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SHORT COMMUNICATION

Plant homologue of human excision repair gene *ERCC1* points to conservation of DNA repair mechanisms

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Summary

Nucleotide excision repair (NER), a highly versatile DNA repair mechanism, is capable of removing various types of DNA damage including those induced by UV radiation and chemical mutagens. NER has been well characterized in yeast and mammalian systems but its presence in plants has not been reported. Here it is reported that a plant gene isolated from male germline cells of lily (*Lilium longiflorum*) shows a striking amino acid sequence similarity to the DNA excision repair proteins human ERCC1 and yeast RAD10. Homologous genes are also shown to be present in a number of taxonomically diverse plant genera tested, suggesting that this gene may have a conserved function in plants. The protein encoded by this gene is able to correct significantly the sensitivity to the cross-linking agent mitomycin C in ERCC1-deficient Chinese hamster ovary (CHO) cells. These findings suggest that the NER mechanism is conserved in yeast, animals and higher plants.

Introduction

Numerous environmental mutagenic agents such as UV light, chemical mutagens and cross-linking agents, fungal and bacterial toxins, and ionizing radiation induce damage of genetic material (DNA) in living cells (Doetsch, 1995; Friedberg, 1985). Such damage, if left unrepaired, can lead to mutations, general deterioration of cell function and cell death. To alleviate these toxic effects of DNA damage and maintain the genetic integrity, all living organisms have evolved a complex network of DNA repair mechanisms (Friedberg, 1985). These mechanisms include DNA excision repair comprising the nucleotide excision repair (NER) and base excision repair (BER) systems, as well as enzymatic photo-reactivation, recombination repair and post-replication repair.

NER is one of the most versatile DNA repair mechanisms operating in prokaryotes and eukaryotes. Unlike other DNA repair processes which repair specific DNA damage, NER is capable of removing various classes of DNA damage including those induced by UV radiation and chemical modification. In the NER process, DNA damage is removed as an oligonucleotide fragment, followed by replacement with new DNA using the undamaged (intact) strand as a template. Based on studies in yeast and mammalian systems, it has been suggested that this DNA repair process involves five steps: recognition of specific damage, dual incision of the damaged strand, lesion removal, synthesis of a repair patch and finally ligation of the new strand (Hoeijmakers, 1994). NER has been well studied in bacterial, yeast and mammalian systems. Isolated NER genes from these organisms show significant sequence similarity (Hoeijmakers, 1993a,b). NER has not yet been characterized in plants, although biochemical evidence suggests that such a DNA repair mechanism is present (Britt, 1996; McClennan, 1987). Molecular characterization of gene products involved in DNA repair in plants has so far been limited to photolyases, the enzymes involved in photo-reactivation (Ahmad *et al.*, 1997).

We have been searching for genes which are exclusively expressed or up-regulated in plant male germline (generative) cells. In flowering plants, the male gametophyte, commonly known as pollen, consists of a large vegetative cell and a small generative cell. The generative cell is wholly enclosed in the vegetative cell, forming a unique 'cell within a cell' structure. The generative cell later undergoes a mitotic division giving rise to two male gametes (sperm cells) which participate in the fertilization process. Generative cells are difficult to isolate from pollen but by using an enzymatic procedure we were able to isolate a sufficient number of generative cells from the pollen of lily (*Lilium longiflorum*) for biochemical and molecular analysis (Blomstedt *et al.*, 1996). Using a differential hybridization approach, a number of clones corresponding to transcripts either specifically expressed or up-regulated in the generative cell were isolated from a generative cell cDNA library. One of the cDNA clones belonging to the up-regulated class was found to have a

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striking amino acid sequence similarity with a human excision repair protein, ERCC1.

ERCC1 (excision repair cross-complementation) is one of the proteins involved in NER in humans (van Duin *et al.*, 1986). ERCC1 homologues are present in yeast, known as RAD10 in *Saccharomyces cerevisiae* and Swi10 in *Schizosaccharomyces pombe* (Reynolds *et al.*, 1985; Rodel *et al.*, 1992; Weiss and Friedberg, 1985). These proteins are known to be each other's homologues on the basis of sequence similarity, the mutant phenotypes, their role in DNA repair and the biochemical/enzymatic functions of the proteins. Significant homology to *E. coli* NER proteins, namely UvrA and UvrC, was also observed at the C-terminus of human ERCC1 (Husain *et al.*, 1986; Sancar *et al.*, 1984; Weeda *et al.*, 1993). ERCC1, functioning as part of a catalytic complex with other proteins including XPF (ERCC4) and ERCC11, is considered to be responsible for endonucleolytic incision at the 5′ side of DNA lesions (Matsunaga *et al.*, 1995; Sijbers *et al.*, 1996a). Similarly in yeast, RAD10 exists as a functional complex with RAD1 protein (Bardwell *et al.*, 1994; Davies *et al.*, 1995; Rodriguez *et al.*, 1996; Tomkinson *et al.*, 1993). Apart from excision repair, yeast homologues of ERCC1 have an additional function in mitotic recombination, a pathway that involves recombination between direct repeats in *S. cerevisiae* and mating type switching in *S. pombe* (Gutz and Schmidt, 1985; Schiestl and Prakash, 1990). Recently, it has been proposed that the human ERCC1 complex also has a function in mitotic recombination (Sijbers *et al.*, 1996b). ERCC1-deficient Chinese hamster mutants are not only sensitive to UV, but also to the cross-linking agent mitomycin C (MMC). In addition to excision repair, recombinational repair is thought to be necessary for repair of inter-strand cross-links.

In this report, we describe the isolation and characterization of a cDNA clone corresponding to a gene which is preferentially expressed in male germline cells (generative cells). This gene was found to share significant sequence similarity with a human excision repair gene, *ERCC1*. Further analysis suggested that the protein encoded by this gene is functional in DNA repair. Isolation of a plant homologue of a human excision repair gene is the first step towards elucidating the NER mechanism in plants.

Results and discussion

Isolation of cDNA clone and sequence analysis

A generative cell cDNA library of lily (*Lilium longiflorum*) was screened by differential hybridization and several cDNA clones showing preferential or specific hybridization to generative cells were obtained. Sequence analysis revealed that one of the clones had a significant sequence similarity with a human NER gene, *ERCC1*. The deduced amino acid sequence of this clone shows 71% similarity (52.2% identity) to human *ERCC1* in 221 amino acids of overlap (Figure 1). We have tentatively named this clone lily *ERCC1*. Lily ERCC1 protein also displays a significant sequence similarity to Swi10 of *S. pombe,* showing 64% (41.8% identity) in 195 amino acids of overlap, and 39% (30% identity) to RAD10 of *S. cerevisiae*. The similarity between lily ERCC1 and human ERCC1 is higher than that observed between human ERCC1 and *S. cerevisiae* RAD10. Thus, by these criteria, the identified cDNA indeed is expected to encode the *Lilium* ERCC1 protein. No significant overall homology was found with other proteins in the accessible databases.

Comparison of the deduced amino acid sequence of the lily *ERCC1* cDNA clone with its human counterpart showed that it lacks a N-terminal region which corresponds to the first 68 amino acid residues of human ERCC1 while it has 49 additional amino acid residues at the C-terminal end (Figure 1). Notably, the most strongly conserved region is the central region which is also highly conserved in yeast Swi10 and RAD10 (Figure 1). This is particularly interesting with respect to DNA repair functions. Earlier deletion analysis has shown that removal of the first 92 N-terminal residues comprising almost one-third of human ERCC1 protein does not affect its DNA repair functions (Sijbers *et al.*, 1996b). It has also been demonstrated that addition of a polyhistidine tag at the C-terminus of human ERCC1 did not interfere with DNA repair functions (Sijbers *et al.*, 1996a). The lily *ERCC1* cDNA clone described here appears to possess all the functional domains. Therefore, we expect the lily ERCC1 polypeptide to be functional in DNA repair.

DNA repair activity of lily ERCC1 in mutant CHO cells

We assayed the protein encoded by the lily *ERCC1* gene for complementation of sensitivity to UV (NER defect) and the cross-linking agent MMC (recombination defect) in ERCC1-deficient Chinese hamster ovary (CHO) cells, 43–3B (Troelstra *et al.*, 1992a,b; Wood and Burks, 1982). Previously, we found that human ERCC1 specifically and efficiently corrects the UV as well as the MMC sensitivity of this cell line (Sijbers *et al.*, 1996b). Transfection of the human ERCC1 gene in representitive cell lines of all 10 other known NER-deficient CHO complementation groups failed to correct their repair defect (Van Duin *et al.*, 1988). Similarly, micro-injection experiments with the human *ERCC1* gene in fibroblasts of all known complementation groups (11 identified) also gave negative results, indicating that the *ERCC1* gene specifically corrects only this mutant cell line (Van Duin *et al.*, 1989).

When the lily *ERCC1* gene was introduced into the 43–3B mutant cell line, it was found that lily ERCC1 protein was able to induce a significant correction of the sensitivity to MMC but we were unable to detect correction of UV

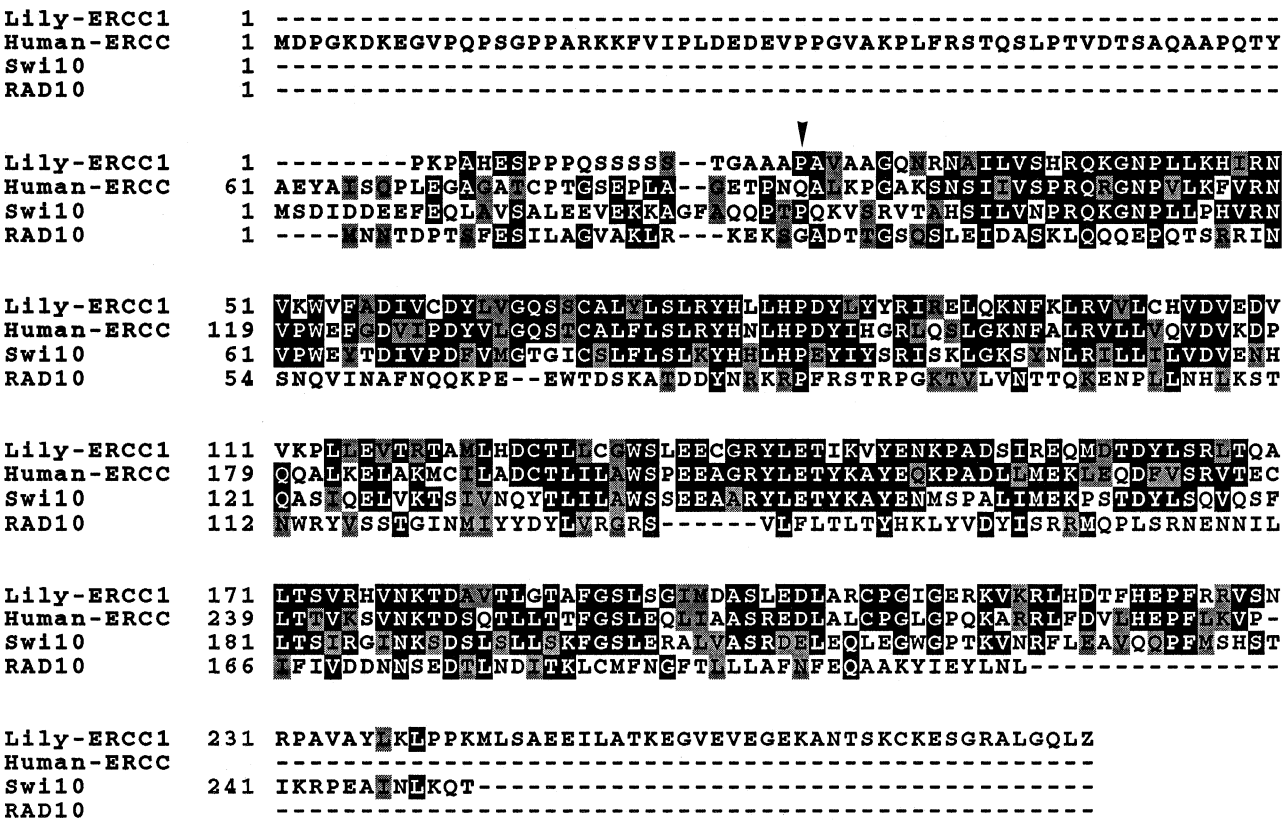

Figure 1. Alignment of the deduced amino acid sequence of lily ERCC1 with human ERCC1, yeast Swi10 and RAD 10.
Amino acid positions are shown on the left of sequences. Gaps are represented by dots. Identical and similar amino acids are represented by shaded letters. The arrow indicates the position corresponding to amino acid residue 92 of human ERCC1. Deletion analysis has shown that amino acid residues 1–92 of human ERCC1 are not required for DNA repair function (Sijbers *et al.*, 1996b).

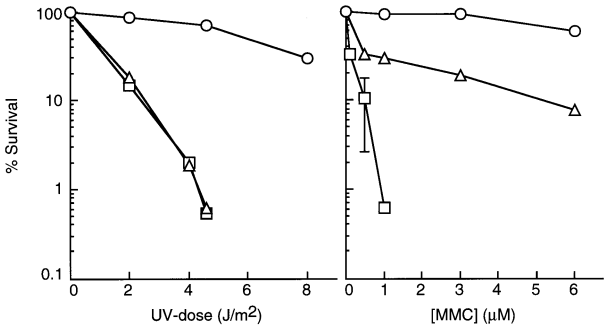

Figure 2. Survival of Chinese hamster group 1 mutant 43–3B transfectants and parental cell line CHO9 after treatment with UV and MMC.
Bars represent standard errors of the mean. (○) Normal Chinese hamster cell line; (□)mock-transfected Chinese hamster group 1 mutant; (△) Chinese hamster group 1 mutant ransfected with lily *ERCC1*.

sensitivity of the CHO *ERCC1* mutant (Figure 2). Experiments with *ERCC1* have shown that there is no significant increase in MMC resistance above wild-type levels upon transfection of the gene into normal wild-type CHO cells. Thus, the observed MMC correction with the lily *ERCC1* gene is specific for this CHO mutant.

These results are remarkably similar to those obtained from human *ERCC1* cDNA missense mutational experiments. When specific amino acids in the conserved region of human ERCC1 were substituted, most of the resultant ERCC1 proteins were unable to complement UV defect in 43–3B cells while the sensitivity to MMC was still corrected (Sijbers *et al.*, 1996b). Moreover, the amount of mutated ERCC1 protein produced in these mutations was found to be significantly reduced. As free ERCC1 protein is highly unstable and rapidly degraded inside the cell, it was concluded that these mutations affect ERCC1 protein stability, probably by interfering with the formation of the ERCC1/XPF complex (Sijbers *et al.*, 1996b). It was further concluded that the removal of cross-links induced by MMC requires less ERCC1 protein than repair of UV-induced damage. In this context, the most likely explanation for the lily ERCC1 protein complementation results is that the sequence divergence in the conserved region of lily ERCC1 protein results in formation of low levels or unstable complexes with Chinese hamster XPF. Consequently, the amount of functional complex formed in lily *ERCC1*-transfected cells is not sufficient for repair of UV damage via the NER pathway although it is sufficient for the repair of MMC-induced cross-links, probably through a recombination repair pathway.

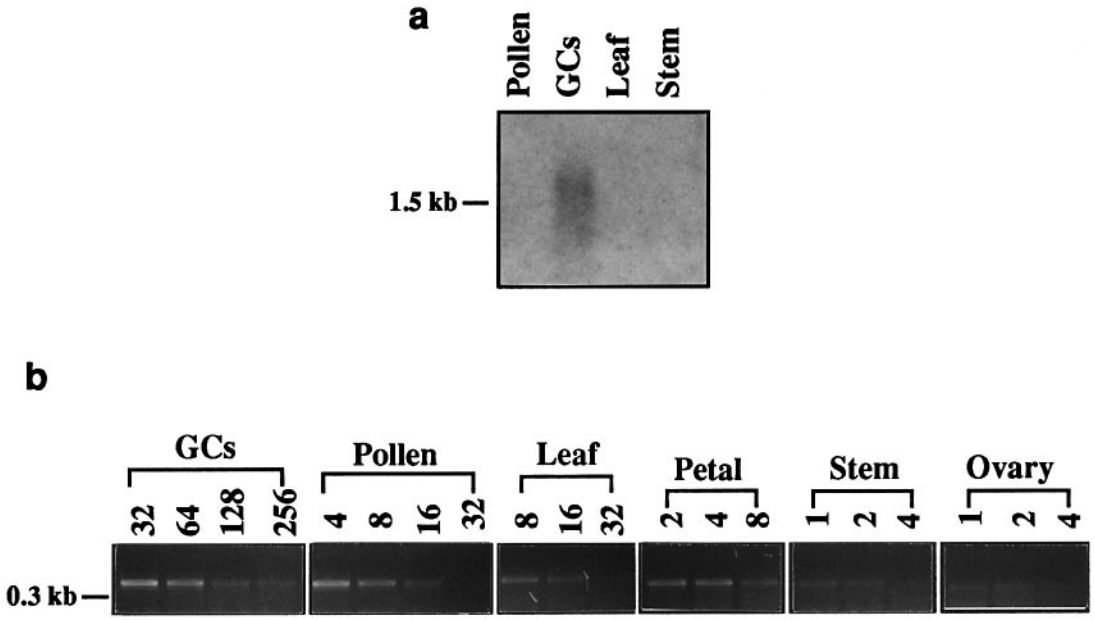

Figure 3. Expression of lily *ERCC1*.
(a) Northern blot analysis showing the expression of lily *ERCC1* in male germline (generative) cells. The levels of lily *ERCC1* mRNA in other tissues are below the detectable limit. Approximately 1.2 μg of mRNA was loaded per lane.
(b) RT–PCR detection of lily *ERCC1* mRNA in different tissues as indicated. Pollen mRNA includes the contribution of both generative cell and vegetative cell. The numbers 1, 2, 4 and 8 represent 1, 1/2, 1/4 and 1/8 of mRNA input, respectively, starting as approximately 6 ng of mRNA. Assay conditions used are within linear range. Molecular sizes are indicated on the left. GCs, generative cells.

There is substantial evidence supporting the idea that, in addition to its role in NER, the ERCC1/XPF complex (and its *S. cerevisiae* homologue RAD1/RAD10) is also implicated in a mitotic recombination repair pathway that is involved in elimination of inter-strand DNA cross-links (Sijbers *et al.*, 1996b). In the case of *S. cerevisiae*, certain RAD1/RAD10 NER-defective mutants show impairment in a type of mitotic recombination that is distinct from the RAD52 pathway of homologous recombination. In *S. pombe*, the ERCC1 counterpart, Swi10, has a function in mating type switching which also involves recombination. In mammals (cell lines, mouse mutants and human patients), a genetic defect in the ERCC1/XPF complex results in sensitivity to DNA cross-linking agents such as MMC, which is unique to such NER-defective mutants. Removal of these types of lesions induced by DNA cross-linking agents probably requires recombination events (Sijbers *et al.*, 1996b). Thus, there are number of lines of evidence which support an additional mitotic recombination role for ERCC1/XPF. Further characterization of capability of lily ERCC1 protein in NER and recombination repair will depend upon the isolation of its plant XPF counterpart.

Expression characteristics of the lily ERCC1 *gene*

The lily *ERCC1* clone corresponds to a transcript of approximately 1.3 kb of very low abundance which was detectable in generative cells but not in leaf, stem and pollen by Northern blot analysis (Figure 3a). However, lily *ERCC1* mRNA was detectable by RT–PCR in all the tissues tested although the levels of expression varied (Figure 3b). The highest level of lily *ERCC1* mRNA was observed in generative cells while the lowest was recorded in ovary and stem tissues, where PCR amplification product was barely detectable. Based on the signal intensity, we estimate that the content of lily *ERCC1* mRNA in the generative cell is approximately 4–8-fold higher than that in entire pollen. Since the generative cell is enveloped within pollen, we consider that a significant part of the PCR amplification product obtained using pollen mRNA input represents the contribution of generative cells to the pool of pollen mRNA. These results suggest that lily *ERCC1* is expressed at an elevated level in the generative cell compared with other tissues.

It is of a great interest to note that lily *ERCC1* expression is up-regulated in the male germline cells of plants. Pollen, as part of its developmental process, is exposed to solar UV radiation and other environmental mutagens after being released from the anther. This exposure could inevitably lead to DNA damage, in both vegetative and generative cell nuclei of pollen. Jackson (1987) also suggested that dehydration of pollen, which often accompanies anther dehiscence, can lead to DNA damage. Any damage in the generative cell nucleus DNA, whether caused by environmental agents or by dehydration, may lead to heritable mutations. Up-regulation of the *ERCC1* homologue in lily generative cells suggests that a highly active DNA repair process exists in male germline cells in order to protect

germline DNA from heritable mutations resulting from damaged DNA.

DNA repair measured as unscheduled DNA synthesis has been demonstrated in pollen from both dicotyledonous and monocotyledonous plants (Jackson and Linskens, 1978, 1979, 1980, 1982). Whether unscheduled DNA synthesis occurs in the generative nucleus and/or in the vegetative nucleus could not be elucidated in these studies. It was also suggested that both dark repair and photoreactivation may be operational in pollen (Jackson, 1987). Recently, a light-inducible photolyase gene *PHR1* has been cloned from *Arabidopsis* (Ahmad *et al.*, 1997). Expression of this photolyase was significantly higher in flowers than in vegetative tissues. Although it was suggested that the high level of photolyase in flowers might reflect a need to protect exposed gametes, it was not shown whether the level of photolyase expression is actually elevated in male germline cells. It can be assumed that the NER pathway may play an important role in maintaining DNA integrity in male germline cells. As such, male germline (generative or sperm) cells appear to be an ideal source for isolation of DNA repair genes from plants.

Conservation of the lily ERCC1 *gene in other plant genera*

Southern blot analysis showed that lily *ERCC1* homologues are present in the genomes of taxonomically diverse plant species including *Arabidopsis thaliana,* oilseed rape (*Brassica napus*), maize (*Zea mays*), rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*) (Figure 4). The presence of homologous genes in all the genera tested implies that the DNA repair role of the *ERCC1* gene may be conserved in higher plants. In this context, it is likely that a complex, comparable to the human ERCC1/XPF and yeast RAD1/RAD10, may be present in plants. Furthermore, it can be expected that genes encoding subunit polypeptides of such a catalytic complex may be up-regulated in male germline cells. Isolation of the putative plant nucleotide excision gene, lily *ERCC1*, may provide a useful tool for obtaining these genes. Further analysis using antisense RNA or functional complementation of *Arabidopsis* mutants should allow ultimate characterization of the excision repair pathway in plants.

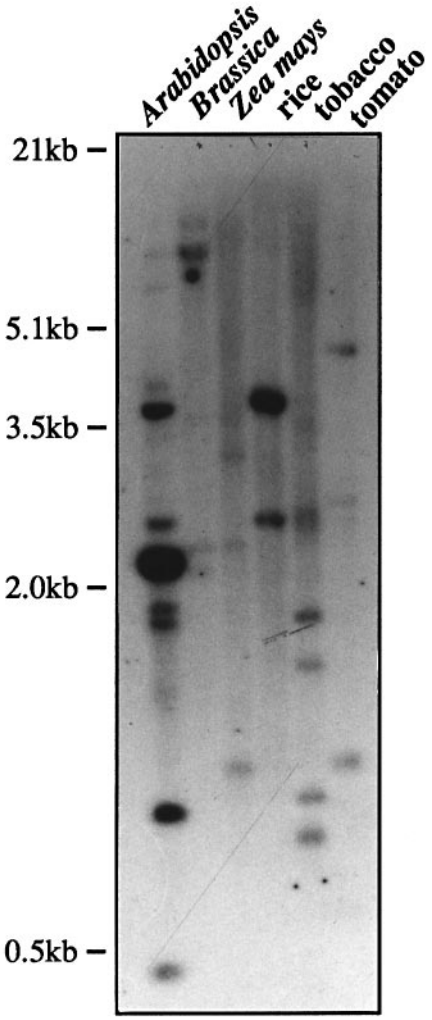

Figure 4. Southern blot analysis of genomic DNA showing conservation of the lily *ERCC1* gene in a number of taxonomically diverse plants as indicated.
Aliquots of 20 μg of genomic DNA were loaded on each lane. Molecular sizes are indicated on the left.

Experimental procedures

Library construction and cDNA isolation

Generative cells were isolated from fresh pollen of lily using the procedure described by Tanaka (1988). Isolated generative cells were stored at –70°C until use. PolyA(+) RNA was extracted directly from generative cells using an mRNA purification kit and cDNA was obtained using a Time Saver cDNA synthesis kit according to the manufacturer's instruction (Pharmacia LKB). A cDNA library was constructed in λgt 11 vector following the manufacturer's specification (Promega). Differential screening was carried out using ^{32}P-labelled first-strand cDNAs prepared from generative cells, leaf, stem and pollen. Putative generative cell specific clones were sequenced with an ABI PRISM^{TM} dye terminator cycle sequencing kit (Perkin-Elmer). Sequence analysis was performed using ANGIS (Australian National Genome Information Service).

Northern and Southern blot analysis and RT–PCR

Northern and Southern blots were prepared as described previously (Xu *et al.*, 1995). Approximately 1.2 μg of mRNA and 20 μg of genomic DNA was used for Northern and Southern blots, respectively. Blots were probed with a 0.5 kb fragment corresponding to the region highly homologous to human *ERCC1* and hybridization was carried out as described by Xu *et al.* (1995).

RT–PCR was performed using a controlled amount of mRNAs from various tissues. For each tissue, mRNA was subjected to

serial two-fold dilutions. Dilutions within the linear range were used for amplifications. RT–PCR amplification products were probed with the 0.5 kb fragment to confirm the specificity of the product.

Complementation assays

The lily *ERCC1* cDNA insert was subcloned into a mammalian expression vector pcDNA-3 (Invitrogen). The construct was transfected into the ERCC1-deficient Chinese hamster ovary (CHO) cell line, 43–3B (Wood and Burks, 1982), as described by Troelstra *et al.* (1992a). Stable transfected mass populations selected on G418 (800 μg ml^{-1}) were checked for the presence of intact lily *ERCC1* cDNA by PCR as described previously (Troelstra *et al.*, 1992b). Survival of wild-type CHO9, mock- or lily cDNA-transfected 43–3B cells after treatment with UV or MMC was determined using the [^{3}H]-thymidine incorporation assay as described elsewhere (Sijbers *et al.*, 1996b).

Acknowledgements

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