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Research article

Identification of a phosphorylation site in cyclobutane pyrimidine dimer photolyase of rice

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ABSTRACT

Cyclobutane pyrimidine dimer (CPD) photolyase monomerises ultraviolet (UV) radiation-induced CPDs present in DNA, using energy from UVA and visible light. In plants, CPD photolyase activity is a crucial factor for determining UVB sensitivity. We previously demonstrated that native rice CPD photolyase is phosphorylated. To determine the phosphorylation site(s), the phosphorylation status of CPD photolyase was analyzed in rice varieties that have amino acid alterations at the potential phosphorylation sites. In wild-rice species, CPD photolyase was phosphorylated. In Poaceae species, CPD photolyase was phosphorylated in wheat but not in maize. Mutant CPD photolyase proteins, in which these putative phosphorylated residues were replaced with alanine residues, were synthesized using an insect cell-free translation system. A slow-migrating band disappeared when the serine residue at position 7 was mutated. A phospho-specific antibody was generated to determine whether this residue is phosphorylated in CPD photolyase. Only the slow-migrating band of native rice CPD photolyase was detected using this antibody, indicating that the serine residue at position 7 is a phosphorylation site in native rice CPD photolyase.

1. Introduction

Ultraviolet (UV) radiation causes DNA damage, characterised by the production of cyclobutane pyrimidine dimers (CPDs) and pyrimidine–pyrimidone (6-4) photoproducts [(6-4) photoproducts], which are formed between adjacent pyrimidines on the same strand [1]. CPDs constitute the majority of DNA damage caused by UV radiation (approximately 75%), and (6-4) photoproducts account for the remainder [2]. Such damage can be lethal or mutagenic to organisms and can impede DNA replication and transcription [3,4]. In rice plants, CPDs are a major cause of UVB-induced growth inhibition [5]. Plants possess mechanisms to cope with UV-induced DNA damage, including photoreactivation (photorepair) and nucleotide excision repair (also referred to as dark repair). In plants, photorepair is mediated by the enzyme photolyase, which absorbs blue/UVA light as an energy source to monomerise CPDs. Photorepair is the major pathway for counteracting UVB-induced DNA damage [6,7]. We previously reported that CPD photolyase is a crucial factor for determining the sensitivity of rice to UVB [8–11].

CPD photolyase is widely distributed in all three kingdoms (archaea, eubacteria and eukaryia), but it is absent from *Bacillus subtilis*, *Schizosaccharomyces pombe* and placental mammals [12]. There are two classes of CPD photolyases that differ in their amino acid sequences [13]; class I is expressed in microorganisms and class II is expressed in higher organisms. Plant CPD photolyase belongs to class II. In contrast to class I, several features of class II CPD photolyase are not fully understood, including its mechanism(s) of regulation. We previously demonstrated that purified native CPD photolyase from rice leaves is phosphorylated [14]. It is unknown whether CPD photolyases are phosphorylated in other organisms.

Phosphorylation controls the functions of many proteins, and many signalling cascades are affected by protein phosphorylation. We previously reported that CPD photolyase is localized to chloroplasts, mitochondria and nuclei, and that this protein efficiently repairs UVB-induced CPDs in all DNA-containing organelles in rice plants [15]. Furthermore, we investigated the phosphorylation status of CPD photolyase within each of these organelles. Mitochondria contain a high proportion of unphosphorylated CPD photolyase, whereas nuclei and chloroplasts have a relatively high proportion of the phosphorylated form [15]. These results suggest the translocation of CPD photolyase to particular organelles may be regulated by its phosphorylation status. However, the effects of phosphorylation on the biochemical and physiological functions of

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CPD photolyase remain unclear. Identification of the phosphorylation site(s) of CPD photolyase may help reveal whether the structure of the protein is modulated by phosphorylation and allow the phosphorylation-mediated signalling cascade to be dissected. In this study, we attempted to identify the phosphorylation site(s) of rice CPD photolyase.

2. Results

2.1. Phosphorylation of CPD photolyase in wild-rice species

Mutation analysis is a useful technique for determining protein phosphorylation site(s). Amino acid residues that can be phosphorylated include serine (S), tyrosine (Y) and threonine (T). There are several potential phosphorylation sites in CPD photolyase of the *japonica* rice cultivar Sasanishiki, which has 32 serine residues, 17 tyrosine residues and 14 threonine residues. Therefore, we first examined natural rice varieties that express CPD photolyase with amino acid alterations at some of these potential phosphorylation sites.

CPD photolyase in wild-rice species has a high degree of genetic variability [11]. CPD photolyase from Sasanishiki is phosphorylated [14]. When the 506 bp full-length amino acid sequence of CPD photolyase from Sasanishiki was compared with that of the wild-rice W1299 strain (*Oryza meridionalis*), ten amino acid differences were found (F114L, Q126R, I250M, D258H, F260Y, C305R, V313I, Q367H, Y434F and P495L) [11]. Among these amino acid residues, tyrosine residue at position 434 is a potentially phosphorylated amino acid residue. A comparison of the amino acid sequence of CPD photolyase from the W1626 strain (*O. meridionalis*) with the amino acid sequence of that from the Sasanishiki strain revealed three amino acid alterations (P13S, Q126R and T416P) [11] and one potentially phosphorylated amino acid residue at position 416 in W1626.

To determine whether CPD photolyase in W1299 or W1629 is phosphorylated, we purified CPD photolyase from the leaves of each strain. Purified CPD photolyase was subjected to SDS-PAGE and western-blot analysis using an anti-rice CPD photolyase antiserum. We previously showed that phosphorylated rice CPD photolyase migrates at 56 kDa (i.e., the slow-migrating band), whereas unphosphorylated rice CPD photolyase migrates at 54 kDa [14]. The 54 kDa and 56 kDa bands were detected in CPD photolyase purified from both W1299 and W1629 (Fig. 1), indicating that CPD photolyase is phosphorylated in these strains, but not at the residues that differ between these strains and Sasanishiki, including tyrosine at position 434 and threonine at position 416.

2.2. Phosphorylation of CPD photolyase of Poaceae species

Next, we analyzed the amino acid sequences of CPD photolyase in various related Poaceae species. The sequences of CPD photolyase in rice (*Oryza sativa* L.), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and maize (*Zea mays*) are known. (GenBank/EMBL/DDBJ accession numbers AB096003, AK330529, AK372010 and BT033850, respectively). When these sequences were aligned, 41 potential phosphorylation sites were found to be conserved, including 17 serine residues, 17 tyrosine residues and seven threonine residues (Fig. 2). To determine whether CPD photolyase is phosphorylated in each of these Poaceae species, we purified CPD photolyase from rice, wheat, barley and maize leaves.

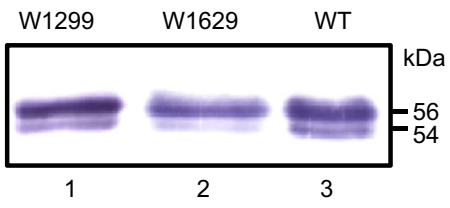

Fig. 1. Western-blot analysis of CPD photolyase purified from leaves of wild-rice species. The purified CPD photolyase was subjected to SDS-PAGE, followed by western-blot analysis using anti-rice CPD photolyase antiserum. Lane 1, W1299; lane 2, W1629; lane 3, Sasanishiki cultivar.

When we treated CPD photolyase purified from rice leaves with lambda protein phosphatase (λ-PPase) and performed SDS-PAGE and western-blot analysis using an anti-rice CPD photolyase antiserum, the slow-migrating band disappeared (Fig. 3A, lane 2). This did not occur when the protein was treated with heat-inactivated λ-PPase (Fig. 3A, lane 3). The slow-migrating band was also detected in purified native wheat CPD photolyase (Fig. 3B, lane 1). This band disappeared when the protein was treated with λ-PPase (Fig. 3B, lane 2), indicating that native wheat CPD photolyase is phosphorylated. In contrast, in barley, which belongs to the same subfamily as wheat, the slow-migrating band was not detected (Fig. 3C, lane 1). The slow-migrating band was also not detected in purified native maize CPD photolyase (Fig. 3D, lane 1). These results indicate that CPD photolyase may not be phosphorylated in barley and maize. Consequently, the phosphorylation sites of CPD photolyase are thought to be located among amino acid residues that are only conserved between rice and wheat, but not among those conserved between maize or barley.

2.3. Mutation analysis of predicted phosphorylation sites

One potential phosphorylation site, serine at position 5, is conserved only between rice and wheat, and is absent from maize and barley. To confirm whether this site is phosphorylated, we performed mutation analysis and employed an in vitro expression system to generate various mutated proteins. CPD photolyase expressed in *E. coli* is not phosphorylated [14] and thus *E. coli* cannot be used to generate recombinant proteins suitable for this study. Instead, we used an insect cell-free translation system, which possesses eukaryotic post-translational modification machinery. The slow-migrating band was detected when rice CPD photolyase was synthesized using the insect cell-free translation system (Fig. 4A, lane 1), and it disappeared when the protein was treated with λ-PPase (Fig. 4A, lane 2). These results indicate that rice CPD photolyase synthesized using an insect cell-free translation system is phosphorylated. Site-directed mutagenesis was next used to change the serine residue at position 5 of rice CPD photolyase to an alanine residue, and the mutated protein was synthesized using the insect cell-free translation system. However, when this protein was analyzed by SDS-PAGE and western-blot analysis, the slow-migrating band was still detected (Fig. 4B, lane 2), indicating that this serine residue is not phosphorylated and that phosphorylation of another residue(s) is responsible for the slow-migrating band.

Next, we studied other potential phosphorylation sites in rice CPD photolyase. Although wheat and barley belong to the same subfamily, and their CPD photolyases share 96.4% amino acid sequence homology, the slow-migrating band was not detected in native barley CPD photolyase (Fig. 3C). We thought it is possible that CPD photolyase was phosphorylated in barley but this form was not recognized by the anti-rice CPD photolyase antiserum. Therefore, we focused on potential phosphorylation sites that are conserved between rice, wheat, and barley. The potentially phosphorylated amino acid residues in rice CPD photolyase at positions 7, 84, 115, 267, 494 and 504 were also found in wheat and barley but not in maize. We constructed plasmids carrying genes encoding rice CPD photolyase in which these residues were replaced with