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CENTRIN2 interacts with the Arabidopsis homolog of the human XPC protein (AtRAD4) and contributes to efficient synthesis-dependent repair of bulky DNA lesions

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Abstract

Arabidopsis thaliana CENTRIN2 (AtCEN2) has been shown to modulate Nucleotide Excision Repair (NER) and Homologous Recombination (HR). The present study provides evidence that AtCEN2 interacts with the Arabidopsis homolog of human XPC, AtRAD4 and that the distal EF-hand Ca^{2+} binding domain is essential for this interaction. In addition, the synthesis-dependent repair efficiency of bulky DNA lesions was enhanced in cell extracts prepared from Arabidopsis plants overexpressing the full length AtCEN2 but not in those overexpressing a truncated AtCEN2 form, suggesting a role for the distal EF-hand Ca^{2+} binding domain in the early step of the NER process. Upon UV-C treatment the AtCEN2 protein was shown to be increased in concentration and to be localised in the nucleus rapidly. Taken together these data suggest that AtCEN2 is a part of the AtRAD4 recognition complex and that this interaction is required for efficient NER. In addition, NER and HR appear to be differentially modulated upon exposure of plants to DNA damaging agents. This suggests in plants, that processing of bulky DNA lesions highly depends on the excision repair efficiency, especially the recognition step, thus influencing the recombinational repair pathway.

Abbreviations: BER, Base Excision Repair; DSB, Double Strand Break; GFP, Green Fluorescent Protein; HR, Homologous Recombination; ICL, Inter/Intra Crosslink; NHEJ, Non Homologous End Joining; NER, Nucleotide Excision Repair; MMR, Mismatch Repair; XP, Xeroderma Pigmentosum

Introduction

The Centrin protein family is highly conserved from yeast to humans and is closely related to the EF-hand Ca^{2+}-binding domain calmodulin family (Salisbury, 1995). AtCEN2 is very similar to the HsCEN2 protein with 55.8% identity and 71.2% similarity within their EF-hand domains (Molinier *et al.*, 2004). At*CEN2* defective Arabidopsis plants are hypersensitive to UV-C and exhibit a reduced efficiency of DNA synthesis-dependent repair of UV induced DNA damage (Molinier *et al.*, 2004). This repair defect is accompanied by enhanced levels of somatic homologous recombination (HR). These observations led to the proposal that AtCEN2 is involved in the NER process and that

a novel interconnection between an early step of NER and HR exists in Arabidopsis (Molinier *et al*., 2004).

Nucleotide Excision Repair (NER) machinery recognises a wide range of bulky DNA lesions including damage formed after UV irradiation and treatment with anti-cancer drugs such as Cisplatin and Mitomycin C (for review Costa *et al*., 2003). The NER process consists of 5 steps: the recognition of the DNA damage, the opening of the pre-incision complex, the dual incision of the lesion, the DNA synthesis followed by the ligation of the newly synthetised DNA strand.

In animal models, defects in NER have been linked to genetic disorders, such as Xeroderma Pigmentosum (XP). Seven XP-genes (XPA-G) with distinct functions in repair have been identified (de Laat *et al*., 1999). A complex consisting of XPC/hHRad23B (XPC and human homolog of Rad23) has been documented to be of critical importance for recognition of DNA adducts (Hey *et al*., 2002). Purification of the XPC complex from Hela cells revealed the existence of a third component, CENTRIN2 (HsCEN2), a Ca^{2+}-binding protein with four EF-hand domains (Araki *et al*., 2001). The human CEN2 (HsCEN2) and XPC proteins have been shown to directly interact and it has been suggested that HsCEN2 may stabilise the XPC/hHRad23B complex. Recently Nishi *et al*. (2005) have shown that HsCEN2 stimulates NER by interacting with the C-terminal region of XPC.

DNA double-strand break (DSB) can also occur in the cell. HR is the major DNA DSB repair process in yeast, whereas non-homologous-end joining (NHEJ) is preferentially used in higher eukaryotes for repairing DSBs (Puchta and Hohn, 1996; Vonarx *et al*., 1998; Britt, 1999; Elliott and Jasin, 2001). The level of HR-dependent repair is affected by defects in NER, as well as in Base Excision Repair (BER) and Mismatch Repair (MMR) in all analysed eukaryotic organisms suggesting the existence of interconnections between these different repair pathways (Liang *et al*., 1998; Paques and Haber, 1999; Ries *et al*., 2000; Elliott and Jasin, 2001; Gherbi *et al*., 2001; Hendricks *et al*., 2002; Villemure *et al*., 2003; Dubest *et al*., 2004; Molinier *et al*., 2004). NER has also been shown to be the initial step in repair of inter/intrastrand crosslinks repair (ICLs), followed by recombinational DNA repair (Cole and Sinden, 1975; Jachymczyk *et al*., 1981; De Silva *et al*., 2000; Saffran *et al*., 2004).

We used biochemical and genetic approaches to analyse the role of AtCEN2 protein in DNA repair and to test the interconnection between NER and recombinational DNA repair. We show that AtCEN2 interacts with the Arabidopsis homolog of human XPC (AtRAD4) in a Ca^{2+} dependent manner and that the distal EF-hand domain of AtCEN2 is required for this interaction. Polyclonal antibodies raised against recombinant AtCEN2 were used to show that the AtCEN2 protein concentration increased rapidly upon UV-C exposure. Following UV-C treatment a functional AtCEN2–GFP fusion protein localised rapidly in the nucleus. The efficiency of DNA synthesis-dependent repair of UV-C and Cisplatin induced DNA damage, known to be predominantly repaired by the NER pathway, was enhanced in Arabidopsis plants overexpressing the full length AtCEN2 as compared to wild type. In contrast, plants overexpressing a truncated AtCEN2 form did not show any differences in synthesis-dependent repair compared to the control. The rate of recombinational DNA repair was found to be low in plants overexpressing the full-length AtCEN2 after treatments with UV-C and Cisplatin whereas HR frequency (HRF) was strongly stimulated in control and plants overexpressing a truncated AtCEN2 form. We therefore suggest that bulky DNA lesions are processed in a different way depending on the efficiency of the NER pathway.

Materials and methods

Protein purification, antibodies and western blotting

The cDNAs of full length AtCEN2 (At4g37010), truncated AtCEN2-C (nucleotides 1 to 432) or AtCEN2-B (nucleotides 1 to 324) were cloned into pGEX3X plasmid (Amersham Biosciences, England) in order to produce GST protein fusions. These fusion proteins were expressed in *E. coli* strain Bl21 and purified on glutathione-sepharose 4B (Amersham Biosciences, England) according to manufacturerÕs instruction. Proteins CEN2, CEN2-C, and CEN2-B were cleaved from GST by factor Xa (Amersham Biosciences, England) and the protein purity was analysed by SDS-PAGE.

AtCEN2 was used to raise polyclonal antibody in rabbit (α-CEN; commercially made by Eurogentec, Belgium). For western blot analysis, plants were ground in liquid nitrogen, Laemmli SDS sample buffer (10% glycerol, 2% SDS, 60 mM Tris–HCl pH 6.8, 2% β-mercaptoethanol, and 0.005% bromophenol blue) was added, vigorously mixed for 30–60 s and incubated for 8 min at 95 °C. Cell debris were removed by centrifugation in a benchtop centrifuge and the supernatant was used for protein analysis. Ten μg of proteins were loaded onto 15% acrylamide-containing SDS gel, and proteins were fractionated by electrophoresis. Proteins were then transferred to nitrocellulose membrane. A dilution of 1:1000 of the α-CEN polyclonal rabbit antibody was used for the western blot. The ECL detection kit was used for chemiluminescence detection (Amersham Biosciences, England).

In vitro *translation and protein–protein interaction*

The cDNA of the C-terminal AtRad4 (At5g16630; last 852 nucleotides) was cloned into plasmid pPET3d. TNT T7-coupled transcription/translation system (Promega, Switzerland) and [^{35}S] Methionine (Amersham Biosciences, England) were used for the *in vitro* translation of [^{35}S]-labelled Rad4-Ct. T7 Luciferase (LUC) DNA provided by the manufacturer (Promega, Switzerland) was used as a control. [^{35}S]-labeled proteins were checked on SDS-PAGE, followed by autoradiography. To analyse protein–protein interaction *in vitro*, [^{35}S]-Rad4-Ct or [^{35}S]-LUC was incubated with GST-CEN2 or-CEN2-C or CEN2-B on glutathione-sepharose 4B in buffer A (20 mM potassium phosphate (pH 7.5), 100 mM potassium chloride, 10% glycerol, 0.01% Triton X-100, 1 mM DTT, 1 mM $CaCl_2$, and complete protease inhibitor tablet without EDTA (Roche Applied Science, Switzerland) or buffer B (buffer A without $CaCl_2$ but with 1 mM EGTA). The incubation was performed at 4 °C for 2 h. Beads were washed 3 times with individual buffers. Proteins were eluted from the beads with one volume of 2×SDS sample buffer and analysed on 12% SDS-PAGE, followed by autoradiography.

Production of transgenic plants

The pOEX4 vector (Molinier *et al*., 2004) containing the mas (mannopine synthase) promoter was used to drive the expression of either the full length AtCEN2 or the truncated AtCEN2-C cDNA into Arabidopsis plants. Arabidopsis IC9 line carrying the intermolecular recombination substrate (Molinier *et al*., 2004) were used to produce AtCEN2 or AtCEN2-C overexpressing plants. T3 homozygous transgenic plants were used to monitore somatic homologous recombination frequency.

The same binary vector was used to drive the expression of a GFP-AtCEN2 protein fusion (C-terminus fusion of the E-GFP, Clontech) and used to transform WT Arabidopsis plants (ecotype Columbia). T3 homozygous transgenic plants were used for further experiments.

The pLOLA vector containing the double 35S promoter, the myc coding sequence and the nos terminator (Ferrando *et al*., 2001) was used to create the AtCEN2 C-terminus myc tagged fusion protein. The AtCEN2 cDNA was cloned between the *Nco*I–*Hin*dIII sites. In order to produced transgenic Arabidopsis plant, the double 35S promoter-coding sequence, AtCEN2 cDNA, myc sequence and terminator were subcloned into the *Kpn*I site of the pOEX6N binary vector (Fritsch, unpublished). This plasmid was mobilised into *Agrobacterium tumefaciens* and used to transform WT Arabidopsis plants (ecotype Columbia). T3 homozygous transgenic plants were used for futher experiments.

AtCEN2 knock-out and AtCEN2 RNAi Arabidopsis plants used in the present study have been described in Molinier *et al*. (2004).

DNA damage treatments and determination of somatic homologous recombination frequency (HRF)

For all experiments plants were grown *in vitro* on solid GM medium (MS elements [Duchefa, The Netherlands], 1% sucrose, 0.8% agar [Agar-agar Ultrapur, Merck], pH 5.8) in a culture chamber under a 16/8 h photoperiod (24/21 °C) during 7 days or 13 days before being subjected to the specific preculture condition established for each treatment For UV-C treatment, 7-day-old plants were transferred to large Petri dishes (160 mm diameter) containing solid GM medium, to a density of 1 plant/cm^2 and grown for an additional week. For the UV-C irradiation (254 nm, 6 Kerg/cm^2) a Mineral light-Lamp (UV-Products Inc.,

San Gabriel, USA) was used. For the Cisplatin treatment 13-day-old plants were subcultured during 24 h in 300 μl of liquid GM medium in 24 cell plates. Before treatment the culture medium was removed and Cisplatin (0, 1 and 5 μM, Sigma, Buchs, Switzerland) diluted in liquid GM medium was applied.

Somatic HRF was measured in the T3 generation of 2-week-old plants in a batch of 24 plants after treatments with either UV-C (0, 10, 30 and 50 Kerg/cm^2) or Cisplatin (0, 1 and 5 μM). We define HRF as the average number of GUS+ spots (recombination events) per plant. Experiments were duplicated.

Microscopy

Roots of vertically grown one-week old *in vitro* germinated plants were irradiated with UV-C at 10 Kergs/cm^2 using a Minerallight-Lamp (UV-Products, San Gabriel, CA) and left to recover in a growth chamber for a period of 30 min or 1 h or 2 h before imaging. For Cisplatin treatment one-week old *in vitro* germinated plants were subcultured in 500 μl of liquid GM medium during 24 h. Before treatment the culture medium was removed and Cisplatin (1 μM) diluted in liquid GM medium was applied. Plants were cultured in a growth chamber for a period of 1 h, 2 h or 4 h before imaging. Images were captured as a stacked series using Olympus Fluoview BX61 laser Scanning confocal microscope and processed using Adobe Photoshop.

In vitro *DNA repair assay*

The *in vitro* repair assay according the method described by Li *et al.* (2002) was performed on cell extracts prepared from AtCEN2, AtCEN2-C overexpressing plants and from the IC9 line as control. The pGEX and pBSK plasmids were linearised using the *Sma*I restriction enzyme and purified using the Gel extraction kit (Qiagen, Germany). The pGEX-linearised plasmid was either UV-C damaged (450 J/m^2) using the Stratalinker (Stratagene) or Cisplatin damaged (5 μM) according Li *et al.* (2002) and used as repair substrate. The non-damaged pBSK-linearised plasmid was used as internal control. Twenty-five μg of protein extracts were used per time point and mixed with 200 ng of damaged plasmid (pGEX) and 300 ng of non-damaged plasmid (pBSK). The reaction was stopped 1 or 2 h after incubation. Plasmids were purified using the Gel extraction kit (Qiagen, Germany) and separate by electrophoresis on a 0.8% agarose gel. DNA was transferred to a nylon membrane (Roche, Manheim, Germany) by capillary transfer and DIG detection procedures were performed using the DIG Nucleic Acid Detection kit (Roche, Manheim, Germany) according the manufacturer's instruction. The Quantitity One software (Biorad, France) was used to normalise the loading of the plasmid and the DIG detection signal relative to the non-damaged control plasmid. The synthesis-dependent repair efficiency from cell extracts of AtCEN2 and AtCEN2-C overexpressing plants was calculated relative to this control.

Results

Characterisation of AtCEN2 expression

In order to analyse the biological function of AtCEN2 in DNA repair, polyclonal antibodies were raised against a purified recombinant AtCEN2 protein. Anti-AtCEN2 serum recognised the purified protein whereas the pre-immune serum did not (Figure 1A). In wild type plants AtCEN2 was detectable at the expected molecular weight of 20 kDa (Figure 1B). The protein could not be detected in plants defective for AtCEN2 expression (Molinier *et al.*, 2004) proving the specificity of the anti-AtCEN2 antibody and the absence of crossreactivity with the endogenous AtCEN1 protein (Figure 1B). The antibody also reacted to epitope-tagged AtCEN2 proteins, such as AtCEN2-GFP and AtCEN2-Myc, expressed in plants (Figure 1C). It has previously been shown that the steady state level of AtCEN2 mRNA increased 2 h after UV-C treatment (Molinier *et al.*, 2004). This antiserum has now permitted us to determine whether or not the AtCEN2 protein content varies in response to DNA damage. The AtCEN2 protein level increased more than 3 fold 30 min after exposure to UV-C and remained constant up to 6 h after UV irradiation (Figure 1D). This rapid increase of AtCEN2 content may reflect an important role for this protein in the early response to UV irradiation. In contrast, in plants treated with bleomycin (BLM),

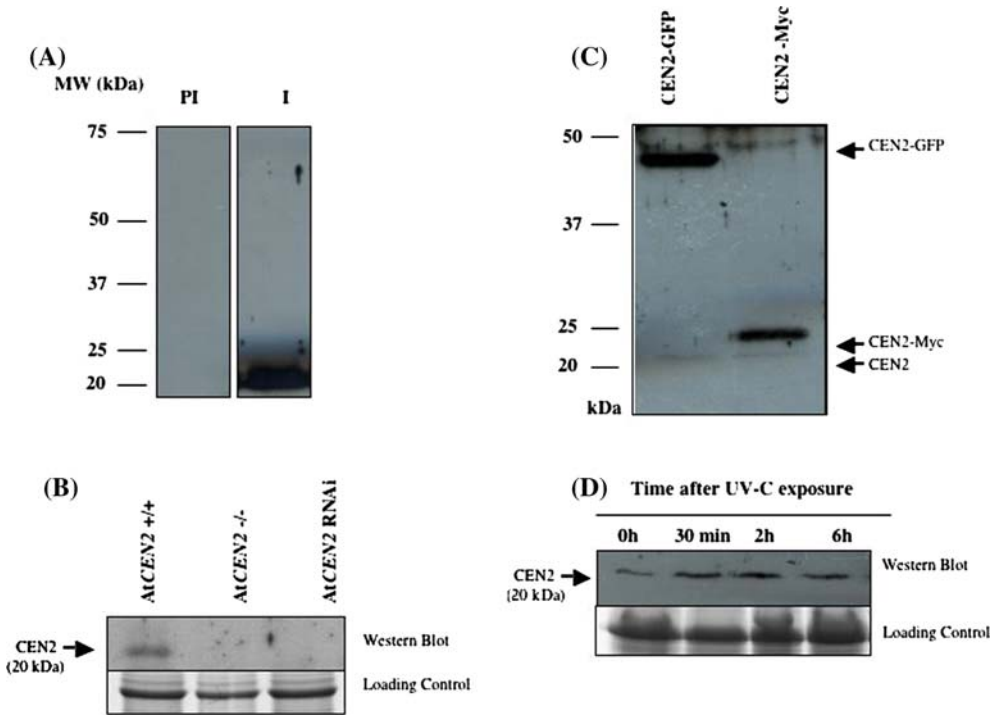

Figure 1. Characterisation of AtCEN2 expression using polyclonal anti-AtCEN2 antibodies. (A) Immunoblot analysis of the purified AtCEN2 protein using pre-immune (PI) and immune (I) sera. The immune serum is able to recognise the purified AtCEN2 protein at 20 kDa. (B) Immunoblot analysis of AtCEN2 protein of wild type (AtCEN2 +/+), knock-out (AtCEN2 −/−) and AtCEN2 RNAi plant cell extracts (See experimental procedures for details). Comassie blue staining was used as loading control. (C) Immunoblot analysis of AtCEN2 protein level in plant overexpressing either a GFP or myc tagged fusion. Endogenous AtCEN2 is detectable at 20 kDa. (D) Immunoblot analysis of AtCEN2 protein of WT plant cell extracts after treatment with UV-C (10 Kerg/cm^2). Comassie blue staining of the Rubisco was used as loading control.

a commonly used anti-cancer drug causing predominantly DSBs, the steady state levels of AtCEN2 mRNA and protein do not change (data not shown). AtCEN2 transcript and protein levels thus specifically respond to UV-C induced DNA damage.

AtCEN2 localises in the nucleus after exposure to UV-C and Cisplatin

In order to study the subcellular localisation of AtCEN2, E-GFP was fused to its C-terminus. Arabidopsis plants overexpressing AtCEN2-GFP under the control of a constitutive promoter were produced and characterised (Figure 1C). Their ability to repair UV damaged DNA using an *in vitro* DNA repair assay was evaluated. Similarly to plants overexpressing a non-tagged AtCEN2 (Molinier *et al*., 2004), AtCEN2-GFP plants exhibit higher synthesis-dependent repair of UV-C induced DNA damage compared to wildtype (data not shown), confirming that the CEN2-GFP fusion is biologically functional and can therefore be used to analyse the intracellular dynamics of AtCEN2 after exposure to various DNA damaging agents such as UV-C and Cisplatin. These analyses were done in roots as the repair of bulky DNA lesions in this tissue is predominantly through the NER pathway (Kimura *et al*., 2004).

In untreated roots, the GFP fluorescence was relatively low and mostly localised in the cytoplasm (Figure 2A and D). After exposure to UV-C (30 min) and Cisplatin (2 h), GFP fluorescence was localised in the nuclei of roots cells and hairs roots, respectively (Figure 2B, C and E).

AtCEN2 interacts with the Arabidopsis homolog of human XPC (AtRad4)

Mammalian CEN2 has been shown to bind to the XPC/hHRAD23B complex (Araki *et al*., 2001). In order to investigate whether AtCEN2 binds to the

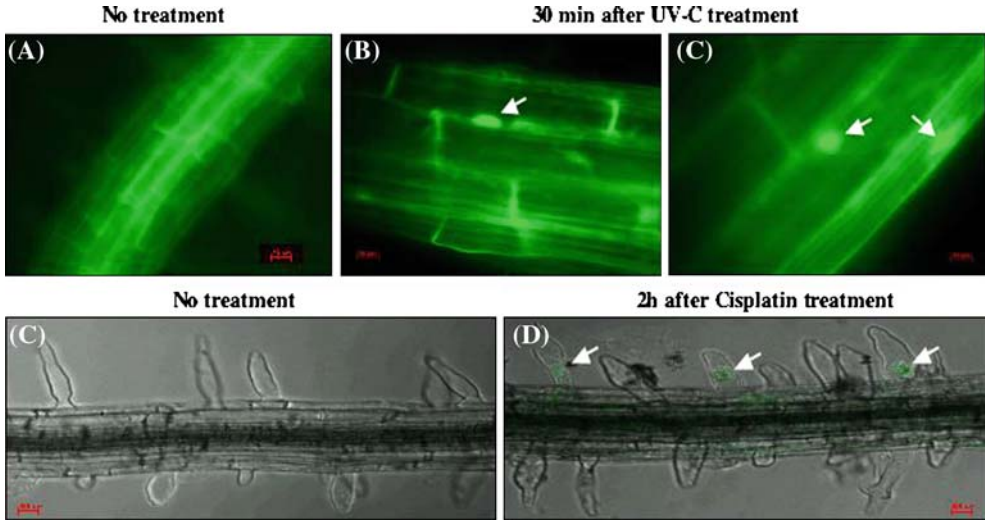

Figure 2. Subcellular localisation of a constitutively expressed AtCEN2-GFP fusion upon UV-C and Cisplatin treatments in Arabidopsis root cells. The AtCEN2-GFP fusion protein was stably expressed in transgenic Arabidopsis plants (see materials and methods for details). Weak fluorescence was observed in roots which were untreated (A and D), 30 min following UV-C irradiation (B and C, 10 Kerg/cm^2) or 2 h following Cisplatin treatment (E, 1 μM). These pictures are representatives of 3 independent experiments. Size bars represent: 75 μm (A), 50 μm (B and C), 100 μm (D and E).

Arabidopsis homolog of human XPC, AtRad4, an *in vitro* affinity pull-down assay was used. Recombinant GST-AtCEN2 fusion protein was produced, purified and coupled onto glutathione-sepharose beads. The part of the AtRad4 gene coding for the conserved C-terminal domain, has been shown to interact with HsCEN2 (Popescu *et al*., 2003) was cloned and the protein was radioactively labeled with ^{35}S-Met using an *in vitro* transcription-coupled translation kit. In the presence of 1 mM of Ca^{2+}, ^{35}S-AtRad4-Ct bound to GST-CEN2, as proven by the single band detected after elution (Figure 3A). In contrast, in the presence of 1 mM of EGTA this interaction was significantly reduced (Figure 3A). ^{35}S-labeled luciferase used as control did not show any binding (Figure 3A). These data show that the Arabidopsis CENTRIN2 binds the homolog of the human XPC, AtRad4 and that calcium is required for this interaction.

In order to map the AtRad4-binding site in AtCEN2, truncated versions of GST-CEN2 were produced and purified (Figure 3B). In CEN2-C, the distal EF-hand domain was removed and in GST-CEN2-B, the two C-terminal EF-hand domains were deleted (Figure 3B). Only the full length CEN2, but neither of the truncated forms were able to bind to Rad4-Ct (Figure 3C). ^{35}S-labeled luciferase did not show binding to any of these CEN2 proteins, neither the full-length nor the truncated AtCEN2 forms, confirming the specificity of the binding (Figure 3D). These data suggest that the C-terminal EF-hand domain of CEN2 is critically required for its binding to AtRad4, an interaction which may stabilise the damage recognition complex AtRAD4/AtRAD23.

The distal EF-hand domain of AtCEN2 is required for efficient NER

It has previously been shown that AtCEN2 defective plants exhibit moderate UV-C sensitivity (Molinier *et al*., 2004) and it may thus be expected to observe a higher tolerance to UV irradiation in AtCEN2 overexpressing plants. However, neither plants overexpressing the full length AtCEN2 nor those overexpressing the truncated form of AtCEN2 exhibited significant altered sensitivity to UV-C and Cisplatin treatments as compared to the control plants (data not shown). AtCEN2 protein concentration is thus not a limiting factor for UV and Cisplatin tolerance.

In order to more directly test the implication of AtCEN2 in DNA repair the repair efficiency of bulky DNA damage such as UV-C and Cisplatin was evaluated using an *in vitro* repair assay. This assay evaluates the excision repair efficiency of plant cell extracts by measuring the efficiency of

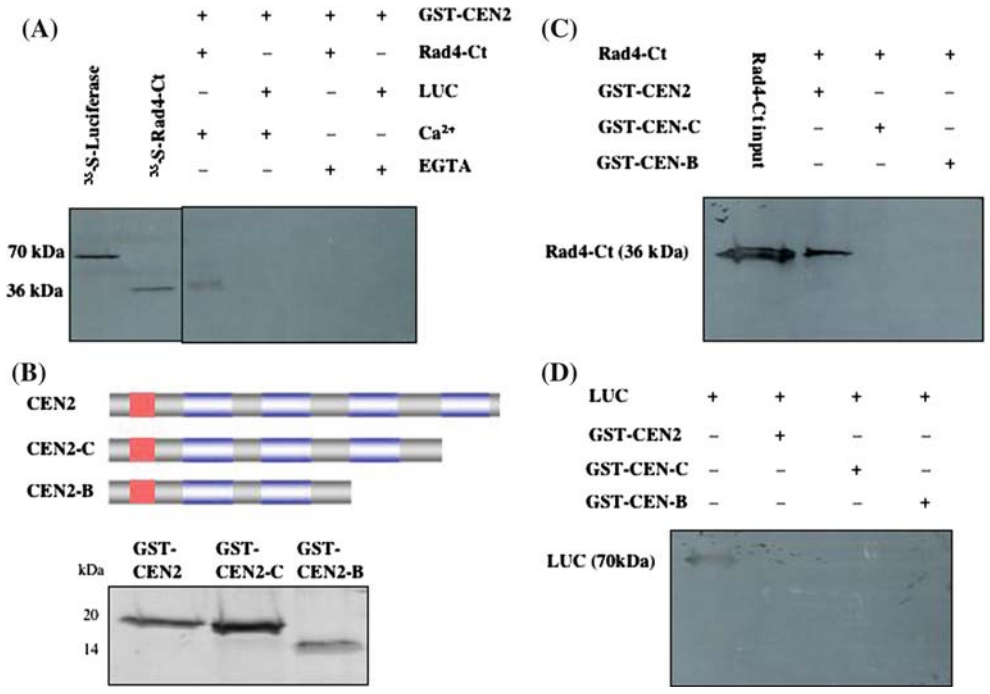

Figure 3. *In vitro* binding assay of AtCEN2 with the C-terminal domain of AtRad4. (A) *In vitro* binding of AtCEN2 with AtRad4. The binding assay was performed using [^{35}S]-labelled Rad4 and GST-CEN2 in the presence of Ca^{2+} or EGTA (see experimental procedures for details). The [^{35}S]-labelled luciferase (LUC) was used as control. (B) Schematic representation of the different AtCEN2 forms: full length AtCEN2; AtCEN-C with deletion of the distal EF-hand domain, AtCEN-B with deletion of the C-terminal 2 EF-hand domains. Red box: ATP-binding site; blue box: EF-hand domain. SDS PAGE gel (15%) of recombinant truncated forms of AtCEN2. (C) *In vitro* binding assay of the different AtCEN2 forms with AtRad4 in the presence of Ca^{2+}. (D) Control of the *in vitro* binding assay using the luciferase protein.

incorporation of DIG dUTP in a UV-C or Cisplatin damaged plasmid. Thereby the efficiency of dark repair of UV-C and Cisplatin induced DNA damage is evaluated for plants overexpressing the full-length and truncated AtCEN2 forms.

DNA synthesis-dependent repair of both UV-C and Cisplatin damage was more efficient in plants overexpressing the full length AtCEN2 compared to the control (Figure 4). In contrast no significant change in the repair efficiency was observed in

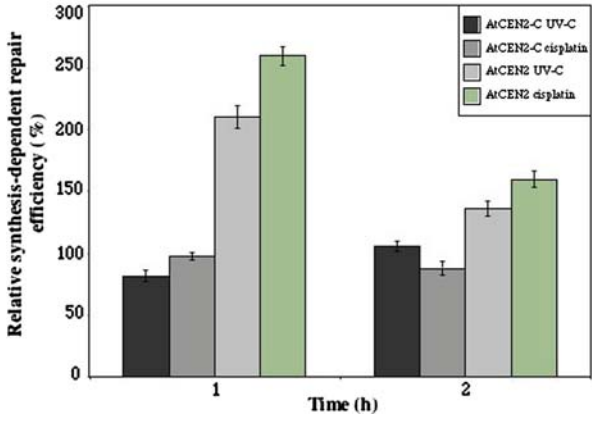

Figure 4. *In vitro* synthesis-dependent repair efficiencies of UV-C and Cisplatin induced DNA damage of cell extracts from AtCEN2, AtCEN2-C overexpressing plants. Synthesis-dependent repair efficiencies (%) of AtCEN2 and AtCEN2-C overexpressing plants are expressed relative to the untreated plasmid control 1 and 2 h after incubation (See materials and methods for details).

plants overexpressing the AtCEN2-C form compared to the control (Figure 4). These data suggest that the distal EF-hand domain of AtCEN2, via its interaction with AtRad4, contributes to improve the NER efficiency and that this interaction is a limiting factor for the DNA synthesis-dependent repair of UV and Cisplatin induced DNA damage as measured *in vitro*. In addition, these results may allow the conclusion that in plants the AtRad4 complex is also involved in the synthesis dependent repair of bulky DNA lesions such as ICL.

Recombinational DNA repair is not or is weakly stimulated in plants overexpressing AtCEN2

In a previous study we have shown that plants deficient for At*CEN2* expression exhibited a reduced efficiency in synthesis dependent repair of UV lesions accompanied by an increased somatic HR frequency (Molinier *et al.*, 2004). These results had suggested the existence of an interconnection between different DNA repair pathways (i.e. NER and HR). Therefore we addressed the question whether up-regulated synthesis-dependent repair of bulky DNA lesions could influence recombinational DNA repair. For this, somatic HR was measured in transgenic plants harbouring a recombination substrate consisting of tandem disrupted β-glucuronidase (GUS) reporter gene in direct repeat orientation (Molinier *et al.*, 2004). Recombination event between the homologous sequences leads to the restoration of an active β-glucuronidase gene and can be monitored using a histochemical assay. These recombination lines have been transformed to overexpress either the full length AtCEN2 or the truncated AtCEN2-C form.

UV-C is known to induce DNA lesions predominantly repaired by the NER pathway but it also induces somatic HR. Cisplatin induces ICLs, which in eukaryotes are known to be repaired by a combination of NER and the HR pathways. Somatic HR was found to be induced in control and 3 independent AtCEN2-C overexpressing plants upon exposure to UV-C and Cisplatin whereas somatic HR was not or only weakly stimulated in 2 independent AtCEN2 overexpressing plants (Figure 5A, B, D and E). Consistent with previous findings (Molinier *et al.*, 2004) plants deficient for At*CEN2* expression exhibited elevated somatic HRF as compared to the control (Figure 5C and F) both in the absence and in the presence of induced DNA damage. It is important to notice that a relative high difference of somatic HRF exists between UV-C untreated and Cisplatin untreated At*cen2* plants. This variability, strictly restricted to At*CEN2* defective plants, is due to the preculture conditions, which are in solid culture medium prior UV-C exposure and in liquid culture medium prior Cisplatin treatment.

Taken together these results show that in plants which overexpress AtCEN2 and thus show enhanced synthesis-dependent repair of bulky DNA damage, the use of somatic HR pathway is accordingly reduced.

Discussion

Using biochemical approach we showed that AtCEN2 interacts with the Arabidopsis homolog of human XPC, AtRAD4 and that its distal EF-hand Ca^{2+} binding domain is essential for this interaction as well as for an efficient synthesis-dependent repair of bulky DNA lesions. In addition, the rapid nuclear localisation of AtCEN2 upon UV-C exposure strengthens its role in the early step of the excision repair process as a component of the plant recognition complex. Finally we showed that AtCEN2 expression level, modulating the NER pathway, differentially influences HR efficiency, suggesting that interconnection between these 2 repair pathways exists.

CENTRIN2 interacts with the Arabidopsis *homolog of the human XPC protein (AtRad4)*

AtCEN2 is closely related to human CEN2 (HsCEN2) with more than 70% similarity within their EF-hand domains (Molinier *et al.*, 2004). The C-terminal half of HsCEN2 shows high affinity to Ca^{2+} and is able to form dimers and oligomers (Durussel *et al.*, 2000; Matei *et al.*, 2003). Binding of Ca^{2+} to the distal EF-hand domain of HsCEN2 stabilises the protein and induces co-operative Ca^{2+} binding by the 3rd EF-hand (Matei *et al.*, 2003). In the present study it was not possible to distinguish whether a dimerisation and/or Ca^{2+}-binding activity of AtCEN2 is/are required for its binding to AtRAD4. The C-terminal half of HsCEN2 containing the 4th EF-hand motif, is involved in both activities and

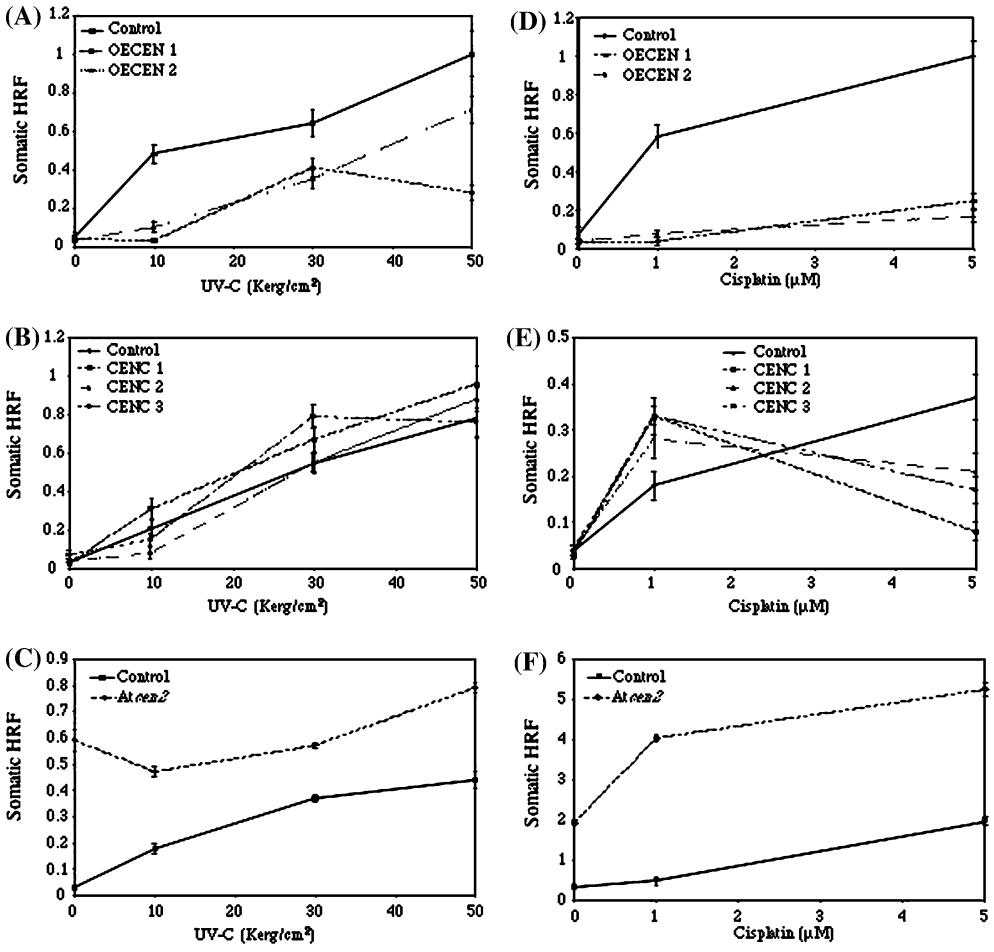

Figure 5. Somatic homologous recombination frequencies (HRF) in independent transgenic plants overexpressing either the full length AtCEN2 (OECEN1, OECEN2; A and D), the truncated AtCEN2-C form (CENC1, CENC2, CENC3; B and E) or in At*CEN2* (C and F) deficient plants upon exposure to UV-C (A, B and C) and Cisplatin (D, E and F). Plants were treated with either UV-C (0, 10, 30 and 50 kerg/cm^2) or Cisplatin (0, 1 and 5 μM). Somatic HRF $\pm$ SD (average number of GUS+ spots/plant) was determined 4 days after each treatment. 24 plants per treatment were used and experiments were duplicated.

AtCEN2 lacking this domain is unable to form dimers (our unpublished data). A recent study has also shown that reversible HsCEN2 self-assembly depends upon its N-terminal part (Tourbez *et al.*, 2004). Although EF-hand domains are identical within the centrin superfamily, AtCEN2 differs from other members by a specific N-terminal ATP binding domain. This domain is able to bind ATP in the presence of EGTA but not of Ca^{2+}, suggesting the existence of another or a plant specific function of the Arabidopsis CEN2 protein (Liang, unpublished data).

HsCEN2 binds the Rad4 domain of the XPC protein (Popescu *et al.*, 2003). The C-terminal domain (Rad4 domain) of XPC is well conserved between species and contains predicted DNA, Rad23 and TFIIH binding sites (Uchida *et al.*, 2002). Recently, Nishi *et al.* (2005) have shown that HsCEN2 binds to a putative alpha-helical region near the C-terminus, and that 3 amino acid substitutions in this domain abrogated this XPC-HsCEN2 interaction. The CEN2 binding motif is also present in AtRad4 (Popescu *et al.*, 2003) and this has permitted analysis of the interaction between the AtCEN2 and AtRad4 proteins. As predicated, the AtCEN2 is able to bind AtRad4 in the presence of calcium in agreement with the work of Popescu *et al.* (2003) showing that HsCEN2-XPC interaction is Ca^{2+}-dependent. Although the CEN2 binding domain of the XPC

protein is well characterised in human nothing was known about the CEN2 domain required for this interaction. We show here that the distal EF-hand domain is absolutely required for AtCEN2-AtRad4 interaction suggesting that this domain may also be involved for the interaction of XPC-CEN2 in human. Thus the XPC-CEN2 binding motif, AtRad4-AtCEN2 in this study, is conserved between animals and plants and the recognition complex of UV damage may be comparable in plants as in human and may work in a similar way in higher eukaryotes.

XPC/Rad4 protein complex stability also requires Rad23 function, which prevents ubiquitin-dependent degradation (Okuda *et al*., 2004). In mouse HRad23A/B double knockout fibroblasts, XPC protein was very unstable (Ng *et al*., 2003; Okuda *et al*., 2004). In Arabidopsis 4 At*Rad23* genes are present, but the corresponding AtRAD4 interactor has not been identified yet. In addition, HsCEN2 has been shown to play a role in stabilisation of XPC in an HRad23-dependent manner (Araki *et al*., 2001). XPC protein level is low under normal growth conditions, but the DNA repair activity of XPC can be induced upon UV-C damage (Ng *et al*., 2003). We show here that after UV-C treatment, the amount of AtCEN2 protein increased and its subcellular localisation was rapidly changed. AtCEN2 relocalised into the nucleus upon UV-C and Cisplatin treatments. Consistently, XPC protein was localised to the damage sites in mammalian cells upon UV-C treatment (Ng *et al*., 2003). This suggests the possibilities that the AtRAD4 protein is also relocalised and stabilized at damage sites as well as in human cells and that AtCEN2 contributes to this process. Ng *et al*. (2003) have shown that overexpression of hHRad23 leads to increased amounts of stabilised XPC and reduced cell viability. AtCEN2 overexpressing plants are viable and did not exhibit developmental aberrations. This suggests that the function of AtCEN2 is different from those of Rad23 responsible for the XPC stability by the regulation of proteolysis via the ubiquitin/proteasome pathway (Schauber *et al*., 1998; Ng *et al*., 2003). Therefore AtCEN2 may be involved in the stabilisation or stimulation of the recognition complex at the damaged sites without influencing significantly AtRAD4 protein amounts.

AtCEN2 expression level differentially influences NER and HR efficiencies

In mammals the defects of the first step of NER (recognition step) lead to dramatic genetic disorders (for review Lehmann, 2003). Rad4/XPC deficient cells exhibit higher susceptibility to bulky DNA lesions. Although Araki *et al*. (2001) have shown using *in vitro* appoach that HsCEN2 contributes to stabilise the XPC complex and that the lack of HsCEN2 leads to reduced synthesis-dependent repair efficiency on UV induced DNA damage, nothing is known about the sensitivity of CEN2 deficient mammalian cells to different DNA damaging agents and about their recombinational DNA repair efficiency. Therefore plants offer a good opportunity to study the role of CEN2 in DNA repair. In Arabidopsis we have recently shown that plants deficient for AtCEN2 expression exhibit UV-C sensitivity, a lower NER efficiency and an enhanced somatic HR frequency (Molinier *et al*., 2004), suggesting an interconnection between the different repair pathways in plants. In the present study we show that AtCEN2 overexpression leads to an increased efficiency of synthesis-dependent repair of UV-C and Cisplatin induced DNA lesions whereas overexpression of the truncated AtCEN2-C form did not affect repair efficiency.

These results also provide evidence that the plant recognition complex, AtRad4-AtCEN2 including also AtRad23, is involved in ICL repair as in other eukaryotes (You *et al*., 2003). In yeast and mammals ICL repair requires both NER and HR (for review Mc Hugh *et al*., 2001). In WT Arabidopsis, somatic HR is induced after Cisplatin treatment, showing that HR also contributes to ICL repair. We demonstrate here that overexpression of AtCEN2 abolishes this enhanced HRF suggesting that increased capacity of synthesis-dependent repair of bulky DNA damage prevents recombinational repair. Recent studies showed that the recognition complex in general and XPC protein in particular play critical roles in Cisplatin DNA repair initiation by preventing high mutation rate (Wang *et al*., 2004a, b). Our results address the question of the potential role of CEN2 in this repair process, which is especially relevant for cancer cell drug resistance in mammalian cells.

The relatively lower induction of somatic HR in AtCEN2 overexpressing plants after treatments with UV-C and Cisplatin suggests that critical points for the regulation of the different repair pathways may include connections between upstream recognition processes, competition of repair factors for substrates and the state of chromatin at the damaged sites.

Our findings are in agreement with the recent results of Garinis *et al*. (2005) who have shown in mammalian cells that UV lesions, such as cyclobutane pyrimidine dimers (CPDs), lead to the accumulation of gamma-H2AX, P53bp1, Rad51 foci and increased amounts of DSBs which are substrates for the HR process. Therefore, Arabidopsis plants either defective for CEN2 expression or overexpressing CEN2 is a material of choice to study the becoming of the UV lesions in plants misregulated in NER and the stimulation or inhibition of the HR process, respectively.

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References

Araki, M., Masutani, C., Takemura, M., Uchida, A., Sugasawa, K., Kondoh, J., Ohkuma, Y. and Hanaoka, F. 2001. Centrosome Protein Centrin 2/Caltractin 1 Is Part of the Xeroderma Pigmentosum Group C Complex That Initiates Global Genome Nucleotide Excision Repair. J. Biol.Chem. 276: 18665–18672.

Britt, A.B. 1999. Molecular genetics of DNA repair in higher plants. Trends Plant Sci. 4: 20–24.

Cole, R.S. and Sinden, R.R. 1975. Repair of cross-linked DNA in Escherichia coli. Basic Life Sci. 5: 487–495.

Costa, R.M., Chigancas, V., Galhardo Rda, S., Carvalho, H. and Menck, C.F. 2003. The eukaryotic nucleotide excision repair pathway. Biochimie 85: 1083–1099.

De Silva, I.U., McHugh, P.J., Clingen, P.H. and Hartley, J.A. 2000. Defining the role of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. Mol. Cell. Biol. 20: 7980–7990.

Dubest, S., Gallego, M.E. and White, C.I. 2004. Roles of the AtErcc1 protein in recombination. Plant J. 39: 334–342.

Durussel, I., Blouquit, Y., Middendorp, S., Craescu, C.T. and Cox, J.A. 2000. Cation- and peptide-binding properties of human centrin 2. FEBS Lett. 472: 208–212.

Elliott, B. and Jasin, M. 2001. Repair of double-strand breaks by homologous recombination in mismatch repair-defective mammalian cells. Mol. Cell. Biol. 21: 2671–2682.

Ferrando, A., Koncz-Kalman, Z., Farras, R., Tiburcio, A., Schell, J. and Koncz, C. 2001. Detection of in vivo protein interactions between Snf1-related kinase subunits with intron-tagged epitope-labelling in plants cells. Nucleic Acids Res. 29: 3685–3693.

Garinis, G.A., Mitchell, J.R., Moorhouse, M.J., Hanada, K., de Waard, H., Vandeputte, D., Jans, J., Brand, K., Smid, M., van der Spek, P.J., Hoeijmakers, J.H., Kanaar, R. and van der Horst, G.T. 2005. Transcriptome analysis reveals cyclobutane pyrimidine dimers as a major source of UV-induced DNA breaks. EMBO J. 24: 3952–3962.

Gherbi, H., Gallego, M.E., Jalut, N., Lucht, J.M., Hohn, B. and White, C.I. 2001. Homologous recombination in planta is stimulated in the absence of Rad50. EMBO Rep. 2: 287–291.

Hendricks, C.A., Razlog, M., Matsuguchi, T., Goyal, A., Brock, A.L. and Engelward, B.P. 2002. The *S. cerevisiae* Mag1 3-methyladenine DNA glycosylase modulates susceptibility to homologous recombination. DNA Repair 1: 645–659.

Hey, T., Lipps, G., Sugasawa, K., Iwai, S., Hanaoka, F. and Krauss, G. 2002. XPC-HR23B complex displays high affinity and specificity for damaged DNA in a true-equilibrium fluorescence assay. Biochemistry 41: 6583–6587.

Jachymczyk, W.J., von Borstel, R.C., Mowat, M.R. and Hastings, P.J. 1981. Repair of interstrand cross-links in DNA of *Saccharomyces cerevisiae* requires two systems for DNA repair: the RAD3 system and the RAD51 system. Mol. Gen. Genet. 182: 196–205.

Kimura, S., Tahira, Y., Ishibashi, T., Mori, Y., Mori, T., Hashimoto, J. and Sakaguchi, K. 2004. DNA repair in higher plants; photoreactivation is the major DNA repair pathway in non-proliferating cells while excision repair (nucleotide excision repair and base excision repair) is active in proliferating cells. Nucleic Acids Res. 32: 2760–2767.

de Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H. 1999. Molecular mechanism of nucleotide excision repair. Genes Dev. 13: 768–785.

Lehmann, A.R. 2003. DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie 85: 1101–1111.

Li, A., Schuermann, D., Gallego, F., Kovalchuk, I. and Tinland, B. 2002. Repair of damaged DNA by Arabidopsis cell extract. Plant Cell 14: 263–273.

Liang, F., Han, M., Romanienko, P.J. and Jasin, M. 1998. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc. Natl. Acad. Sci. USA 95: 5172–5177.

Matei, E., Miron, S., Blouquit, Y., Duchambon, P., Durussel, I., Cox, J.A. and Craescu, C.T. 2003. C-Terminal Half of Human Centrin 2 Behaves like a Regulatory EF-Hand Domain. Biochemistry 42: 1439–1450.

McHugh, P.J., Spanswick, V.J. and Hartley, J.A. 2001. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. Lancet Oncol. 2: 483–490.

Molinier, J., Ramos, C., Fritsch, O. and Hohn, B. 2004. CENTRIN2 modulates homologous recombination and nucleotide excision repair in Arabidopsis. Plant Cell 16: 1633–1643.

Ng, J.M.Y., Vermeulen, W., van der Horst, G.T.J., Bergink, S., Sugasawa, K., Vrieling, H. and Hoeijmakers, J.H.J. 2003. A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. Genes Dev. 17: 1630–1645.

Nishi, R., Okuda, Y., Watanabe, E., Mori, T., Iwai, S., Masutani, C., Sugasawa, K. and Hanaoka, F. 2005. Centrin 2 stimulates nucleotide excision repair by interacting with xeroderma pigmentosum group C protein. Mol Cell Biol. 13: 5664–5674.

Okuda, Y., Nishi, R., Ng, J.M., Vermeulen, W., van der Horst, G.T., Mori, T., Hoeijmakers, J.H., Hanaoka, F. and Sugasawa, K. 2004. Relative levels of the two mammalian Rad23 homologs determine composition and stability of the xeroderma pigmentosum group C protein complex. DNA Repair 3: 1285–1295.

Paques, F. and Haber, J.E. 1999. Multiple pathways of recombination induced by double-strand break in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. 63: 349–404.

Popescu, A., Miron, S., Blouquit, Y., Duchambon, P., Christova, P. and Craescu, C. T. 2003. Xeroderma Pigmentosum Group C Protein Possesses a High Affinity Binding Site to Human Centrin 2 and Calmodulin. J. Biol. Chem. 278: 40252–40261.

Puchta, H. and Hohn, B. 1996. From centimorgans to base pairs: homologous recombination in plants. Trends Plant Sci. 1: 340–348.

Ries, G., Heller, W., Puchta, H., Sandermann, H., Seidlitz, H.K. and Hohn, B. 2000. Elevated UV-B radiation reduces genome stability in plants. Nature 406: 98–101.

Saffran, W. A., Ahmed, S., Bellevue, S., Pereira, G., Patrick, T., Sanchez, W., Thomas, S., Alberti, M. and Hearst, J.E. 2004. DNA repair defects channel interstrand DNA crosslinks into alternate recombinational and error-prone repair pathways. J. Biol. Chem. 279: 36462–36469.

Salisbury, J.L. 1995. Centrin, centrosomes, and mitotic spindle poles. Curr. Opin. Cell Biol. 7: 39–45.

Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W. and Madura, K. 1998. Rad23 links DNA repair to the ubiquitin/proteasome pathway. Nature 391: 715–718.

Tourbez, M., Firanescu, C., Yang, A., Unipan, L., Duchambon, P., Blouquit, Y. and Craescu, C.T. 2004. Calcium-dependent self-assembly of human centrin 2. J. Biol. Chem. 279: 47672–47680.

Uchida, A., Sugasawa, K., Masutani, C., Dohmae, N., Araki, M., Yokoi, M., Ohkuma, Y. and Hanaoka, F. 2002. The carboxy-terminal domain of the XPC protein plays a crucial role in nucleotide excision repair through interactions with transcription factor IIH. DNA Repair 1: 449–461.

Villemure, J.F., Abaji, C., Cousineau, I. and Belmaaza, A. 2003. MSH2-deficient human cells exhibit a defect in the accurate termination of homology-directed repair of DNA double-strand breaks. Cancer Res. 63: 3334–3339.

Vonarx, E.J., Mitchell, H.L., Karthikeyan, R., Chatterjee, I. and Kunz, B.A. 1998. DNA repair in higher plants. Mutat. Res. 400: 187–200.

Wang, G., Dombkowski, A., Chuang, L. and Xu, X.X. 2004a. The involvement of XPC protein in the Cisplatin DNA damaging treatment-mediated cellular response. Cell Res. 14: 303–314.

Wang, G., Chuang, L., Zhang, X., Colton, S., Dombkowski, A., Reiners, J., Diakiw, A. and Xu, X.S. 2004b. The initiative role of XPC protein in Cisplatin DNA damaging treatment-mediated cell cycle regulation. Nucleic Acids Res. 32: 2231–2240.

You, J.S., Wang, M. and Lee, S.H. 2003. Biochemical analysis of the damage recognition process in nucleotide excision repair. J. Biol. Chem. 278: 7476–7485.