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The tomato DDI2, a PCNA ortholog, associating with DDB1-CUL4 complex is required for UV-damaged DNA repair and plant tolerance to UV stress

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ABSTRACT

CULLIN 4 (CUL4)-DAMAGED DNA binding protein 1 (DDB1)-based ubiquitin E3 ligase modulates diverse cellular processes including repair of damaged genomic DNA. In this study, an uncharacterized gene termed as DDB1-Interacting protein 2 (DDI2) was identified in yeast two-hybrid screening with bait gene *DDB1*. The co-immunoprecipitation (co-IP) assays further demonstrated that DDI2 is associated with tomato DDB1-CUL4 complex *in vivo*. It appears that DDI2 encodes an ortholog of proliferating cell nuclear antigen (PCNA). Confocal microscope observation indicated that DDI2-GFP fusion protein was localized in nuclei. The expression of *DDI2* gene is constitutive but substantially enhanced by UV-C irradiation. The transgenic tomato plants with overexpression or knockdown of *DDI2* gene displayed the increased or decreased tolerance, respectively, to UV-C stress and chemical mutagen cisplatin. The quantitative analysis of UV-induced DNA lesions indicated that the dark repair of DNA damage was accelerated in *DDI2* overexpression lines but delayed in knockdown lines. Conclusively, tomato *DDI2* gene is required for UV-induced DNA damage repair and plant tolerance to UV stress. In addition, fruits of *DDI2* transgenic plants are indistinguishable from that of wild type, regarding fresh weight and nutrient quality. Therefore, overexpression of *DDI2* offers a suitable strategy for genetic manipulation of enhancing plant tolerance to UV stress.

1. Introduction

The DNA of all creatures is subjected to damage by environmental and chemical agents including ultraviolet (UV) light; ionizing radiation; and chemical mutagens [1]. As sessile organisms, plants are inevitably affected by UV radiation and other environmental stresses. UV light can penetrate cells and cause DNA damage; in particular cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts [(6–4)PPs], that blocks or renders inaccurate replication and transcription; and leads to the mutagenic consequences [2]. For surviving, plants have evolved a number of adaptive strategies to overcome the stresses; such as physiological adaptation like biosynthesis of UV-absorbing compounds [3]; photoreactivation [1]; nucleotide excision repair (NER) [4] and translesion synthesis (TLS) [5]. In light, plants primarily use photoreactivation to repair UV-induced DNA lesions. Without light, plants can also exert the mechanism of nucleotide excision repair (NER) and translesion synthesis (TLS) to remove or bypass DNA damage independently from light energy. This process is commonly termed as dark repair [6]. The NER mechanism is employed in two pathways called transcriptional coupled repair (TC-NER) and global genome repair (GG-NER) [7,8]. As the first step of GG-NER, the recognition of DNA lesions is mediated by DDB1-DDB2 complex.

Abbreviations: CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; NER, nucleotide excision repair; OE, overexpression; ORF, open reading frame; UV, ultraviolet ray; WT, wild-type.

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Damaged DNA binding protein 1 (DDB1) and DDB2 were initially isolated as a heterodimeric protein complex which binds UV-induced DNA lesions [9]. Recent investigations indicated that DDB1 was assembled with CULLIN4 (CUL4) to form a DDB1-CUL4-based E3 ubiquitin ligase (CRL4) complex [10]. CRL4-mediated ubiquitination and degradation of DDB2 is indispensible for recruiting NER factor XPC to UV-damaged DNA lesions and subsequent DNA repair [11]. In plant, CRL4 ligase participates in various developmental processes such as photomorphogenesis [12] and plastid division [13], and in the response to biotic [14] and abiotic stresses [15]. Also, plant $CRL4^{DDB2}$ E3 ligase is a key factor in DNA damage response and DNA repair [16] and in maintaining the genome integrity upon UV stress, as demonstrated by the facts that the genetic mutants of *CUL4*, *DDB1a*, and *DDB2* in *Arabidopsis* exhibited hypersensitivity to UV-C stress [7,8].

Considering that DDB1 serves as the adaptor of ubiquitin ligase CRL4 and is bound by substrate proteins, we performed yeast-two-hybrid screening with bait gene DDB1 to uncover the potential DDB1-CUL4-associated factors and target proteins. Our previous work characterized the DDB1-Interacting protein 1 (DDI1) which interacts with CUL4-DDB1 complex and plays a role in plant response to abiotic stresses including UV irradiation [17]. In this study, we identified another DDB1-interacting protein (termed as DDI2). Co-IP assays further confirmed that DDI2 is associated with DDB1-CUL4 complex *in vivo*. Genetic manipulation of *DDI2* gene affected UV-induced DNA damage repair and plant tolerance to UV-C stress.

2. Materials and methods

2.1. Plant growth conditions

Tomato plants were germinated and grown in the greenhouse under standard conditions (26 °C day, 18 °C night; 16-h light:8-h dark). Primary transformed plants (T_0) and transgenic generation 1 (T_1) plants were sown in the greenhouse and transplanted into the field 35 days later. T2 homozygous transgenic seeds and controls were germinated on 1/2MS medium plates under white light (16-h light:8-h dark) or continuous darkness at 25 °C to ensure that expression of the *DDI2* gene was induced by UV-C. Two-week-old seedlings were radiated with 600 J/m^2 UV-C and then whole seedlings were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. Yeast two-hybrid screening

The LexA-based Y2H screening was conducted to identify proteins interacting with DDB1. The LexA-DDB1 bait was transformed into the yeast EGY48 stain containing a report plasmid pSH18-34. The expression of the LexA-DDB1 fusion protein was verified and no auto-activation was detected. A tomato cDNA library generated in the prey vector pJG4-5 was further introduced to screen DDB1-interacting proteins. The protein–protein interaction was determined on yeast medium containing X-gal or without leucine supply. Primers used in Y2H cloning are listed in following table.

2.3. Co-immunoprecipitation analysis

The C-terminal HA-tagged and FLAG-tagged constructs were generated by PCR amplification of tomato DDI2, DDB1 or CUL4 cDNA and consequently cloned into the binary vector pBTEX. The constructed vectors are introduced into the *Agrobacterium tumefaciens* GV2260. The agrobacterial infiltration into *Nicotiana benthamiana* leaves was performed at OD_{600} = 0.4. The infiltrated leaf tissues were collected at 36 h after infiltration and used for co-immunoprecipitation assay as described previously [18].

2.4. Plasmid construction, tomato transformation and transgenic analysis

DNA manipulations were carried out by using standard procedures (Sambrook and Russell, 2001).

Tomato *DDI2* cDNA (Genome locus number *Soly*06g076660.2.1) was amplified by RT-PCR for construction of the 35S-DDI2-RNAi and 35S-DDI2 over-expression vectors. An inverted-repeat fragment was constructed in vector pSKint (a chemical-regulated inducible RNAi system in plants) and transferred into pBI121 at the *Bam*HI and *Sac*I restriction sites by PCR using primers DDI2Ri-ZF (5′-CTCGAGGGATCCCGCCACTGGATTCTCTCTG-3′), DDI2Ri-ZR (5′-AAGCTTTATAACAGTGGCTTCTTCAG-3′), DDI2Ri-FF (5′-GAGCTCCG-CCACTGGATTCTCTCTG-3′), DDI2Ri-FR (5′-GAATTCTATAACAGT-GGCTTCTTCAG-3′), to introduce unique restriction sites at the product ends. The complete open reading frame (ORF) of DDI2 was amplified from cDNAs by using PCR primers DDI2-OE-F (5′-GGATCCATGTTGGAACTACGTCTTGTTCA-3′) and DDI2-OE-R (5′-GAGCTCTCAAGGCTTGGTTTCCTCTT-3′). These fragments were then inserted into the PBI121 vector (driven by the 35S promoter) at the *Bam*HI and *Sac*I restriction enzyme sites. The direction of insertion was confirmed by sequencing. The resulting constructs were called CaMV35S-DDI2-RNAi and DDI2 over-expression (DDI2-OE).

Transgenic plants were generated by *A. tumefaciens*-mediated transformation according to the method described by Fillatti et al. [19]. Transformed lines were first selected for kanamycin (70 mg/L) resistance and then analyzed by PCR to determine the presence of T-DNA. Primers were designed for the NPTII (Kana) marker of pBI121 and used to confirm integration: 5′-GGCAATTACCTTATCCGCAA-3′ and 5′-AGAACTCGTCAAGAAGGCGA-3′.

2.5. Molecular analysis

Total RNA was extracted from roots, stems, leaves, flowers and fruits at various developmental stages using the RNA prep Pure Plant Kit (TIANGEN BIOTECH, DP432). The semi-quantitative RT-PCR used the internal reference *SlUBI3* (GenBank accession number X58253) to co-amplify with the target gene in every PCR reaction. Forward and reverse primers for RT-PCR analysis were designed for *UBI3* (forward, 5′-AGAAGAAGACCTACACCAAGCC-3′, reverse, 5′-TCC CAAGGGTTGTCACATACATC-3′); *DDI2* (forward,

Primer	Sequence	Purpose
DDB1F *Eco*RI	5′ GATGAATTCATGAGTGTATGGAACTAC-3′	Forward primer for generating pEG202::DDB1 construct
DDB1R *Xho*I	5′-GTCCTCGAGCTAATGCAACCTTGTCAAC-3′	Reverse primer for generating pEG202::DDB1 construct
CUL4F *Eco*RI	5′-CAGAATTCATGAAGAAAGCTAAGTCAC-3′	Forward primer for generating pJG4-5::CUL4 construct
CUL4R *Xho*I	5′-ATTCTCGAGCTAAGCAAGGTAGTTGTATA-3′	Reverse primer for generating pJG4-5::CUL4 construct
DDI2F *Eco*RI	5′-GAATTCATGTTGGAACTACGTCTTGTTCA-3′	Forward primer for generating pJG4-5::DDI2construct
DDI2R *Xho*I	5′-CTCGAGTTAACTTCTCCTGGATTTAT-3′	Reverse primer for generating pJG4-5::DDI2 construct
DDI2F *Kpn*I	5′-GGTACCATGTTGGAACTACGTCTTGTTCA-3′	Forward primer for generating pBTEX::DDI2-FLAG,
DDI2R *Stu*I	5′-AGGCCTTTAACTTCTCCTGGATTTAT-3′	Reverse primer for generating pBTEX::DDI2-FLAG,
CUL4F *Bam*HI	5′-CAGGATCCATGAAGAAAGCTAAGTCAC-3′	Forward primer for generating pCB302::CUL4-HA construct
CUL4R *Xba*I	5′-ATTTCTAGAAGCAAGGTAGTTGTATA-3′	Reverse primer for generating pCB302::CUL4-HA construct
DDB1F *Kpn*I	5′-GTCGGTACCATGAGTGTATGGAACTAC-3′	Forward primer for generating pBTEX::DDB1-HA/FLAGconstructs
DDB1R *Stu*I	5′-GAAAGGCCTATGCAACCTTGTCAACTC-3′	Reverse primer for generating pBTEX::DDB1-HA construct