A Cytosolic Arabidopsis d-Xylulose Kinase Catalyzes the Phosphorylation of 1-Deoxy-d-Xylulose into a Precursor of the Plastidial Isoprenoid Pathway

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Plants are able to integrate exogenous 1-deoxy-d-xylulose (DX) into the 2C-methyl-d-erythritol 4-phosphate pathway, implicated in the biosynthesis of plastidial isoprenoids. Thus, the carbohydrate needs to be phosphorylated into 1-deoxy-d-xylulose 5-phosphate and translocated into plastids, or vice versa. An enzyme capable of phosphorylating DX was partially purified from a cell-free Arabidopsis (Arabidopsis thaliana) protein extract. It was identified by mass spectrometry as a cytosolic protein bearing d-xylulose kinase (XK) signatures, already suggesting that DX is phosphorylated within the cytosol prior to translocation into the plastids. The corresponding cDNA was isolated and enzymatic properties of a recombinant protein were determined. In Arabidopsis, xylulose kinases are encoded by a small gene family, in which only two genes are putatively annotated. The additional gene is coding for a protein targeted to plastids, as was proved by colocalisation experiments using green fluorescent protein fusion constructs. Functional complementation assays in an Escherichia coli strain deleted in the cytosolic enzyme could exclusively phosphorylate xylulose in vivo, not the enzyme that is targeted to plastids. Xk activities could not be detected in chloroplast protein extracts or in proteins isolated from its ancestral relative Synechocystis sp. PCC 6803. The gene encoding the plastidic protein annotated as “xylulose kinase” might in fact yield an enzyme having different phosphorylation specificities. The biochemical characterization and complementation experiments with DX of specific Arabidopsis knockout mutants seedlings treated with oxo-clomazone, an inhibitor of 1-deoxy-d-xylulose 5-phosphate synthase, further confirmed that the cytosolic protein is responsible for the phosphorylation of DX in planta.
While there is only weak evidence that DX has a functional role in vivo, it was already substantially used as a stable or radioactive isotope-labeled precursor for studies of the biosynthetic pathway in plants (Schwender et al., 1997; Sagner et al., 1998; Arigoni et al., 1999; Hoeffler et al., 2002; Luan and Wüst, 2002), as well as in bacteria (Hill et al., 1989; Broers, 1994; Giner et al., 1998; Rosa-Putra et al., 1998b; Spiteller et al., 2002). The assumption that in plants the MVA and the MEP pathways somehow conspire was based on incorporation studies (Schwarz, 1994; Adam et al., 1999; Kasahara et al., 2002; Hemmerlin et al., 2003a; Hampel et al., 2005), which also revealed some catalytic properties of enzymes involved in the MEP pathway. Furthermore, DX has been successfully used to partially complement the \( \text{Cla}^{+} \) phenotype, an Arabidopsis \(( \text{Arabidopsis thaliana} \) DXS knockout mutant (Estevez et al., 2000), which shows an albino phenotype (Mandel et al., 1996).

Previously, an enzyme-catalyzed formation of DX (1-deoxy-\( \delta \)-threo-2-pentulose) was described in several bacteria (Yokota and Sasajima, 1984). The E1 component (pyruvate decarboxylase) of the pyruvate dehydrogenase complex was identified to catalyze the decarboxylation of pyruvate and the condensation of the enzyme-bound hydroxyethyl-thiamine diphosphate with glyceraldehydes (Yokota and Sasajima, 1986). In plants, the localization of this complex is mitochondrial and plastidial (Tovar-Mendez et al., 2003); however, the physiological relevance or even presence of these secondary activities remains obscure. Along with DX, cell-free extracts from \( \text{Escherichia coli} \) and \( \text{Klebsiella planticola} \) are able to catalyze the formation, from pyruvate and \( \delta \)-glyceraldehyde, of 5-hydroxypentane-2,3-dione (laurencione), a product resulting from the dehydration of DX (Rosa-Putra et al., 1998a). This latter compound is a natural product that accumulates, among others, in the red alga \( \text{Laurencia spectabilis} \) (Bernart et al., 1992).

\[ \text{Figure 1. Isopentenyl diphosphate synthesis via the MEP pathway. Intermediates involved in the biosynthesis are as follows: 1, pyruvate; 2, \( \delta \)-glyceraldehyde 3-phosphate; 3, DXP; 4, MEP; 5, 4-diphosphocytidyl-2-C-methyl-o-erythritol; 6, 2-phospho-4-(cytidine 5’-diphospho)-2C-methyl-o-erythritol; 7, 2C-methyl-o-erythritol 2,4-cyclodiphosphate; 8, (\( \epsilon \))-4-hydroxy-3-methylbut-2-2-enyl diphosphate; 9, isopentenyl diphosphate; 10, dimethylallyl diphosphate; 11, DX; and 12, ME. Genes encoding the enzymes of the pathway are as follows: dks, 1-deoxy-\( \delta \)-xylulose 5-phosphate synthase; dxr, 1-deoxy-\( \delta \)-xylulose 5-phosphate reductoisomerase; ygbP, 4-diphosphocytidyl-2C-methyl-o-erythritol synthase; ychB, 2-phospho-4-(cytidine 5’-diphospho)-2C-methyl-o-erythritol kinase; ygbB, 2C-methyl-o-erythritol 2,4-cyclodiphosphate synthase; gcpE, (\( \epsilon \))-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; lytB, (\( \epsilon \))-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; and idi, isopentenyl diphosphate isomerase.} \]
but was also proposed to serve as a pyridoxol precursor (Wolf et al., 1997).

It has been established that the nonphosphorylated DX is not a substrate for 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR), the next enzyme downstream in the isoprenoid pathway (Kuzuyama et al., 2000). This suggests that for integration into the plastidial MEP pathway, a phosphorylation is needed (Fig. 1) by a DX kinase (DXK) and a transport across the plastidial envelope. The sequence of these two steps remained unknown, as well as the identities of the enzyme and/or of the transporter implied in this process. Recently, Flügge and Gao (2005) showed that a plastidial D-xylulose 5-phosphate (XP) transporter is able to accept DXP as a substrate. These findings argue in favor of a phosphorylation of DX in the cytosol, followed by transport of DXP across the plastidial envelope. In addition, it has been reported that D-xylulose kinase (XK; EC 2.7.1.17), an enzyme that participates in the pentose phosphate pathway, phosphorylates DX in *E. coli* (Wungsintaweekul et al., 2001). In a more recent study, Hemmi et al. (2002) identified a nonannotated gene encoding a protein possessing high homologies with the IIC component of the phosho(enol)pyruvate-dependent phosphotransferase system. Its expression in *E. coli* led to an increase in the incorporation of DX by *E. coli* cells, but, nonetheless, the identity of the enzyme really responsible for DX phosphorylation in planta still remained enigmatic. Here, we describe the isolation and characterization of a cytosolic enzyme from Arabidopsis having DXK activity. The identified corresponding gene belongs to a family of only two members, encoding a cytosolic form and a plastidial one. To verify or not the hypothesis that this second enzyme could also be involved in DX phosphorylation, different strategies were applied: among others, functional complementation in *E. coli* or analysis of Arabidopsis knockout mutants. In this way, we could irrevocably validate the identity of DXK as corresponding to the protein initially purified from crude plant extracts.

**RESULTS**

**Identification of 1-Deoxy-d-Xylulokinase Purified from Arabidopsis Protein Extracts**

Enzyme-synthesized [2-14C]DX (Hemmerlin et al., 2003a) was used to detect DXK activity in crude cell extracts isolated from different organisms (*Sphingobacterium multivorum*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, Arabidopsis, tobacco [Nicotiana tabacum] BY-2, and radish [Raphanus sativus]). A recombinant His-tagged version of *E. coli* XK (Trisch et al., 2004), shown to bear DXK activity (Wungsintaweekul et al., 2001), was used as a positive control (Fig. 2, lane 2). DXK activity could not be detected in *S. cerevisiae*, an organism synthesizing isoprenoids exclusively via MVA, while all other protein extracts did catalyze the formation of DXP (Fig. 2). As DXP is incorporated into the plastidial MEP pathway, chloroplast protein extracts were tested as well, but no activity could be evidenced (Fig. 2, lane 9). A primary screening of various enzyme sources allowed us to identify Arabidopsis and radish total protein extracts as the best candidates to start with plant DXK purification (Fig. 2, lanes 6 and 8). To facilitate the later cloning of the gene encoding DXK and its characterization, Arabidopsis was chosen as the source of proteins.

For purification, stability of apparent enzyme activity was tested in preliminary experiments. DXK from Arabidopsis was revealed to be heat stable. Further attempts to purify the enzyme to a higher degree after the Sepharose-Q column were unsuccessful and led to loss of activity. Therefore, we decided to identify all proteins contained in the mixture by a proteomic approach. The Coomassie Blue-stained gel revealed about 20 major bands (Fig. 3A). All spots, identified by MALDI-TOF-MS, are listed in Figure 3B and corresponded to proteins encoded by the Arabidopsis genome (Arabidopsis Genome Initiative, 2000). However, only one (in spot no. 5) contained the conserved motifs FGGY-N (pfam00370.11) and FGGY-C (pfam02782.11) characteristic of a carbohydrate pentose kinase. This enzyme corresponds to a putative XK (accession no. Q9IT51) with a calculated molecular mass of 61,830 D (Fig. 3B).

\[ \text{Figure 2. Screening for DXK activity in protein extracts from different plants and microorganisms. Proteins were incubated for 4 h in the presence of 50 mM Tris-HCl, 20 mM MgCl}_2, 10 mM KF, 20 mM ATP, and 3.5 mM [2-14C]DX. After incubation, an aliquot (2 µL) of the reaction mixture was directly spotted on a silica plate, and the product DXP was separated from the substrate DX by TLC. The plate was developed in (6:1:3, v/v/v) n-propyl alcohol:ethyl acetate:water and analyzed by phosphor imaging. Lane 1, Negative control without enzymes; lane 2, positive control with purified *E. coli* xylulose kinase; lane 3, *S. multivorum*; lane 4, *S. cerevisiae*; lane 5, *S. aureus*; lane 6, Arabidopsis; lane 7, tobacco BY-2; lane 8, radish; lane 9, radish chloroplast protein extract; lane 10, [2-14C]DXP.} \]
Enzymatic Characterization of a Recombinant Deoxy-D-Xylulose Kinase from Arabidopsis

To confirm that the identified protein bears DXK activity, a recombinant His-tag enzyme (His<sub>6</sub>-At-DXK) and as its homolog from <i>E. coli</i> (His<sub>6</sub>-Ec-DXK) were purified using Ni<sup>2+</sup> columns. The purity of both recombinant enzymes was greater than 98% as judged by SDS-PAGE (Fig. 4A), with apparent molecular masses being close to the calculated values (53,414 D for the <i>E. coli</i> enzyme and 62,119 for the Arabidopsis enzyme, respectively). Following the technique described in Figure 2, a DXK enzyme assay was performed (Fig. 4B). In the presence of ATP and Mg<sup>2+</sup>, the recombinant enzymes were able to catalyze the conversion of DX into a more polar compound migrating with authentic DXP on a thin-layer chromatography (TLC) plate. The formation of DXP was proven by a coupled spectrometric assay with DXR (Fig. 1), as described by Kuntz et al. (2005).

To compare His<sub>6</sub>-At-DXK with His<sub>6</sub>-Ec-DXK, some enzymatic properties were determined. We observed that His<sub>6</sub>-At-DXK, initially purified from protein extracts, was heat stable. Thus, in a first series of experiments, using [2-<sup>14</sup>C]DX, we evaluated enzyme stability of recombinant His<sub>6</sub>-Ec-DXK versus His<sub>6</sub>-At-DXK at various temperatures (Fig. 4C). Both enzymes showed more than 50% of the optimum activity between 10°C and 50°C, confirming temperature stability within a broad range. Overall, in comparison to His<sub>6</sub>-Ec-DXK, His<sub>6</sub>-At-DXK is the more efficient enzyme (Fig. 4D). His<sub>6</sub>-At-DXK showed specific XK activity in the presence of ATP as a cosubstrate but also accepted DX, albeit at greatly reduced efficiency (175-fold), like its homolog from <i>E. coli</i> (Fig. 4D). A 5-fold higher affinity was observed for the plant enzyme when D-xylulose was used as a substrate, and 2-fold when DX was used (Fig. 4D). While enzyme activity remained stable for several weeks, it has to be noted that the K<sub>m</sub> values have to be determined with freshly prepared enzyme fractions, as the values slightly increased over storage time. Next, substrate specificities for nucleoside triphosphate were tested (Fig. 4, E and F). The given activities were obtained using the radiometric assay, and values represent initial velocities calculated from time-stop measurements. Generally, each nucleotide was accepted as a cosubstrate when D-xylulose was used as a substrate; however, their relative efficiency appears quite different. The plant enzyme showed a clear preference for ATP, as compared to His<sub>6</sub>-Ec-DXK. In the presence of DX instead of D-xylulose, the activity decreased for both enzymes. However, as compared to His<sub>6</sub>-Ec-DXK, His<sub>6</sub>-At-DXK used exclusively ATP as a substrate. It

### Table

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**Figure 3.** Arabidopsis DXK protein purification analyzed by SDS-PAGE. A, The partially purified protein extract was concentrated, then separated by SDS-PAGE on a 12% acrylamide gel and stained with Coomassie Blue. Black arrowheads indicate sections of the gel, which were cut off and treated for protein identification by mass spectrometry. Identified proteins are listed in B. The position of spot number 5, in which the presence of a protein containing a pentose kinase motif was identified, is highlighted. Molecular mass standards (Bio-Rad; low-range SDS-PAGE standards) are as indicated (phosphorylase B, 97 kD; bovine serum albumin, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21 kD; and lysozyme, 14 kD).
has to be noted that after a longer incubation time (>16 h), DXP can be formed even if $V_i$ is low (<1%).

A Small Gene Family Encodes XK Proteins with Differential Subcellular Localization in Arabidopsis

A second gene (At2g21370), encoding a putative XK (Swiss-Prot accession no. P42826), was found in the Arabidopsis genome. Alignment of protein sequences having homologies with XK from different organisms, by the aid of ClustalX software (Thompson et al., 1997; downloaded at ftp://ftp-igbmc.u-strasbg.fr), indicated there is no significant similarity between both XKs. Furthermore, this protein is more closely related to the well-characterized E. coli XK (Wungsintaweekul et al., 2001) than the enzyme we purified from plant extracts. Consequently, the corresponding gene was referred to as Atxk-1, in comparison to the E. coli gene, while the gene encoding the purified DXK was named Atxk-2. A phylogenetic tree (Fig. 5), constructed with ClustalX using the neighbor-joining method, showed that they have evolved independently. The analyses distinguished three distant groups: the first one assembled eukaryotes, the second grouped mainly cyanobacteria, and the third one joined eubacteria. The Arabidopsis proteins confine to two divergent groups: AtXK-1 clusters with the cyanobacteria group, while AtXK-2 clusters with proteins present in higher eukaryotic cells.
In addition, Atxk-1 bears a 5' extension in the open reading frame, which encodes an additional peptide of 43 amino acids as compared to the Synechocystis protein. It was identified as a potential targeting peptide by Predotar (http://urgi.inra.fr/predotar/predotar.html) for prediction of subcellular localization, with a probability of 40% for chloroplastic targeting and only 2% for mitochondrial targeting. The Psort software (http://psort.imb.u-tokyo.ac.jp/form.html) predicted the protein to be targeted to the stroma of the chloroplast with a probability of 51%, 36% to the mitochondrion and 29% to peroxisomes. Indeed, the protein contained at its C terminus a SGL motif, similar to a SKL-like peroxisome targeting signal type 1 motif. Furthermore, the hypothesis of Atxk-1 being targeted to peroxisomes was investigated by colocalization experiments, either with the truncated form of Atxk-1 fused to the C-terminal side of GFP or its mutated version in which the SGL motif was removed (Fig. 6). None of these chimeric proteins colocalized with an expressed RFP-SKL protein (Hemmerlin et al., 2006), which was taken as reference for peroxisomal labeling (Fig. 6). When fused to the N terminus of GFP, the truncated form of the protein (without transit peptide), starting with MSGNKTGN, was found in the cytosol (Fig. 6), and some cells contained a few protein aggregates within the cytosol. Furthermore, GFP fusion proteins at either the N terminus or C terminus of Atxk-2 confirmed that the protein was cytosolic, as was expected by the sequence analysis and the purification experiment (Fig. 6). In summary, the colocalization experiments proved that Atxk-1 is a plastidial protein, whereas Atxk-2 corresponds to a cytosolic one. Further, Atxk-1 presents no membrane domains and apparently corresponds to a stromal protein.

Functional Complementation of an *E. coli* XK Deletion Mutant

If we consider Atxk-1 to putatively encode a protein with XK activity, the question comes up as to why we did not identify this protein as a DXK. Furthermore, the possibility of DX phosphorylation by this enzyme would change the sequence of events compared to those expected with the isolated Atxk-2. In this case, DX would first be transported into the chloroplasts and then become phosphorylated. Does this enzyme bear higher substrate specificity for D-xylulose than for DX or is even the catalytic activity completely different?

In vitro activity assays with the recombinant enzyme did not provide evidence for any carbohydrate kinase activity. We tested nearly 40 sugars (monosaccharides and disaccharides, including D-xylulose and DX) as potential substrate for Atxk-1, but none of them
Figure 6. Subcellular localization of At-XK isoforms fused to GFP. The constructs used for colocalization experiments are represented at the top of the pictures. Bars represent the gene contained in the construct used for bombardment experiments (green: gfp; yellow: Atxk-2; blue: Atxk-1 transit peptide; red: rfp; orange: truncated form of Atxk-1; chloro: coding for PTS-RFP targeting specially the RFP into chloroplasts). The truncated form of Atxk-1 was fused either with its C terminus SGL motif or without (symbolized by an orange triangle). Pictures, depicting 5- to 16-h-old cells after transformation of tobacco cv xanthi (containing chloroplasts) or cv Bright Yellow-2 cells (all other cases), are documented. Green pictures are representative of images recordings made with a LSL510 confocal microscope after excitation at 488 nm and recording of the emission between 505 and 530 nm (top right picture) for the detection of GFP. Red pictures are representative of acquisitions made after excitation at 543 nm and emission of the natural red fluorescence of chlorophyll or RFP. The bright-field image is represented in the top right picture. When indicated, the bottom right picture is a superposition of both green and red channels, which shows colocalization of the fusion protein with the red standard, if any. The white scale bars represent 10 μm.
was significantly phosphorylated (data not shown). Because this might have been due to the lack of some unknown cofactor(s) or interaction with other proteins, the capacity of AtXK-1 to phosphorylate d-xylulose in vivo was investigated by functional complementation assays. We took advantage of the fact that E. coli contains a unique gene (xylB) encoding XK (Rosenfeld et al., 1984). A strain was created in which xylB was deleted and replaced by a CAT cassette. The deletion did not affect Xyl isomerase activity in vivo (data not shown), which was essential for complementation assays. While no obvious phenotype could be detected on rich Luria-Bertani (LB) medium, the growing capacity of the mutant strain was strongly affected on minimum M9 medium supplemented with 0.2% Xyl (Fig. 7). As a control, when the medium was supplemented with 0.2% Glc, the mutant grew, as did the wild-type DY329 strain (Fig. 7).

Functional complementation assays were performed with the Arabidopsis isogenes Atxk-1, Atxk-2, and xylB (positive control). With Xyl as a carbon source and in the presence of 1 mM IPTG, AtXK-2 and XylB could rescue the xylB deficiency, whereas the strain transformed with the construct coding for AtXK-1 did not grow, like the mutant strain transformed with an empty vector (Fig. 7). These results suggest that Atxk-1 does not code for a xylulose kinase. To prove that AtXK-1 is not responsible for the phosphorylation of DX into DXP in planta, additional strategies had to be developed, as described below.

Chloroplasts and Synechocystis sp. PCC 6803 Protein Extracts Do Not Phosphorylate Xylulose or DX

Because our studies suggested that At-XK1 corresponds to a plastidial enzyme, we tested the hypothesis that chloroplast protein extracts catalyze the conversion of d-xylulose into XP or DX into DXP. Being genetically close to Arabidopsis, radish, which produces considerable plant material within a few days, was chosen to verify this hypothesis. Stromatal protein extracts were assayed, but neither apparent-DXK (Fig. 2) nor XK activity (data not shown) could be evidenced. As AtXK-1 bears strong homologies with the Synechocystis sp. PCC 6803 protein (NP_442817) annotated as XK, we tested protein extracts from this cyanobacterium for enzyme activities. Neither XK (Fig. 8A) nor DXK (Fig. 8B) activities could be detected, but enzymatically synthesized [14C]D-XP was converted into other compounds with distinct Rf and, therefore, different polarities (Fig. 8, C and D). Different NTPs were tested, but none of them could be utilized to catalyze the formation of DXP or XP (Fig. 8, A and B). These results demonstrate that in cyanobacteria, as well as in chloroplasts, there is no XK activity, at least not in form of a soluble enzyme. However, it cannot be excluded that the protein is not expressed or that it is regulated by an unknown mechanism, even though the transcripts are detected by reverse transcription (RT)-PCR in wild-type plants (Fig. 9C).

Analysis of Insertional Arabidopsis Mutants

To further prove AtXK-2 to be responsible for DX phosphorylation in planta, we characterized Arabidopsis T-DNA insertional mutants. Mutants were identified in the database from the Salk collection (http://signal.salk.edu). Homozygous plants were selected by PCR, and sequence analysis of the junction fragments obtained with T-DNA and gene-specific primers identified the T-DNA insertion in exon 7 for At2g21370 and in exon 2 for At5g49650 (Fig. 9, A and B). RT-PCR analysis indicated that the RNAs were not expressed in the corresponding homozygous mutant plants (Fig. 9C). No phenotype could be observed under standard culture conditions. Using the Arabidopsis microarray database Genevestigator (Zimmermann et al., 2004), we checked the expression pattern of the RNAs transcripts of Atxk-1 and Atxk-2: Atxk-1 is expressed in all organs and tissues, though predominantly in leaves and pedicels. Atxk-2 is more ubiquitously expressed, with a slightly higher expression level in petals and stamens. Analysis on the eFP browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) confirmed these results and showed that Atxk-1 expression is enhanced by drought, genotoxic stress, or oxidative stress, while Atxk-2 expression is stimulated by osmotic stress.

For close inspection of biochemical properties in vivo, biochemical characterizations were initiated. The presence of XK and DXK activities in cytosolic extracts and plastid fractions for each plant type was verified (Fig. 10). While in wild-type and Atxk-1::T-DNA cytosolic plant extracts apparent activities could be detected, Atxk-2::T-DNA plant extracts were not able to phosphorylate d-xylulose or DX (Fig. 10). None of the
chloroplast fractions was able to phosphorylate the sugars, which confirmed the result presented in Figure 2. The tests were performed using ATP, GTP, CTP, or UTP as a phosphate donor and resulted each time in a negative enzyme assay. The results are consistent with the fact that XK is missing in plant chloroplasts and that AtXK-2, annotated as xylulose kinase, encodes a protein with different, yet unknown, enzyme activity.

As a control, it was verified that the chloroplast protein extract was metabolically active using XP as substrate (Fig. 10), as was the *Synechocystis* extract.

Biochemical Phenotype and Chemical

Complementation with DX of OC-Treated Plants

To further characterize the biochemical phenotype of mutant plants, we tested their capacity to metabolize exogenous sugar added to the culture medium. In vivo, *Atxk-1* knockout mutants, as well as wild-type Columbia (Col)-0, were able to use Xyl as a carbohydrate source in dark culture conditions (Fig. 11A). In contrast to this, *Atxk-2* knockout mutants did not germinate in the presence of Xyl, which was even found toxic as compared to seeds that were allowed to germinate without any sugar (Fig. 11A). To assess the role of AtXK-2 in the phosphorylation of DX in Arabidopsis, we tested the capacity of wild-type, *Atxk-1::T-DNA*, and *Atxk-2::T-DNA* plants to phosphorylate DX in planta. For this purpose, o xo-clomazone (OC; also referred to as keto-clomazone), a bleaching herbicide affecting DXS activity (Müller et al., 2000; Ferhatoglu and Barrett, 2006), was used to inhibit the MEP pathway and, thereby, the formation of β-carotene normally protecting photosynthetic reaction centers from photooxidation. We tested the capacity of OC-treated wild-type and mutant plants to phosphorylate DX into DXP, allowing after incorporation the bypass of the inhibition of DXS and, consequently, a reversion of the bleaching effect of OC (Fig. 11B). In the first series of experiments, we determined the minimal concentration of OC causing a bleached phenotype on Arabidopsis seedlings and the DX concentration needed to overcome, at least partially, the effect (data not shown). Reference concentrations were 0.2 μM OC and 2 mM DX. Under those conditions, a pale green complexion was recovered with wild-type, *Atxk-1::T-DNA* plants remained albino (Fig. 11B) and *Atxk-1* was unable to take over the function of AtXK-2 in the *Atxk-2::T-DNA* knockout mutant. In summary, these investigations led us to conclude that AtXK-2 is the sole enzyme able to phosphorylate DX in planta.

DISCUSSION

Plants incorporate at rather high efficiencies different carbohydrates providing sources of energy and carbon scaffolds. Along with other polyols, sugars that are taken up by the cells (Böttner and Sauer, 2000; Reinders et al., 2005) need to be activated by phosphorylation for their integration into metabolic pathways. DX and ME are known to be phosphorylated and used as precursors for the biosynthesis of isoprenoids synthesized via the MEP pathway (Rohmer,

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**Figure 8.** Measurement of XK and DXK activities in *Synechocystis* sp. PCC 6803 protein extracts. The protein extracts were incubated for 4 h in the presence of assay buffer (Tris-HCl [50 mM], MgCl2 [20 mM], KF [10 mM], NTP [20 mM] and 3.5 mM [1 mCi mmol-1] 2-14C DX or 3.05 mM [U-14C] Xyl (1.4 mCi mmol-1). After incubation, a fraction (2 μL) of the reaction mixture was directly spotted on silica plates and the product DXP (or XP) was separated from the substrate DX (or D-xylulose) by developing in (6:1:3, v/v/v) n-propanol:ethyl acetate:water; 30:5:15). D, XK products separated on a silica plate. C, Metabolism of XP developed on silica plates. B, XK products separated on a silica plate. A, DXK products separated on a silica plate. C, Metabolism of XP developed on silica plates. C, Metabolism of XP developed on silica plates.
The uptake mechanism for DX is still unknown, whereas ME seems to be transported via the bacterial sorbitol phosphotransferase system (Testa et al., 2004). Nothing is known about the uptake system in plant cells, but Wolfertz et al. (2004) estimated the rates of deuterated DX incorporation into the MEP pathway from the labeling degree found in isoprenoid end products and speculated that the labeling is due to kinase activity. These observations stand for high DX incorporation/phosphorylation in plants. In a previous study, it was shown that, in plants, DX incorporation is sufficient to overcome the absence of...
we did not isolate in the crude soluble extract, or the activity simply does not exist. In plants, sorbitol transporters do not bear phosphotransferase activity (Gao et al., 2003) as do bacterial transporters (Testa et al., 2004). Therefore, it is reasonable to assume that plants are not able to phosphorylate ME, which might explain the rather toxic effect of an accumulation of this polyol. As a consequence, DX is the sole exogenous precursor that can be used for integration into the plastid isoprenoid biosynthetic pathway.

Phosphorylations of carbohydrates are known to be carried out by the sugar-kinase protein family. Compared to other sugar kinases like ribulokinase (with specificities for Glc, Fru, ribulose, xylulose, and Fuc), the ribokinase-like family (with specificities for Rib and Fru), and the galactokinase-like family (with specificity for Gal, N-acetylgalactosamine; Bork et al., 1993). The identification of a XK being able to phosphorylate DX in planta is a comparable result to what was observed in prokaryotic cells (Wünsintaweekul et al., 2001). Nevertheless, the identified protein was not identical to that predicted by the same research group after sequence homology analysis with the Arabidopsis genome.

So far, only low interest had been focused on plant XK. In a quite early study, XK activity was evidenced in pea, spinach, corn, and wheat protein extracts (Zahnley and Axelrod, 1964). XK (ATP: D-xylulose 5-phosphotransferase; EC 2.7.1.17) is predicted to be implicated in the ARACyc pathway (http://www.arabidopsis.org:1555/ARA/NEW-IMAGE?type=PATHWAY&object=XYLCAT-PWY&detail-level=3), and catalyzes the formation of xp (dí-threo-2-pentulose 5-phosphate) from D-xylulose. XP represents a central metabolite within the oxidative pentose phosphate pathway that comprises three enzymatic reactions that convert Glc-6-phosphate into ribulose-5-phosphate and generates two molecules of NADPH. Therefore, this pathway plays a key function in the cell for the production of NADPH. It also provides intermediates for other biosynthetic pathways (Kruger and von Schaewen, 2003), for instance, for fatty acid biosynthesis, nitrate reduction, and the shikimate pathway (Emes and Neuhaus, 1997). In plants, it is compartmentalized within the cytosol and the plastids (Schnarrenberger et al., 1995). Both pathways are linked via a pentose phosphate translocator that is ubiquitously expressed in the various plant tissues (Eicks et al., 2002). In bacteria, as well as in plants, D-xylulose is produced from D-Xyl1 by the action of Xyl isomerase (EC 5.3.1.5). This substrate is obtained after degradation of the primary cell wall followed by transport within the cell (Sherson et al., 2003), a process activated in response to stress or during fruit ripening (Labavitch, 1981).

Compared to other sugar kinases like ribulokinase (Lee et al., 2001), XK shows stronger substrate specificities in S. cerevisiae (Richard et al., 2000) or in E. coli (Wünsintaweekul, 2001). Nevertheless, the enzyme

Figure 11. Chemical complementation of Arabidopsis knockout mutants in At-xk-1 and At-xk-2 alleles. A, Phenotype of wild-type Col-0 plants (WT), Atxk-1::T-DNA (A1), and Atxk-2::T-DNA (A2) knockout mutants growing on D-Xyl as a carbon source as compared to a mock experiment containing no carbon source. Seeds (about 25) were bleach sterilized, transferred into 100-ml Erlenmeyer flasks containing liquid Murashige and Skoog (20 ml) medium, and allowed to germinate in the dark for 3 weeks under constant shaking. After this period, the Erlenmeyer flasks were transferred into a culture room at 23°C, with 16-h-light and 8-h-darkness cycles for 2 further weeks. The content was suction filtered and documented by taking photographs on white filter paper. B, In vivo complementation by DX of wild-type Col-0 plants (WT), Atxk-1::T-DNA (A1), and Atxk-2::T-DNA (A2) knockout mutants treated with OC compared to untreated seedlings (Control) and seedlings that were germinated on OC. Seeds were bleach sterilized and allowed to germinate on solid Murashige and Skoog medium containing 2% Suc. Note that only in the wild-type plants and the Atxk-1::T-DNA (A1) knockout mutant DX could partially overcome the bleaching effect of OC, a clear proof that absence of plastidial XK had no impact on greening, whereas the absence of cytosolic XK (A2) did not allow chemical complementation of OC inhibition by DX.

Our studies demonstrated DXK activities in different plant protein extracts. When we used radiolabeled ME as substrate, no kinase activity could be detected independently of the source of plant extracts (Arabidopsis, tobacco cell suspensions, tobacco plants, pea, bean, radish, etc.; data not shown). These results corroborate our earlier observations on the toxicity of ME on tobacco BY-2 cells (Hemmerlin et al., 2003a). Two hypotheses can explain this observation: either this activity is catalyzed by a membrane protein, which
and are annotated as XK, the results in Figures 7 and 11 both enzymes contain carbohydrate kinase signatures, in accordance with the compartmentalization of the pentose phosphate pathways. Although both enzymes contain carbohydrate kinase signatures and are annotated as XK, the results in Figures 7 and 11 show that the plasmoidal enzyme is unable to overcome the lack of the cytosolic enzyme in planta and cannot functionally complement an E. coli knockout mutant. In addition, Xyl isomerase, which in bacteria is always associated with XK, in Arabidopsis is encoded by a single annotated gene (http://www.expasy.org/uniprot/Q9FKK7) with the prediction of a cytosolic protein. In fungi, which lack Xyl isomerase, the combined action of d-Xyl reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) is required to achieve conversion of Xyl into xylulose (Jackson and Nicholoz, 2002). Whereas in Arabidopsis a gene that codes for a putative cytosolic aldose reductase (At5g01670) occurs, the corresponding orthologs of Xyl reductase and xylitol dehydrogenase could not be identified. Moreover, as chloroplasts derived by endosymbiosis from ancestral cyanobacteria (Curtis and Clegg, 1984), the presence of these genes was checked in *Synechocystis*. None of the aforementioned genes or Xyl isomerase could be identified (http://www.kazusa.or.jp/cyano/Synechocystis/index.html). In fact, there

Table 1. Oligonucleotides used in this study

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accepts DX as a substrate. Interestingly, in yeast, an organism using exclusively MVA for isoprenoid biosynthesis, XK activity could be detected in total protein extracts (Tritsch et al., 2004) but DXK could not (this study). In other organisms, there was a clear correlation between XK and DXK activities. The question that still remains open is whether high concentrations of DX interfere with the general metabolism of the cell, especially whether the carbohydrate metabolism is affected. For instance, DX incorporation also stimulates XK activities in tobacco BY-2 cells, but not in Arabidopsis seedlings (data not shown). Furthermore, in Arabidopsis XK is encoded by a small gene family with two members (At2g21370 and At5g49650) coding for two enzymes that evolved independently and are targeted to different subcellular localizations, cytosolic versus plastidial, as it was established in this study. This is in accordance with the compartmentalization of the pentose phosphate pathways. Although both enzymes contain carbohydrate kinase signatures and are annotated as XK, the results in Figures 7 and 11 show that the plasmoidal enzyme is unable to overcome the lack of the cytosolic enzyme in planta and cannot functionally complement an E. coli knockout mutant. In addition, Xyl isomerase, which in bacteria is always associated with XK, in Arabidopsis is encoded by a single annotated gene (http://www.expasy.org/uniprot/Q9FKK7) with the prediction of a cytosolic protein. In fungi, which lack Xyl isomerase, the combined action of d-Xyl reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) is required to achieve conversion of Xyl into xylulose (Jackson and Nicholson, 2002). Whereas in Arabidopsis a gene that codes for a putative cytosolic aldose reductase (At5g01670) occurs, the corresponding orthologs of Xyl reductase and xylitol dehydrogenase could not be identified. Moreover, as chloroplasts derived by endosymbiosis from ancestral cyanobacteria (Curtis and Clegg, 1984), the presence of these genes was checked in *Synechocystis*. None of the aforementioned genes or Xyl isomerase could be identified (http://www.kazusa.or.jp/cyano/Synechocystis/index.html). In fact, there
is no real need for a XK activity in the plastid. Although the substrate specificity of At-XK1 could not be identified, we clearly showed that this enzyme was not able to phosphorylate D-xylulose or DX in vivo and that the cytosolic AtXK-2 was solely responsible for this enzyme activity. Therefore, it is now reasonable to propose that Atxk-1 codes for a chloroplast-targeted protein with different functions. Alternative biochemical techniques have to be adopted to solve the functional problem.

Our results point to a clear sequence of events needed for DX incorporation into the MEP pathway. DX is phosphorylated within the cytosol, followed by translocation from the cytosol into the plastids. Plastids contain numerous metabolite transporters within their envelope (Ferro et al., 2003; Knappe et al., 2003; Weber et al., 2005). The possibility of DXP to be transported by the well-characterized xylulose 5-phosphate/phosphate translocator (Eicks et al., 2002) was already evidenced by Flügge and Gao (2005). This transporter accepts other sugar-phosphates, but under physiological conditions xylulose 5-phosphate and triose-phosphate correspond to those that are favored (Eicks et al., 2002). This transporter, acting as an antipporter exchanging sugar-phosphate against inorganic phosphate, accepts neither D-xylulose (Eicks et al., 2002) nor DX (Flügge and Gao, 2005). This is an additional argument in favor of DXP phosphorylation within the cytosol, prior to transport into the plastid and incorporation into the MEP pathway.

In conclusion, our work clarifies where phosphorylation of DX takes place, which was previously a matter of debate (Wünszentaweekeel et al., 2001; Wolfrertz et al., 2004).

MATERIALS AND METHODS

Chemical Materials

Ni^{2+} spin columns were purchased from Qiagen. D-Xylulose and D-Xyl were purchased from Fluka and the MitoTracker Red CMXRos probe from Molecular Probes Europe BV. DX was chemically synthesized as described by Hemmerlin et al. (2003a). OC was provided by Dr. Klaus Grossmann (BASF). Silica (silica 60 F254) and polyethyleneimine (PEI)-cellulose F plates, utilized Hemmerlin et al. (2003a). OC was provided by Dr. Klaus Grossmann (BASF). Molecular Probes Europe BV. DX was chemically synthesized as described by Eicks et al. (2002) nor DX (Flügge and Gao, 2005). This is an additional argument in favor of DXP phosphorylation within the cytosol, prior to transport into the plastid and incorporation into the MEP pathway.

In conclusion, our work clarifies where phosphorylation of DX takes place, which was previously a matter of debate (Wünszentaweekeel et al., 2001; Wolfrertz et al., 2004).

Growth Conditions for Bacterial and Yeast Strains

E. coli strain XL1-Blue was purchased from Stratagene, whereas BL21(DE3) and Rosetta-gami(DE3) pLacI were purchased from Novagen. The E. coli DH5α strain used for genetic engineering was obtained from Dr. Donald Court (National Cancer Institute). All strains were grown on LB broth medium (Gibco-BRL) supplemented with ampicillin (100 μg/mL) if needed, or on M9 minimum medium supplemented with 0.05% (w/v) yeast extract. S. cerevisiae strains were handled as described by Tritzsch et al. (2004).

Identification by Mass Spectrometry and Database Searches

SDS-PAGE gels were scanned (GS-800; Bio-Rad) and analyzed with QuantityOne software (Bio-Rad). Proteins bands were excised and in-gel digested with trypsin according to published methods (Jeno et al., 1995), modified

Plant Material and Culture Conditions

Radish (Raphanus sativus) seeds were germinated on tap water for 4 d. Seeds of the T-DNA insertion lines SALK_042149 and SALK_079018, generated at the Salk Institute Genome Analysis Laboratory (Alonso et al., 2003), were obtained from the Arabidopsis Biological Resource Center under the names N542149 and N519643, respectively. When not specifically indicated, wild-type Arabidopsis (Arabidopsis thaliana; ecotype Col-0) was used. Seeds were ethanol and bleach sterilized, and then transferred into liquid Murashige and Skoog medium, pH 5.7. Cultures were grown under a 16-h-light/8-h-dark photoperiod under continuous shaking (100 rpm). Seedlings were recovered after 21 d and stored at −80°C. When grown on soil, Arabidopsis plants were transferred to growth chambers (at 21°C under a 16-h-light/8-h-dark photoperiod). For complementation experiments, seeds were surface sterilized and plated on agar-solidified Murashige and Skoog medium containing different chemicals as described for each experiment. Tobacco (Nicotiana tabacum) BY-2 cells (Nagata et al., 1992) were cultured as described in detail (Hemmerlin et al., 2003a), while tobacco cv xanthi cells were grown as described by Hemmerlin et al. (2003b).

Isolation of Total and Chloroplast Plant Protein Extracts

Several plant, bacterial, and yeast extracts were used to screen for DXK activity. The extraction of total proteins was performed as described by Tritzsch et al. (2004). Cytosolic fractions were obtained after centrifugation at 100,000g for 1 h at 4°C. Chloroplasts from radish and Arabidopsis were purified using a Percoll gradient as described by Gerrick et al. (2001). For enzymatic assays, chloroplasts were lysed by hypotonic treatment with Tris-HCl (62.5 mM), pH 8.0, and MgCl₂ (2 mM).

Partial Purification of an Enzyme with 1-Deoxy-D-Xylulokinase Activity from Arabidopsis

All procedures were conducted at 4°C unless noted otherwise. Frozen 3-week-old Arabidopsis seedlings (50 g) were ground in a mortar in presence of liquid nitrogen. The powder was suspended in 40 mL of ice-cold purification buffer (50 mM Tris-HCl buffer, pH 7.5, 5 mM β-mercaptoethanol) supplemented with 4% (w/v) polyvinylpyrrolidone (Sigma). Heat-labile proteins were precipitated by incubation at 50°C (20 min). The supernatant was cleared by centrifugation at 17,000g; then mixed with PEG6K, to a final 20% (w/v) concentration. The homogenate was stirred for 30 min, then incubated for 2 h on ice. Precipitated proteins were removed by centrifugation at 17,000g for 20 min and the supernatant was loaded on a DEAE-Sephrose column (2.4 × 15 cm) equilibrated with the buffer (flow rate: 25 mL h⁻¹). The column was carefully washed with the same buffer to elute unbound proteins and PEI. Proteins were eluted with a linear gradient of NaCl (400 mM, 0–0.4 M in the buffer). Fractions (5 mL) were collected and assayed for DXK activity. Active fractions were pooled and desalted by dialysis against purification buffer (3 × 1 L), followed by loading onto a Sepharose Q column (1 × 8 cm) equilibrated with the same buffer. After washing, bound proteins were eluted with a linear gradient (60 mL) of increasing concentration of NaCl (0–0.3 M) in the buffer. Fractions (1 mL) were collected, assayed for DXK activity, and analyzed by SDS-PAGE. Those with the highest activity were pooled and concentrated using ultrafiltration Centricron 30 concentrators (Millipore).

Protein Concentration Determination and SDS-PAGE

Protein concentration was estimated using the protocol of Bradford (1976), with the Bio-Rad protein assay reagent (Bio-Rad) and bovine serum albumin as a standard. Vertical SDS-PAGE was done according to Laemmli (1970) using a minigel system (Hoefer Scientific Instruments) and 12% (w/v) acrylamide/0.1% (w/v) SDS gel (0.75 mm) gels.


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for use with a robotic digestion system (MassPREP; Micromass). MALDI-TOF-MS measurements were carried out on a UltraflexTOF/TOF (Bruker Daltonics). Samples were handled as described by Hwang et al. (2006). The resulting peptide mass fingerprints were compared to a local copy of the database Swissprot and TrEMBL using MASCOT (Perkins et al., 1999) search software. The parameters used in the search were as follows: peptide mass tolerance 50 ppm, one missed cleavage, carbamidomethyl Cys, Met oxidation, and N-terminal acetylation.

**Isolation of Arabidopsis cDNAs Coding for Putative Xylose Kinases**

Total RNA was isolated from seedlings (0.8 g) according to the protocol described by Chomczynski and Sacchi (1987). The final RNA pellet was dissolved in DEPC-treated water (100 μL). mRNAs were purified using the Dynabeads mRNA kit (Dynal A.S.) according to the manufacturer’s protocol. Double-strand cDNA synthesis was carried out using 2 μg of mRNA, 1 μg of the oligonucleotide (dT)20, and the SuperScript system for cDNA synthesis (Life Technologies S.A.R.L.) according to the manufacturer’s instructions. PCR amplification (50 μL) was achieved using one-twentieth of double-strand cDNA, 0.2 μL of the specific forward primer (AtXk1F, His6-AtXk1F, or His6-AtXk2F), the reverse primer (AtXk1R or Atlas2R), and the 2 x High Fidelity PCR Master system (Roche Diagnostics). The sequences of the primers are listed in Table I. The reaction was set up for 30 cycles with an annealing temperature of 45°C, an elongation temperature of 72°C for 45 s, and a denaturation temperature of 94°C for 45 s. The PCR products were purified using the QIAEX II kit (Qiagen), cloned into pCMT (Promega), and sequenced from the 5’ end of the cDNA under the conditions and with the reagents for PCR sequencing (T3 dye primers; Applied Biosystems).

**D-DXK and D-XK Enzyme Assays**

The kinetic parameters (Vmax, Km) of XK using either t-xylulose or DX as substrate were determined using a coupled spectrophotometric assay. The assays (500 μL) were carried out at 37°C in the assay buffer (Tris-HCl buffer, pH 7.5 [50 mM], containing MgCl2, 5 mM, KCl [10 mM], and DTE [2 mM]). Initial concentrations of ATP, phosphoenolpyruvate, and NADH were 2.5, 0.6, and 0.25 mM, respectively. The concentration of t-xylulose was increased from 0.15 to 0.8 mM for the E. coli enzyme and from 0.03 to 0.2 mM for the Arabidopsis enzyme. The concentration of DX was increased from 0.4 to 2 mM for the E. coli enzyme and from 0.3 to 1.2 mM for the Arabidopsis enzyme. After addition of pyruvate kinase (8 U) and lactate dehydrogenase (8 U), the mixture was preincubated for 5 min to follow unspecific formation of ADP. XK reaction was stopped by adding 100 μL of 10% trichloroacetic acid. The resulting peptide mass fingerprints were compared to a local copy of the

**Functional Complementation in E. coli Knockout Mutants**

The xylB gene is part of an operon (xylAB operon: 3.725,940-3.728,922 min) containing two genes: xylA encoding Xyl isomerase, an enzyme catalyzing the conversion of d-Xyl into t-xylulose, and xylB, respectively (http://regulodb.ccg.unam.mx/index.html). To generate an E. coli strain deleted in d-Xyl, xylB was replaced by the chloramphenicol resistance cassette. For this, the cassette was amplified by pLYsE (Novagen) using CAT1 and CAT4 as primer sets (Table I). The cassette was cloned into pGEMT (Promega), sequenced, and bacteria transformed with these plasmids were allowed to grow on selective LB medium. The E. coli DY329 strain was engineered as described by Yu et al. (2000) using the primers sets XCKATF and XCKATR, listed in Table I. Deletion of the gene was examined by isolation of genomic DNA and PCR amplification with XF and XR. It was verified that xylB (coding for Xyl isomerase), the first enzyme constituting the operon, remained functional. Total protein extracts isolated from the strain were subjected to SDS-PAGE. Functional complementation was determined as follows: bacterial transformants were allowed to grow on selective LB medium. The E. coli strain was streaked onto an LB plate containing the antibiotic and the selective markers. The colonies were transferred to a plate containing the antibiotic but not the selective markers, and the susceptibility to the antibiotic was determined.

**Transcript Expression of GFP Fusion Proteins**

At-xk1 and At-xk2 were 3’ and 5’ fused to GFP using the primers listed in Table I and transformed into tobacco BY-2 or xanthi cells. The open reading frame corresponding to the first 43 amino acids of Aoxk-1 was modified by PCR using PT-Aoxk1GFP-F and PT-Aoxk1GFP-R (Table I) as primers and cDNAs as template as described in the preceding section. The amplified fragment was digested with NcoI and cloned in-frame into the NcoI site of the GFP expression vector (pGFP; Hemmerlin et al., 2006) to yield a fusion of the putative transmembrane peptide with the N terminus of GFP (pIpGFP). Two further constructs were made to obtain an N-terminal fusion protein. For this, the primer sets TcAoxk1GFPNcoI/TcAoxk1GFPNcoI and TcAoxk2GFPNcoI/TcAoxk2GFPNcoI were cloned into pRFP (Hemmerlin et al., 2006).

The resulting plasmids were named pTcAoxk1-GFP and pTcAoxk2-GFP. Finally, in the same way, constructs containing inserts coding for C-terminal GFP fusion proteins were made. The primer set TcAoxk1GFPFSlac/TcAoxk1GFPPRBsl, TcAoxk1GFPFSlac/TcAoxk1GFPMutRxBsl, or TcAoxk2GFPFSlac/TcAoxk2GFPMutRxBsl was utilized to amplify a DNA fragment used to clone Atxk1-tr in-frame at the 3’ site of GFP, after digestion, into the SacI and XbaI sites of pGFP. The resulting plasmids were named pGFP-TcAtxk1, pGFP-TcAtxk1-MutSGI, and pGFP-Atxk2. To obtain the plasmid pTPS-RFP, the chloroplast-specific transit peptide PTS (Hemmerlin et al., 2003b) was digested with NcoI and cloned into pRFP (Hemmerlin et al., 2006). Tobacco suspension cells were used for transformation by tungsten particle shot loading with an inflow gun containing the different plasmids constructs as indicated. After incubation (5-20 h at 23°C), transformed cells were examined using a Zeiss LSM510 confocal laser scanning microscope equipped with an inverted Zeiss Axiosvert 100M microscope and a 63 x 1.2 numerical aperture water immersion objective. GFP was excited at 488 nm using an argon laser and emission spectra were recorded from 505 nm to 530 nm. Chlorophyll as well as RFP were excited at 543 nm using a helium laser and emission was recorded at wavelength higher than 585 nm. Images were handled with the Zeiss LSM Image Browser Version 2.50,0929 software and exported as TIFF files before processed for printing using Photoshop 5.0 (Adobe Systems).

**Cloning of Kinase Genes into the Prokaryotic pET3-a Expression Vector, Protein Overexpression**, and Purification

The genes encoding putative XK from Arabidopsis were digested with the endonucleases Ndel and BamHI, for which restriction sites were introduced by PCR amplification using the primers listed in Table I, then ligated into the corresponding cloning sites of the prokaryotic expression vector pET3-a (Novagen) prepared by the same way. This allowed us to obtain the plasmids designated as pET-(His)6-Aoxk-1 and pET-(His)6-Aoxk-2. The E. coli gene cloned into pET3-a expression vector was obtained as described by Trutsch et al. (2004).

E. coli BL21-DE3 or Rossetta-gami cells were transformed with pET-(His)6-Ex-XK, pET-(His)6-Aoxk-1, and pET-(His)6-Aoxk-2. Overnight cultures were 100 U diluted into LB medium and grown at 37°C until the OD600 reached 0.3, at which time IPTG was added to a final concentration of 0.5 mM. After 12 h of incubation at 23°C, the cells were harvested. Purified protein used for enzyme characterization was obtained using the Ni2+ spin columns, according to the manufacturer’s protocol. The enzymes were dialyzed overnight against Tris-HCl buffer (50 mM, pH 7.5) containing 5 mM β-mercaptoethanol. The degree of purification of the fractions was analyzed by SDS-PAGE.

The xylB gene is part of an operon (xylAB operon: 3.725,940-3.728,922 min) containing two genes: xylA encoding Xyl isomerase, an enzyme catalyzing the conversion of d-Xyl into t-xylulose, and xylB, respectively (http://regulodb.ccg.unam.mx/index.html). To generate an E. coli strain deleted in d-Xyl, xylB was replaced by the chloramphenicol resistance cassette. For this, the cassette was amplified from pLYsE (Novagen) using CAT1 and CAT4 as primer sets (Table I). The cassette was cloned into pGEMT (Promega), sequenced, and bacteria transformed with these plasmids were allowed to grow on selective LB medium. The E. coli strain was engineered as described by Yu et al. (2000) using the primers sets XCKATF and XCKATR, listed in Table I. Deletion of the gene was examined by isolation of genomic DNA and PCR amplification with XF and XR. It was verified that xylB (coding for Xyl isomerase), the first enzyme constituting the operon, remained functional. Total protein extracts isolated from the strain were subjected to SDS-PAGE.
Characterization of the At-\(\text{atk-}x\)-1 and At-\(\text{atk-}x\)-2 Alleles

Both Arabidopsis knockout mutants were generated at the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu; Alonso et al., 2003). Attk-1 (SALK_094661, N542149) and the Attk-2 knockout mutants (SALK_079018, N519643) were obtained from the Nottingham Arabidopsis Stock Centre (http://Arabidopsis.info/). Using the primer sets SA1/AA1 and SA1/LIB1, homozygous plants Attk-1:T-DNA were selected in the same way, using the SA2/AA2 and SA2/LIB1 primer pairs, Attk-2:T-DNA plants were selected. PCR amplifications were performed with Taq polymerase (Invitrogen) according to the manufacturer’s instructions, using 250 pM of each sense (S) and antisense (A) primer. An initial denaturation step for 3 min at 94°C was followed by 30 cycles of 45 s at 94°C, 45 s at 52°C, and 45 s at 72°C. For RT-PCR experiments, 1 μg of total RNA was extracted with TRizol reagent (Invitrogen), DNase I treated, and finally reverse transcribed by the aid of SuperScript II (Invitrogen) using 500 ng of oligo(dt). A fraction (about one-twentieth) of the first-strand cDNAs was used as a template for PCR with gene-specific primers (in a volume of 20 μl with 1 unit of Taq polymerase, 250 μM each dNTP, and 250 μM of each primer [RTAAK1F/RTAAK1R or RTAAK2F/RTAAK2R]). An initial denaturation step for 3 min at 95°C was followed by 35 cycles of 45 s at 95°C, 45 s at 55°C, and 1 min at 72°C. Amplification of actin 2 (At3g18780) cDNA using primers Act2F2 and Act2R was followed by 35 cycles of 45 s at 95

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