experiments were repeated three times. All assays were conducted in duplicate, the results were averaged, and the error was determined.

DNA sequence analysis

Based on the Southern blot analysis (data not shown), DNA fragments of the appropriate size were cut from the gel, purified, and subcloned into pBluescript II KS-. The individual constructs were propagated in the *E. coli* strain, DH5α. Plasmid DNA was isolated using the Wizard miniprep kit (Promega, Madison, WI) and sequenced (Molecular Genetics Core Facility, Department of Microbiology and Molecular Genetics, UT-Houston Medical School, Houston, TX). Sequence analysis was conducted using the University of Wisconsin Genetic Computer Group software (Program Manual for the WISCONSIN PACKAGE, version 8, Genetics Computer Group, Madison, WI). Database homology searches were conducted using BLASTX programs (NCBI).

Complementation of *Burkholderia* oxalate defective (Bod)1

The *obcA* ORF was amplified by PCR using gene-specific primers 5′-ATGACATCGCTATACATCACGGCAG-3′ and 5′-TCAGCCCGCCGCGGTCTGGGGGTCG-3′. The PCR reaction was conducted using the PCRx enhancer kit (Invitrogen Life Technology) according to the manufacturer's instructions. All hybridization steps were performed on a PTC-200 thermal cycler (MJ Research, Watertown, MA) using the following parameters: 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min. After completion of the 30 cycles, a 5-min extension was run at 72 °C. The amplified ORF was TA cloned using the Qiagen TA cloning kit (Qiagen Inc., Valencia, CA). The *obcA* ORF was then isolated by restriction digestion with EcoRI and subcloned into the corresponding site in the pRK415 vector (Wang *et al.*, 2006) for complementation of the Bod1 mutant. For complementation with the C1E2 fragment, a 9-kb EcoRI genomic DNA fragment was cloned into the corresponding site in the pRK415 vector and transformed into a Bod1 mutant.

Deletion analysis and expression

Deletions were made of the 9-kb C1E2 genomic DNA fragment using the available restriction sites and PCR. The C1E2 EcoRI fragment was subcloned into the EcoRI site of pBluescript II KS-. To generate C1E2S2, the C1E2 construct was digested with SacI and religated. To generate the C1E2S2C1, the C1E2S2 construct was digested with ClaI

and religated. To generate the C1N2 construct, an NotI fragment was isolated from C1E2 and subcloned into the NotI site of pBluescript II KS-. To generate the NH3 construct, PCR was used to add back a short fragment to the 3′-end of the NotI fragment. The primers, 5′-AGGATCGAGATCTTCGAC-3′ and 5′-AAGCTTACACGGGGCGGCCACACC-3′ were used to amplify the short DNA fragment. This fragment was then digested with BglII and HindIII and used to replace a slightly smaller sized fragment, which was removed upon digesting the CIN2 construct with the same restriction enzymes.

For expression analysis, the *obcA* ORF was amplified by PCR using the primers, 5′-TCATATGACATCGCTATACATCACGGCAG-3′ and 5′-AAGATATCAGCCCGCCGCGGTCTGGGGGTCG-3′. The N-terminal primer contained an NdeI and the C-terminal primer contained an EcoRV restriction site, respectively. The *obcB* ORF was amplified by PCR using the primers 5′-AACCATGGCGATTTATCGACTCGGGG-3′ and 5′-AAGGATCCACACGGGGCGGCCACACC-3′. The N-terminal primer contained an NcoI and the C-terminal primer contained a BamHI restriction site, respectively. Each *obc* fragment was then unidirectionally cloned into the same or a separate pDUET vector (Novagen, EMD Biosciences Inc.) to generate the three different constructs. To create the construct containing both ORFs on one continuous DNA fragment, the primers 5′-TCATATGACATCGCTATACATCACGGCAG-3′ and 5′-AAGATATCACACGGGGCGGCCACACC-3′ were used in the amplification of this continuous DNA fragment. The amplified fragment was subsequently cloned into the pDUET using the NdeI and EcoRV restriction sites. The resulting expression constructs were transformed into BLR (DE3) competent cells and grown in LB at 30 °C. The expressions of the encoded proteins were elicited by induction with 1 mM of isopropyl-β-D-thiogalactopyranoside.

Preparation of enzyme extracts

Cultures of *B. glumae* were grown in LB overnight at 30 °C. The cells were then diluted 1/50 and grown for an additional 30 h. The cells then were pelleted, the supernatant was discarded, and the pellet was stored at −70 °C until used. Crude extracts were prepared by resuspending the cells in 10 mL of 20 mM Tris (pH 8.0), 150 mM NaCl, and 0.2 mM $CaCl_2$ (TBS). Lysozyme was added to a final concentration of 200 μg mL^{-1} and the cells were incubated on ice for 20 min. The suspension was disrupted by sonic oscillation using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburg, PA) and then centrifuged for 20 min at 16 000 ***g***. The crude extract was recovered and the pellet was discarded.

Oxalic acid biosynthetic activity assay

Oxalic acid biosynthetic activity assays were performed using a modified protocol of assay 2 (Li *et al.*, 1999). In brief, assay 2 was carried out for 10 min at 37 °C in a 200-μL reaction volume (100 mM Tris, pH 8.0, 50 μM EDTA, 350 μm $CoCl_2$, 360 μM acetyl-CoA, 1.25 mM oxaloacetate, and the indicated amount of enzyme extract). Upon completion of the assay, aliquots were quick frozen in liquid nitrogen and stored at −20 °C. The oxalate generated was determined as described above. Experiments were repeated at least three times. Assays were conducted in duplicate, the results were averaged, and the error was determined.

Isolation of genomic DNA and total RNA

Genomic DNA was isolated using the MasterPure Complete DNA and RNA purification kit (Epicentre) according to the manufacturer's directions. Total RNA was isolated using the SV RNA isolation kit (Promega) according to the manufacturer's instructions.

Reverse transcriptase (RT)-PCR

RT-PCR was performed modeling a previously published report (Suzuki *et al.*, 2004). The following modifications were used. The affinity script multiple temperature cDNA synthesis kit (Stratagene) was utilized along with a gene-specific primer P2 in first-strand synthesis from total RNA. The PCRx enhancer system (Invitrogen Life Technology) was used according to the manufacturer's recommendation in the amplification step. The primers used were as follows: P1, 5′-CATGGCTCGCCGCGCTGTCG-3′; P2, 5′-CGCTGGTCGGCATAGAACTC-3′; P3, 5′-CAGCGATCGTCGGCGCATCG-3′; and P4, 5′-GACCAGGCTGTAGTCGCCGA-3′.

Results and discussion

As an initial step toward identifying the molecular determinants of the oxalic acid biosynthetic pathway in *B. glumae*, a transposon-mutagenized library (Nakata, 2002) was screened for mutants in oxalate production. Cultures of individual colonies from the mutagenized library were grown in liquid media and the cultures were assayed for the presence of oxalic acid using a colorimetric detection system. After screening approximately 3000 colonies, four mutants were identified that failed to produce and secrete detectable levels of oxalic acid into the media (Fig. 1a and b). These mutants were named Bod1.

To assess whether the Bod1 knockout phenotype was due to the lack of the biosynthetic step, oxalic acid biosynthetic enzyme assays were conducted. Protein extracts were prepared from control and mutant cells and dialyzed to remove endogenous oxalic acid and other metabolites. Control extracts showed the ability to produce oxalic acid from oxaloacetate and acetyl-CoA *in vitro*, while all four mutants lacked this biosynthetic activity (Fig. 1c).

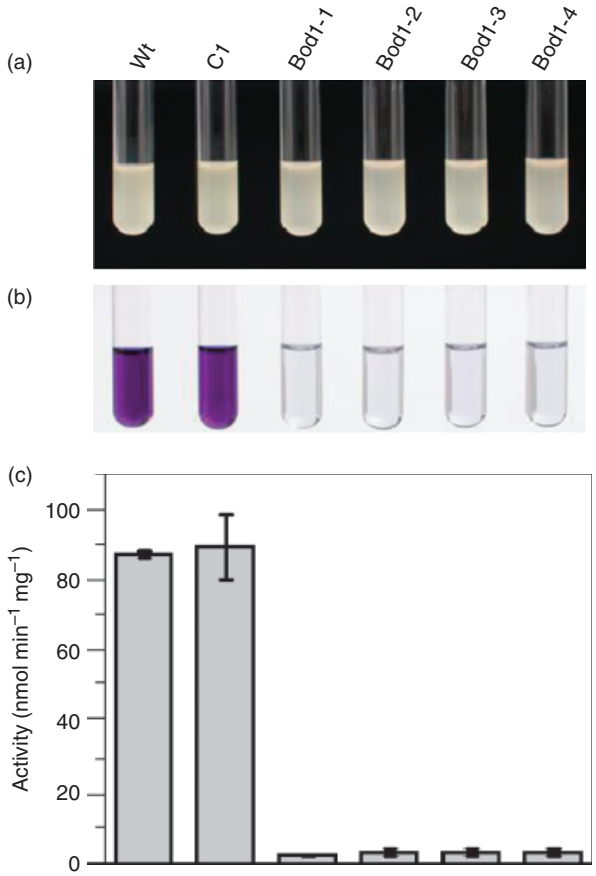

Fig. 1. Isolation of Bod mutants. A transposon-mutagenized *Burkholderia glumae* library was prepared and screened for mutants in oxalic acid production. (a) Comparison of control and Bod mutant cell phenotypes. (b) Colorimetric screen of cultures for the presence of oxalic acid (purple color). (c) Oxalic acid biosynthesis assay using protein extracts prepared from each culture. All mutants that failed to produce oxalic acid lacked oxalic acid biosynthetic enzyme activity. wt, ATCC no. 49703; C1, transposon control.

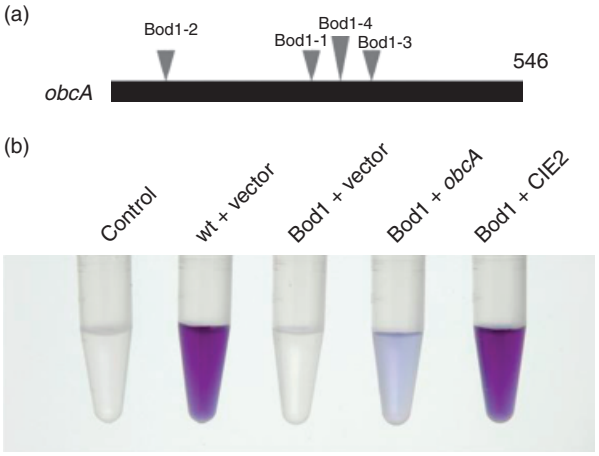

Fig. 2. Identification of the *obcA* gene. (a) DNA sequence analysis revealed that each Bod1 mutant contained a single insertion event (denoted by arrowheads) in the same ORF. This ORF is referred to as the *obcA*. (b) Complementation of the Bod1 mutant with the *obcA* gene resulted in partial restoration of the oxalic acid positive phenotype, while transformation with a larger DNA fragment encompassing the *obcA* gene resulted in a higher level of oxalic acid production. This observation indicated the requirement of other ORF(s) for maximal oxalate production. Control, Bod1 alone; wt, ATCC no. 49703; C1E2, 9-kb fragment. GenBank ACR32205.1.

Southern blot analysis was performed to determine whether the Bod1 mutants resulted from a single or multiple insertion events. Southern blots using different restriction enzymes revealed that each Bod1 mutant contained a single insertion in a restricted fragment of similar size (data not shown). The results indicated that a single essential gene may have been affected. To determine whether this was indeed the case, the area flanking the insertion site of each Bod1 mutant was sequenced. DNA sequence analysis revealed that each Bod1 mutant was a result of an independent insertion event into the same ORF (Fig. 2a). The identified ORF was named *obcA*. A BLAST search, using the *obcA* sequence, against the GenBank database revealed three matches with significant homology. An identity of approximately 67% was found between *obcA* and an ORF from *Burkholderia ubonensis* and about 53% identity was found between the *obcA* sequence and an ORF found in two related human pathogenic bacteria: *Burkholderia pseudomallei* and *Burkholderia mallei*.

To confirm the essential role of the *obcA* gene in oxalic acid biosynthesis, a complementation study of the Bod1 mutant with the *obcA*-coding region was performed. Expression of the *obcA* gene was able to restore the ability of the mutant to produce oxalic acid (Fig. 2b). The observed level of oxalic acid production, however, was much less than the wild type, suggesting that another essential component(s) was missing. This hypothesis was confirmed upon complementation of the Bod1 mutant with a larger 9-kb DNA fragment (C1E2) containing the *obcA* locus (Fig. 2b).

In an effort to identify the missing component(s), deletion analysis was performed on the 9-kb C1E2 fragment (Fig. 3a). Using the available restriction sites present on this DNA fragment, deletions were made to both the 5′ and the 3′ ends. Using this strategy, a second ORF was identified, which we refer to as the *obcB* locus. BLAST searches conducted using this gene revealed a 70% identity to an ORF from *B. ubonensis* as well as similarities to other bacterial acetyltransferases. This is in agreement with the proposed enzyme reaction mechanism and biochemical assay that has a requirement for acetyl-CoA (Li *et al*., 1999).

To verify the role of both genes in oxalic acid production, four different constructs were generated and expressed in *E. coli* (Fig. 3b). *Escherichia coli* is a bacterium that does not normally biosynthesize oxalic acid. As with the

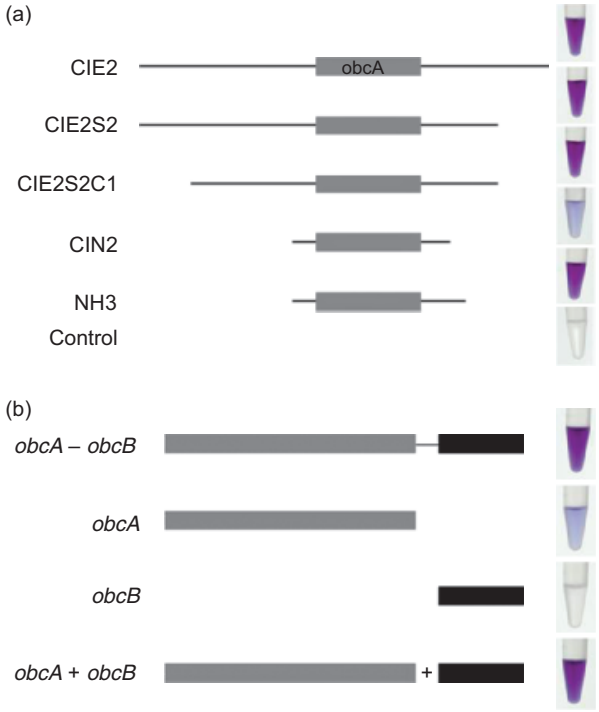

Fig. 3. Identification of the *obcB* gene. (a) 5′ and 3′ deletion analysis of the 9-kb C1E2 fragment indicates that a second ORF downstream of the *obcA* locus is important for higher levels of oxalate production. Oxalic acid colorimetric phenotype corresponding to each deletion mutant is shown to the right. (b) Verification of the coexpression of both *obcA* and *obcB* in oxalic acid biosynthesis. GenBank ACR32204.1.

complementation assay, expression of the *obcA* locus alone resulted in the production of some oxalic acid, while expression of the *obcB* alone did not result in any detectable acid. Coexpression of *obcA* and *obcB* either as one continuous DNA fragment (*obcA–obcB*) or as separate DNA fragments (*obcA*+*obcB*) contained on the same vector resulted in increased oxalic acid levels, and thus confirmed the importance of both ORFs in oxalic acid production (Fig. 3b).

Because both *obcA* and *obcB* are important in the biosynthesis of oxalic acid, are in close proximity to each other, and are encoded in the same transcriptional direction, it seemed likely that both genes could be encoded on a single polycistronic message. Such an arrangement of transcriptional control would also provide a plausible explanation for why complementation of the Bod1 (*obcA* knockout) with a functional copy of the *obcA* gene was not enough to fully restore the oxalate phenotype (Fig. 2b). To test this operon hypothesis, we performed a transcriptional analysis using RT-PCR and gene-specific primers (Fig. 4a and b). Genomic DNA was used as a positive template control and total RNA (without running the RT reaction) was used as a negative template control. All primer pairs used in the RT-PCR experiment resulted in the generation of a DNA fragment of the expected size, indicating that the *obcA* and *obcB* genes

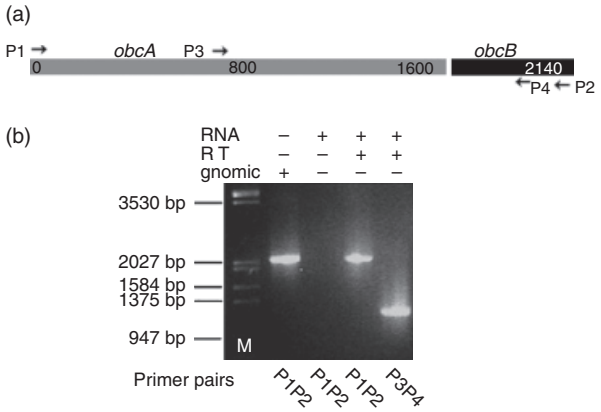

Fig. 4. The *obcA* and *obcB* genes are encoded by an operon. (a) Schematic drawing of the putative oxalic acid biosynthetic operon and the locations of the primers used in the RT-PCR experiment. (b) Transcriptional analysis using RT-PCR and gene-specific primer pairs. M, size markers.

were indeed encoded on a single polycistronic message and were thus structured into an operon.

Overall, it appears that a molecular-genetic approach will be useful in deciphering the oxalic acid biosynthetic pathway in bacteria. Thus far, this approach has led to the identification of two essential components of the oxalic acid biosynthetic machinery from *B. glumae*. It is anticipated that the identification of these first molecular components will expedite the discovery of additional genes and begin to provide us with a better understanding of the regulatory mechanisms controlling oxalate biosynthesis in bacteria and other organisms. It is our hope that this knowledge will prove useful, in the future, to design new strategies to combat oxalic acid-secreting phytopathogens and in the development of desirable fermentative processes for the production of this useful industrial acid.

Acknowledgements

The contents of this publication do not necessarily reflect the views or policies of the US Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. This research was supported in part by the US Department of Agriculture, Agricultural Research Service, under Cooperative agreement number 58-6250-6-001. Thanks are due to Keri Wang for providing the pRK415 vector and Michele McConn, John Knight, and Ross Holmes for their comments on the manuscript.

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