CEO1, a new protein from *Arabidopsis thaliana*, protects yeast against oxidative damage[1]

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Abstract The *Saccharomyces cerevisiae* strain WYT, deficient in the YAP1 transcription factor, was used in a molecular screen to identify genes from *Arabidopsis thaliana* that could overcome the oxidative stress-sensitive phenotype of these yeast cells. A cDNA named CEO1 increased the tolerance to oxidative damage caused by *tert*-butylhydroperoxide of both the Yap1$^-$ mutant and the wild-type yeast. Additionally, in Yap1$^-$ yeast, CEO1 also induced cross-tolerance to oxidative damage caused by hydrogen peroxide and diamide. CEO1 was assigned as being part of a small gene family that, until now, is exclusively restricted to plants. In *Arabidopsis*, CEO1 was produced in all organs, especially in roots and stems. By using the yeast two-hybrid system, proteins that specifically interact with CEO1 in yeast were identified, and putative DNA-binding proteins were consistently recovered.

Key words: DNA-binding protein; Cross-tolerance; Oxidative stress; *tert*-Butylhydroperoxide; *Arabidopsis thaliana*

1. Introduction

During normal cellular activity and in particular under conditions of environmental stress, molecular oxygen can be converted in reactive oxygen species (ROS) [1]. ROS are highly reactive molecules that can oxidize proteins, DNA, and lipids and can lead to extensive injury to cellular components and cell death [2]. The imbalance in the redox status of cells towards an oxidized state is known as oxidative stress and, during evolution, protective systems have been elaborated to cope with such a stress. Molecular defences include low molecular weight antioxidants, such as reduced glutathione, ascorbic acid, and carotenoids as well as ROS-scavenging enzymes, such as catalases, superoxide dismutases, and peroxidases. The activities of ROS-scavenging enzymes and the turnover rates of low molecular weight antioxidants are increased upon oxidative stress [3]. Oxidative stress response regulators that are controlled by specific signal transduction cascades have been characterized in both lower and higher eukaryotes [4,5].

The activities of antioxidant proteins might be a tolerance-limiting factor of an organism against stress conditions [6]. Pleiotropic phenotypes characteristic of cells with loss-of-function or gain-of-function mutations support this idea [7,8]. Plant cells are particularly prone to environmentally induced oxidative stress [9]. Genetic engineering of antioxidant defences has been proven to be a fruitful approach to improve stress tolerance of model and crop plant species [5]. Screening for gain-of-function phenotypes by at random overproduction of proteins followed by the active selection for oxidative stress tolerance might be a tool to identify new components of antioxidant defences and genes of potential biotechnological interest. Previously, we have carried out a gain-of-function screening of plant proteins for their ability to improve oxidative stress tolerance of the budding yeast *Saccharomyces cerevisiae*. By using the sulfhydryl-oxidizing drug diamide to simulate oxidative damage, several plant proteins were identified and characterized, the expression of which improved the diamide tolerance of yeast [10,11].

Similarly, a gain-of-function screening was used here to identify plant proteins that confer increased tolerance of yeast toward *tert*-butylhydroperoxide (tBuOOH), a compound that induces phospholipid peroxidation [12] and single DNA strand breaks [13]. We show that the production of the plant-specific protein CEO1 in yeast results in a cross-tolerance of the engineered yeast cells against hydroperoxides. The production and the putative protein–protein interaction of CEO1 with other plant proteins were further characterized.

2. Materials and methods

2.1. Bacterial and yeast strains, media, and chemicals

The *Escherichia coli* strain XL1 (Stratagene, La Jolla, CA, USA) was used for molecular cloning. Bacteria were grown on standard Luria Broth medium supplemented with antibiotics when required.

The near isogenic *S. cerevisiae* strains DY (MATa *his3 can1-100 ade2 leu2 trp1 ura3::*(3xSV40AP1-lacz)) and WYT (YAP1$^-$ mutant) (MATa *his3 can1-100 ade2 leu2 trp1 ura3 yap1::TRP1*) were used for the screening tests of drug tolerance. Yeast strains were grown on either nutrient-rich YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) supplemented with 50 mg l^{-1} of adenine or in minimal SD medium (0.67% yeast nitrogen base; Difco, Detroit, MI, USA), 2% glucose supplemented either with 0.37% casamino acids or individual amino acids. Dishes contained the same media with 1.5% agar. Hydrogen peroxide (H_2O_2), tBuOOH, and diamide were purchased from Sigma (St. Louis, MO, USA).

2.2. Drug tolerance screening

An *Arabidopsis thaliana* (L.) Heynh. cDNA library constructed in

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[1] CEO1 was deposited in the EMBL databank with the accession number AJ251578.

Abbreviations: AT, 3-aminotriazole; CEO, clone eighty-one; NLS, nuclear localization signal; ORF, open reading frame; ROS, reactive oxygen species; SD URA$^-$, minimal medium without uracil; tBuOOH, *tert*-butylhydroperoxide

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the yeast expression vector pFL61 was a gift of Dr. M. Minet [14]. The tBuOOH resistance screening was carried out as follows. WYT cells were transformed with the cDNA library and uracil prototrophic colonies were selected on minimal SD URA$^-$ media plates for 4 days. The transformed cells were harvested and stored in 60% glycerol. Aliquots of cells were plated onto SD URA$^-$ medium supplemented with 0.2 or 0.5 mM tBuOOH. Putative clones that were resistant against tBuOOH appeared after 4–7 days of incubation at 30°C and were inoculated in 2 ml of SD URA$^-$ liquid medium. Cultures were rescreened for the resistance phenotype in a semi-quantitative drug tolerance test. For that test, 10 μl of 3-fold serial dilutions of each yeast culture were spotted onto SD URA$^-$ plates supplemented with or without tBuOOH. WYT cells transformed with the vector pFL61 were used as control. Individual clones which grew on a medium supplemented with 0.2 mM tBuOOH were further analyzed.

Shuttle plasmids from individual clones were rescued into *E. coli* as described [15]. To confirm the drug tolerance phenotype, either the WYT or the DY strains were transformed with the selected plasmids. Three independent transformants were used for the drug tolerance by using a semi-quantitative dilution test. Yeast cells were grown in liquid SD URA$^-$ medium at 30°C until logarithmic or stationary phase. Cell suspensions corresponding to an OD_{600} of 0.45 were serially diluted (1/3, 1/9, 1/27, 1/81, and 1/243) in 0.3% NaCl and 10 μl of dilutions were spotted on SD URA$^-$ plates supplemented with or without drugs. After 3–4 days of incubation at 30°C, growth was estimated. The drug tolerance of WYT cells was tested with the following concentrations of drugs: 0.2 and 0.3 mM tBuOOH, 0.5 and 1.5 mM diamide, and 0.5 mM H_2O_2. DY transformants were spotted on SD plates supplemented with 0.2 and 0.4 mM tBuOOH.

2.3. Two-hybrid screening

The screening for protein–protein interaction was carried out using the Matchmacker Two-Hybrid System (Clontech, Palo Alto, CA, USA) and the CEO1 protein as a bait. The *Arabidopsis* cDNA library, prepared from 3 weeks old plants, was purchased from Clontech. To prepare the CEO1 bait vector, the CEO1-coding region was amplified by PCR using the *Pfu* DNA polymerase, a pair of gene-specific primers, and the linearized pC81 plasmid as a template. The 5′ ends of PCR primers included *Eco*RI and *Sma*I recognition sites and their sequences were: 5′-AAGCGGCCGCGAATTCATGGAAGCCAAGATCGTC-3′ and 5′-AACCCGGGCGGCCGCCAATCCACCTGCACC-3′.

Screenings were carried out in 20 mM 3-aminotriazole (AT) because the GAL4-CEO1 protein showed slight *trans*-activating properties in the HF7c yeast. Genetic verification of in yeast protein interactions was done with the set of control plasmids supplied by the manufacturer (Clontech). For the deletion analysis of the CEO1 protein, respective parts of the cDNA were amplified by PCR and used to prepare translational fusions between Gal4 and the following parts of the CEO1 protein: amino acid residues from 1 to 280 (ΔCEO1-1), 1–401 (ΔCEO1-2), 221–589 (ΔCEO1-3), 345–589 (ΔCEO1-4), and 221–401 (ΔCEO1-5).

2.4. Additional methods

Total RNA of *A. thaliana* was extracted and hybridized as previously described [10]. DNA sequencing was done on both strands on an ABI373A automatic DNA sequencer (Applied Biosystems, Fullerton, CA, USA) using gene-specific primers and fluorescent dye terminators. The DNA and protein sequences were analyzed with a software package from Genetics Computer Group (GCG, Madison, WI, USA).

3. Results

3.1. Isolation of an Arabidopsis protein that increases the oxidative tolerance of Yap1$^-$ and wild-type yeast

An *A. thaliana* cDNA library from young seedlings that was constructed in the yeast high copy number expression vector pFL61 [14] was used to transform the WYT strain of *S. cerevisiae*. This yeast strain is hypersensitive to hydroperoxides because of the deficiency in the YAP1 transcription regulator [16,17]. Transformant cells were plated on SD URA$^-$ medium containing 0.2 or 0.5 mM tBuOOH. Primary resistant colonies were grown in liquid medium, serially diluted, and spotted onto SD URA$^-$ plates supplemented with tBuOOH. Two clones, named C81 and C90, were consistently more resistant to 0.2 mM tBuOOH than the control WYT strain transformed with the vector pFL61. The plasmids from these two clones, named pC81 and pC90, were rescued and partially sequenced.

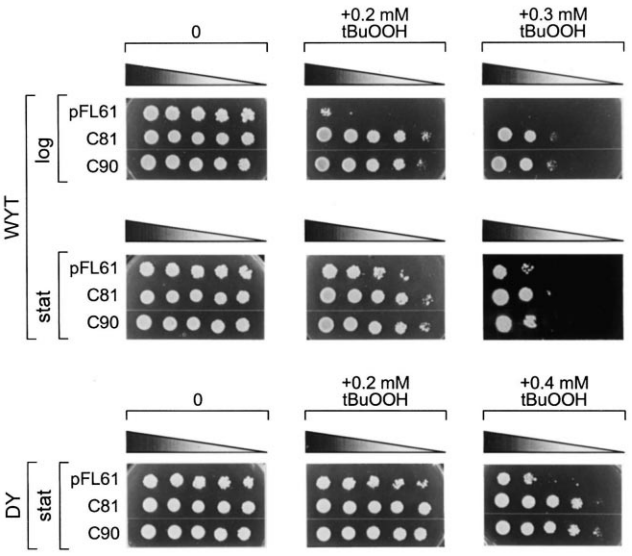

Fig. 1. Increased tolerance of yeast against tBuOOH by overproduction of CEO1. The oxidative stress tolerance of the Yap1$^-$ (WYT) and the wild-type (DY) yeast strains was assessed by growing cells to logarithmic (log) or to stationary phase (stat). Yeasts were transformed with the empty vector pFL61 (pFL61) or with the vector pFL61 carrying the cDNAs *C81* (C81) and *C90* (C90) (both coding for CEO1). Cell cultures corresponding to an OD_{600} of 0.45 were diluted serially (1/3, 1/9, 1/27, 1/81, 1/243) and 10 μl of the dilutions were spotted onto SD medium (0) or onto SD medium supplemented with 0.2, 0.3, and 0.4 mM tBuOOH. Drug tolerance was recorded after 4 days growth at 30°C. The triangles indicate the gradation from higher (dark gray) to lower (light gray) cell density aliquots.

To verify the ability of the selected cDNAs to confer yeast tolerance against oxidative stress, the mutant WYT strain was transformed with the candidate plasmids and the survival of the transformants was evaluated in tBuOOH tolerance assays. WYT (pC81) and WYT (pC90) exponentially growing cells were largely resistant to 0.2 and 0.3 mM tBuOOH, concentrations, which virtually suppressed the growth of the control WYT (pFL61) cells (Fig. 1, top). In stationary phase, though basal stress resistance is increased in yeast [18], again a slight growth improvement was observed in the cells transformed with the C81 and C90 cDNAs (Fig. 1, middle).

To analyze whether the observed biological activity of the isolated plant cDNAs is Yap1 dependent, the DY yeast strain, which is isogenic to WYT and contains a functional Yap1 gene [17], was transformed with the selected plasmids. The sensitivity to tBuOOH was assessed in stationary phase transformant cells. As expected, the DY strain could tolerate higher concentrations of tBuOOH than the WYT strain and the DY (pC81) and DY (pC90) transformants were more tolerant to tBuOOH than the control DY (pFL61) cells, in particular when high concentrations (0.4 mM) were used (Fig. 1, bottom).

Because the plasmids pC81 and pC90 were isolated from

independent clones, conferred identical levels of protection, and contained the same open reading frame (ORF), only pC81 was used for additional studies. We then tested whether the isolated *Arabidopsis* cDNA can confer a cross-tolerance to other elicitors of oxidative damage, in particular to H_2O_2 and diamide. Cell cultures from the logarithmic phase showed that WYT (pC81) transformants are more resistant to 1.5 mM diamide and to 0.5 mM H_2O_2 than WYT (pFL61) cells (Fig. 2 top). Similarly, WYT (pC81) in stationary phase was slightly more tolerant to 0.5 mM diamide and 0.5 mM H_2O_2 than the control (Fig. 2, bottom).

3.2. *CEO1 is a plant-specific protein*

The DNA sequencing of pC81 and pC90 showed that they contain an identical cDNA, which will be referred to as *CEO1* hereafter (acronym for clone eighty-one). *CEO1* is 2471 bp long and is nearly full length because a major transcript of approximately 2.4 kb was detected in an RNA gel blot of total *Arabidopsis* RNA (Fig. 3). Additionally, *CEO1* could also cross-hybridize to a second messenger, which might represent a close homologous gene (Fig. 3). Although expressed at different levels in *Arabidopsis* organs, more in stems and roots than in leaves and flowers, *CEO1* gene would probably have a constitutive expression (Fig. 3).

To analyze *CEO1* expression under oxidative stress conditions, *Arabidopsis* plants were infiltrated with the oxidants H_2O_2 and tBuOOH, the redox-cycling compounds methyl viologen and menadione, and the thiol-oxidizing drug diamide, as previously described [11]. The *CEO1* mRNA levels were evaluated by RNA gel blot analysis. Under the experimental conditions used, the expression of *CEO1* was unresponsive to oxidative stress (data not shown).

CEO1 cDNA encodes a polypeptide of 589 amino acids (CEO1) with a deduced molecular mass of 65.7 kDa. The ORF of this polypeptide is preceded in the 5′ leader sequence of the cDNA by three short ORFs of 19 (ORF-1 from nucleotide 30 to 86), 21 (ORF-2 from nucleotide 249 to 312), and eight (ORF-3 from nucleotide 314 to 337) amino acids and by seven in-frame stop codons. Because only the longest ORF (CEO1) showed some homology to proteins in the databank, we presumed that this was the protein encoded by the *CEO1* gene. Additionally, in yeast tolerance assays, the longest ORF was sufficient to confer tolerance to tBuOOH (data not shown).

A databank search with the putatively encoded CEO1 polypeptide did not reveal significant homology with any protein

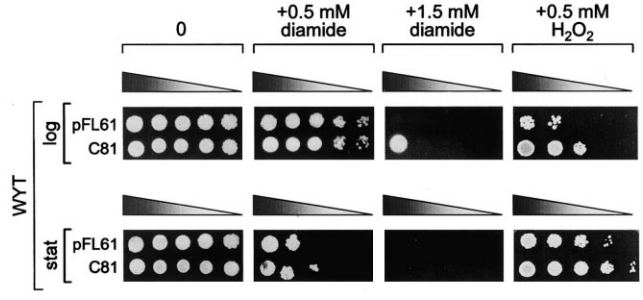

Fig. 2. Effects of the overproduction of CEO1 in Yap1$^-$ yeast cells grown in SD medium containing diamide and hydrogen peroxide. For details, see legend to Fig. 1. In this test, 0.5 mM and 1.5 mM diamide (diamide) and 0.5 mM hydrogen peroxide (H_2O_2) were used to induce oxidative stress.

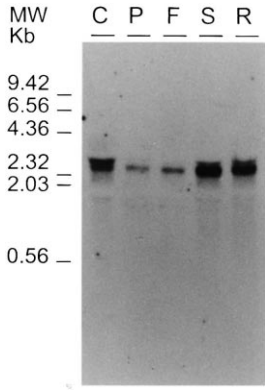

Fig. 3. Tissue-specific expression analysis of *CEO1* in *Arabidopsis* plants. Total RNA (10 μg/lane) from *Arabidopsis* cell suspension (C), aerial parts before bolting (P), flowers (F), stems (S), and roots (R) were hybridized with a probe prepared from *C81* (*CEO1*). ^{35}S-radiolabeled DNA fragments of phage λ were used as molecular weight markers (MW). Their positions on the membranes are indicated on the left. The numbers correspond to molecular masses expressed in thousands of base pairs (kb).

of known function. CEO1 belongs to a small multigene family of *Arabidopsis* composed of at least five members (Fig. 4). The 568 amino acid long polypeptide named CEO2 was probably a close homologue of CEO1 because both proteins were 65.0% identical and 72.3% similar (Table 1). CEO3, CEO4, and CEO5 shared significantly less homology at the amino acid level (Table 1) and lacked the conserved amino-terminal part of CEO1 and CEO2 (Fig. 4).

Sequence analysis using the program PSORT version 6.4 (http://psort.nibb.ac.jp) indicated the presence of three putative nuclear localization signals (NLSs). NLSs are defined amino acid sequences, characterized by a core peptide enriched in arginine (R) and lysine (K), which are required for an active import of proteins with a molecular mass larger than 40–60 kDa into the nucleus [19]. These three NLSs were located at positions 19, 54, and 319 in the CEO1 polypeptide (Fig. 4) and these putative NLSs could also be found at conserved positions in CEO2 (Fig. 4).

3.3. *CEO1 may interact with putative Arabidopsis transcription factors*

Because CEO1 had no typical DNA-binding consensus sequences but weakly activated the expression of reporter genes in yeast (data not shown), the CEO1 function might involve

Table 1
Similarities between the members of the CEO1 family

	CEO1	CEO2	CEO3	CEO4	CEO5
	% Identity				
CEO1	–	65.0	32.2	28.8	30.5
CEO2	72.3	–	29.6	29.0	26.8
CEO3	43.6	44.3	–	62.7	34.1
CEO4	42.2	41.7	71.4	–	28.7
CEO5	41.6	36.9	48.5	46.0	–
	% Similarity				

The amino acid sequences of CEO1, CEO2 (new exon prediction from gene GI, 3608137), CEO3 (GI, 4056438), CEO4 (GI, 3176692), and CEO5 (GI, 4741189) and the GCG software program package were used to calculate the protein identity and similarity.

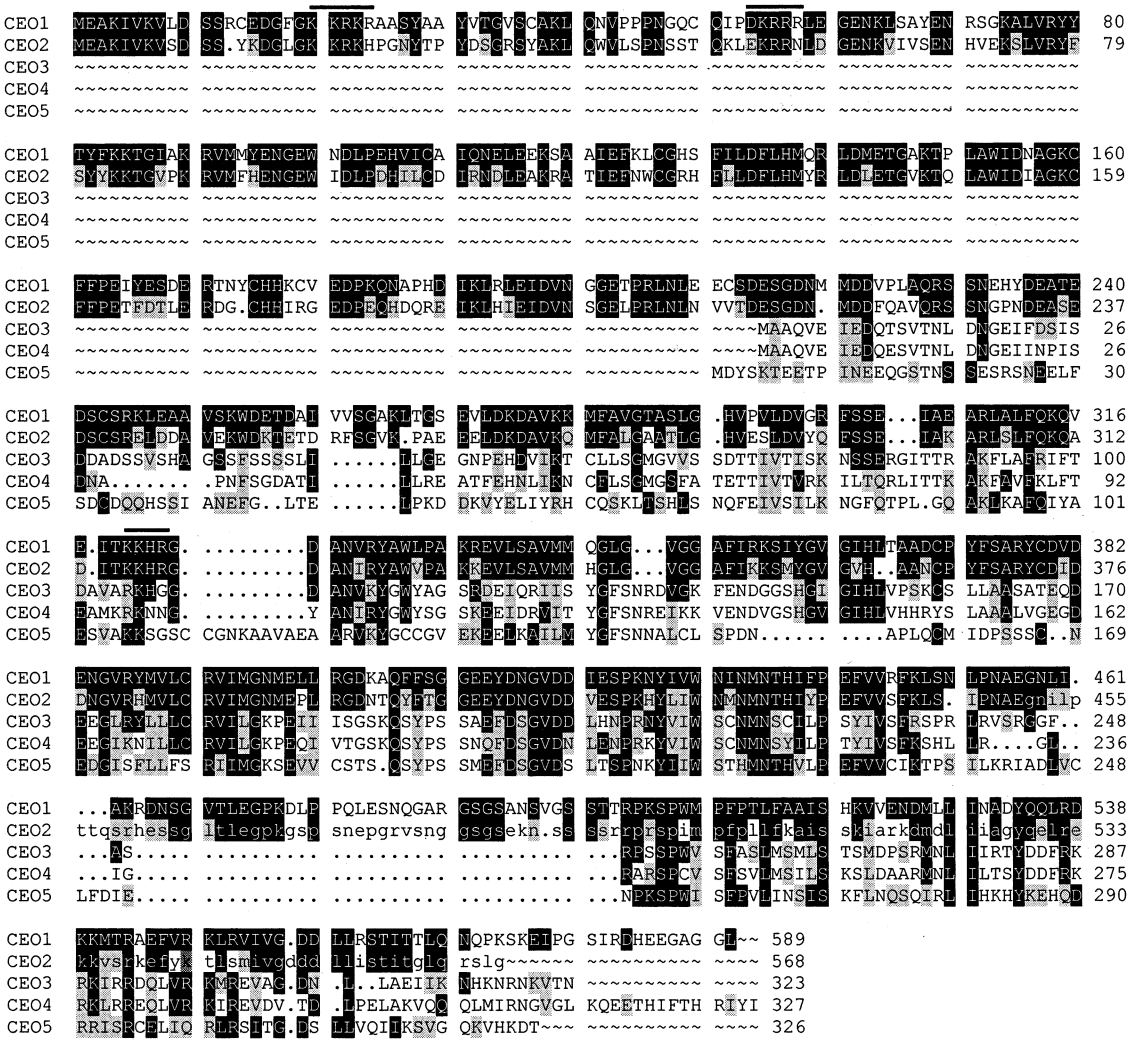

Fig. 4. Sequence alignment of the CEO1 protein and its four *A. thaliana* homologues. The amino acid sequence of CEO1 was aligned with the *Arabidopsis* sequences CEO3 (GI, 4056438), CEO4 (GI, 3176692), and CEO5 (GI, 4741189). For the *CEO2′* gene (GI, 3608137), a new exon prediction was made using the program NNetGene2 (version 2.4; www.cbs.dtu.dk/services/NetGene2) and the resulting sequence was named CEO2. The additionally predicted amino acids are written in lower case letters (positions 451–568) in the sequence CEO2. Identical amino acid residues with respect to CEO1 are shown on black background and similar amino acids are shaded in gray. Dots correspond to gaps introduced to improve the alignment. The three putative NLS sequences are overlined. The sequences were aligned and boxed using the GCG software program package.

the cooperation of other (DNA-binding) proteins. Therefore, and based in the presumption that protein–protein interaction data might contribute to understand the role of CEO1, we carried out a two-hybrid screening. To this end, the reporter HF7c yeast cells were transformed with the bait plasmid pGBT9-*CEO1* and with a plasmid cDNA library from *A. thaliana*, which encodes proteins as carboxyl-terminal fusions with the transcriptional activation domain of GAL4. The screening was done on SD media supplemented with 20 mM AT, which completely abolished background growth of the HF7c (pGBT9-*CEO1*) strain. The His3, β-Lac-positive clones were isolated and further analyzed. After genetic reconfirmation of interaction in yeast and subsequent DNA sequence analysis, 16 different plant cDNAs were identified. Most of these identified cDNAs encoded putative polypeptides of unknown function. Two cDNAs, however, coded for proteins that shared homology with DNA-binding proteins from the putative transcriptional factor families CONSTANS (CO) and ethylene-responsive element-binding protein (EREBP), respectively.

One of the cDNAs encoded the protein STO (X95572) from *A. thaliana*, a protein that confers salt tolerance to yeast [20]. STO is highly similar to putative zinc finger-containing proteins, such as CO (Gi 2695703). These proteins are characterized by the presence of two highly conserved putative zinc finger domains each consisting of two pairs of cysteine residues separated by 16 amino acids (Cys-X_2-Cys-X_{16}-Cys-X_2-Cys). The organization of this motif was similar to that found in the transcription factors from the GATA-1 subfamily, which have two zinc finger domains with the structure Cys-X_2-Cys-X_{17}-Cys-X_2-Cys [21].

The second cDNA coded for a putative protein that was homologous to a large group of proteins that contain the DNA-binding motif AP2. In particular, the strongest homology was found in the DNA-binding domain of several 'related to AP2' (RAP2) proteins from *Arabidopsis* and EREBP pro-

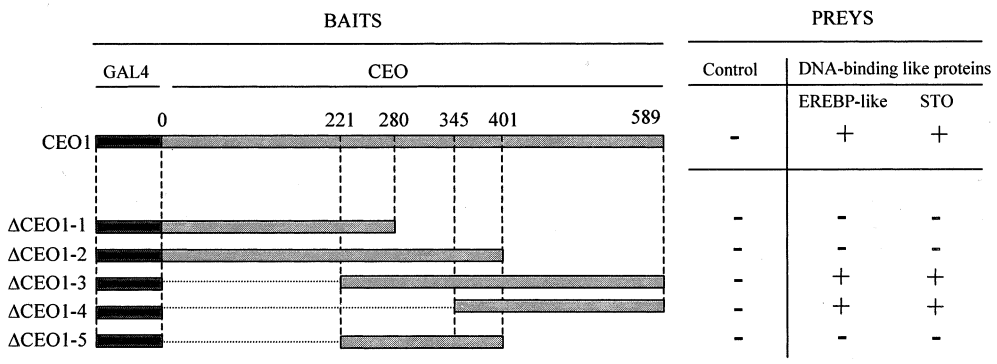

Fig. 5. Functional dissection of the interacting domains of CEO1. To identify physical interactions between different domains of the CEO1 protein and the isolated preys, the DNA-binding (bd) proteins (STO protein and an *Arabidopsis* EREBP-like protein), several deletions in the sequence of CEO1 were generated by PCR. These fragments were cloned in the pGBT9 vector carrying the yeast GAL4-binding domain (GAL4), generating the bait vectors ΔCEO1-1, ΔCEO1-2, ΔCEO1-3, ΔCEO1-4, and ΔCEO1-5. The pGBT9-*CEO1* vector was used as control. Yeast HF7c was transformed with all the possible combinations of the five deletions and the isolated preys. The interactions were determined in SD His^- medium plates supplemented with 20 mM AT and by a β-galactosidase filter assay.

teins from *Nicotiana tabacum*. These proteins are classified as members of the EREBP subfamily of the AP2/EREBP superfamily of transcription factors [22].

Next, we carried out the deletion analysis of the CEO1-coding sequence. The two-hybrid assay was also used to identify domains of interaction of CEO1 with its preys. For this purpose, five deletions in the coding region of CEO1 were generated by PCR (see Section 2) and cloned into the pGBT9 vector as carboxyl-terminal fusions with the DNA-binding domain of GAL4. Yeast HF7c was transformed with all combinations of the CEO1 deletions and the isolated preys. The protein–protein interactions were assessed by growth of the transformants in SD His^- medium with or without 20 mM AT and by subsequent β-galactosidase staining. As summarized in Fig. 5, the interacting domain of CEO1 was located in its carboxyl-terminal part, between the amino acids 345 and 589.

4. Discussion

To identify novel plant genes participating in the tolerance to oxidative stress, we have performed a functional screening in yeast. In this screen we have isolated an *Arabidopsis* cDNA named *CEO1* that, when overexpressed, rendered both the $Yap1^-$ mutant and the wild-type DY yeast strains more tolerant to the lipid-peroxidizing drug tBuOOH. Additionally, the overexpression of *CEO1* in the $Yap1^-$ strain also conferred cross-tolerance to H_2O_2 and diamide, compounds that generate hydroxyl radicals and oxidize thiol groups, respectively.

The previous results indicate that the function of CEO1 in yeast is independent of the stress-responsive regulator YAP1 and that CEO1-like proteins might play a role in oxidative stress responses. On the other hand, we could not detect any change in *CEO1* mRNA levels in plants exposed to oxidative stress, indicating that *CEO1* regulation does not occur at the transcriptional level. The presence of the three short ORFs found in the leader sequence of the *CEO1* cDNA suggests that the expression of CEO1 could be regulated at the translational level. Therefore, to better understand the role of *CEO1*, the expression levels of the CEO1 protein in plants subjected to stress conditions should be further investigated.

Although the function of CEO1 cannot be deduced from an amino acid sequence comparison, we believe that the phenotype of cross-tolerance found in this study strongly suggests that CEO1 affects the yeast stress response systems. This conclusion is supported by numerous studies of yeast, which demonstrate that the overproduction of regulatory proteins confers cross-tolerance to a variety of toxic compounds [23,24].

The physiological role of CEO1 in plants is still unclear. We found that both CEO1 and its close homologue CEO2 have three conserved monopartite NLS sequences and, therefore, a nuclear localization for these proteins is predicted. Additionally, CEO1 interacts physically in the yeast two-hybrid system with DNA-binding-like proteins, which are putative transcription factors from *Arabidopsis*. The first isolated prey that interacted with CEO1 was STO that contains two putative zinc fingers similar to those found in the transcription regulators GATA-1 and CO [25,26]. Expression analyses show that CEO1 and STO share similar features. Both proteins are ubiquitously synthesized in *Arabidopsis* and CEO1 and STO mRNA levels are not increased in *Arabidopsis* plants exposed to oxidative and salt stress conditions, respectively. The second CEO1-interacting protein is a putative protein similar to members from the EREBP subfamily of AP2/EREBP plant transcription factors. Members of this protein subfamily are known to be involved in the induction of defence genes as a response to biotic and abiotic stress [27–29].

The previous results suggest that CEO1 could participate, alone or presumably in combination with other regulatory proteins, in the mechanism of adaptation to stress conditions. Therefore, the association of CEO1 with different transcription modulators would serve to regulate the transcription of specific genes in response to different environmental stimuli. Recent reports support the idea that the activity of transcription regulators can be modulated by the interaction with activator or repressor proteins. In this sense, it has been shown that the GATA-1 protein can interact with two transcriptional cofactors called FOG (for friend of GATA-1) [30] and FOG-2 [31]. These cofactors modulate the activity of GATA factors by acting as activators or repressors depending on promoter and cell type. In conclusion, our data show that the plant-specific protein CEO1 may constitute a cofactor of transcription factors involved in responses to biotic and abiotic stress conditions.

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