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Genome-wide analysis of cytosolic and chloroplastic isoforms of glutathione reductase in plant cells

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Abstract

In recent years regarding the climate change and subsequent environmental stresses, there has been an increasing interest in finding and characterizing of new antioxidant enzymes. Glutathione reductase (GR) is an antioxidant enzyme with central role in maintaining the reduced glutathione pool during stress. So far, however, there has been little discussion on genome-wide analysis of this enzyme. In this study, different computational biology approaches (EST analysis, feature selection, and evolutionary analysis) were exploited to identify the key protein properties influencing on cytosolic and chloroplastic isoforms of glutathione reductase in plants. A specific targeting signal peptide was found in chloroplastic isoforms, while cytosolic isoforms carry a cytosolic domain. This domain may affect the biochemical properties of different GR isoforms. Moreover, specific its functionl motifs were discovered in cytosolic and chloroplastic isoforms implying a link between subcellular localization of GR and functional. Phylogenetic analysis of GR nucleotide and protein sequences showed that diversification of this gene family could be dated back to the early stage of plant evolution, possibly by duplication event before the divergence of monocot and dicot. A high degree of gene structure conservation of localized isoforms in the same subcellular compartment also reflects this process providing an evidence for a close relationship among proteins located in the same subcellular compartment. Study of glutathione reductase expression by EST analysis highlighted cytosolic isoforms as the main isoforrm responding to stress condition.

Keywords EST; Bioinformatics; Signal peptide; Cytosolic domain; Subcellular location.
Abbreviations: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; O_{2^-} , superoxide radical; OH, hydroxyl radicall; RO, alkyl radical; GSSG, glutathione disulfide; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GPx, Glutathione peroxidase; GR, glutathione reductase; UTR, unteranslated region; EST, expressed sequence tag.

Introduction

Aerobic reactions lead to the accumulation of reactive oxygen species, which can be toxic to the cells (Fink and Scandalios, 2002). Biotic and abiotic stresses are known to act as a catalyst in producing free radical reactions resulting in oxidative stress in various organisms where reactive oxygen species (ROS) such as superoxide radical (O_{2^-}), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and alkyl radical (RO) are produced (Fridovich, 1975). ROS rapidly inactivate enzymes, damage vital cellular organelles in plants, and destroy membranes by inducing the degradation of pigments, proteins, lipids and nucleic acids which ultimately results in cell death. In this context, plants possess a complex battery of enzymatic and non-enzymatic antioxidative defense systems that can protect cells from oxidative damage. The enzymatic systems include a set of gene products such as superoxide dismutases (SOD), catalase (CAT), ascorbate peroxidases (APX), glutathione peroxidises (GPX), and glutathione reductases (GR) (Karuppanapandian et al., 2011). Glutathione reductase (GR; EC 1.6.4.2) is a major cellular antioxidant enzyme that widely distributed both in eukaryotes and prokaryotes catalyzing the reduction of oxidized glutathione disulfide (GSSG) to the reduced glutathione (GSH) using NADPH as an electron donor. Mmaintaining high GSH/ GSSG ratio in cells is critical (Meister and Anderson, 1983). It has been reported that GR up-regulates under stresses such as salinity, drought, high light intensity, mechanical wounding, chilling, exposure to heavy metals, and herbicides (Foyer et al., 1991; Romero-Puertas et al., 2006). Isoforms of GR have been reported in many species: in rat liver (Yoshida et al., 1986), mustard (Drumm-Herrel et al., 1989), eastern white pine needles (Anderson, 1992), pea (Creissen et al., 1992), *Chlamydomonas reinhardtii* (Zaffagnini et al., 2008), and red spruce (Belmonte and Stasolla, 2009). GRs in higher plants are encoded by small multigene families classified according to their subcellular localization. Soluble isoforms are found in chloroplasts, cytosol, mitochondria and peroxisomes (Dalkin et al., 1990). However, only two forms of GR genes have been identified to date, a cytosolic isoform (cGR) and an isoform dualytargeted (dtGR) to both chloroplasts and

mitochondria (Chew et al., 2003). In comparison with the other antioxidant enzymes, few studies have been devoted to the description of evolutionary history, gene expression, or gene structure of the GR isoforms. Considering the different subcellular localization and the roles of GR isoforms, it seems likely that the structural diversity of GR genes has been resulted from a complex process of molecular evolution. Bioinformatics and comparative genome analysis are now providing powerful new tools for the molecular dissection of vital phenomena. Rapid increase in the number of published sequences now makes it possible to reconstruct the phylogenetic history of GRs in the green plants. In addition, depositing microarray and EST data in different databanks, such as Gene Index Project (http://compbio.dfci. harvard.edu/tgi) of Harvard University, provides reliable and robust tools for studying the expression patterns of different GR isoforms in stress and non-stress conditions, since it is not clear which isoform responds more during stress conditions. In the other hand, comparative structural analysis of cytosolic and chloroplastic through protein bioinformatics can provide valuable information. Protein function is a direct outcome of its structural amino acid characteristics, so study a large number of amino acid features can provide a comprehensive view of underlying architecture of a specific protein function (Ebrahimi et al., 2011). Recently, machine learning (data mining) techniques have been used widely for understanding the structural properties of a protein molecule. In our previous studies, different feature selection, decision tree, and neural networks algorithms were applied to discover critical features and model thermostability (Ebrahimi et al., 2009; Ebrahimi and Ebrahimie, 2010), ammonium transporters (Tahrokh et al., 2011), and heavy metal protein pumps (Ashrafi et al., 2011). The aims of this study were: (1) to investigate the phylogenetic relationships of GR in plants; (2) to determine the effects of subcellular localization on various properties of GR isoforms by statistical methods; (3) to compare expression of GR proteins in stress conditions; and (4) finding the main structural protein features differing between cytosolic and chloroplastic isoforms by statistical methods.

Results and Discussion

Structural organization of glutathione reductase genes

A detailed comparison of GR genes across plant species (Fig. 1) revealed a high degree of structural gene conservation for GR isoforms localized in the same subcellular compartment. The cytosolic GR genes showed more genomic comprehensive structures than its chloroplastic counterparts (Fig. 1). The cytosolic GR genes are composed of 17 exons interrupted by 16 introns in the transcribed region, but chloroplastic GR genes are composed of 11/10 exons/introns (Fig. 1). Moreover, the length and nucleotide sequence of exons and intron positions in the genes of the same isoform type were very similar. We found that total length of exons in green algae is the nearly same as chloroplastic and cytosolic GRs, while the length and position of introns are different (data not shown). A 5′ non-coding intron with high A + T content was found in the genes for cytosolic GR gene but not in the chloroplastic GR genes (Fig. 1). It has been suggested by Gadea et al. (1999) that the 5′ non-coding intron can be involved in regulating transcription of the corresponding gene because it contains intronic enhancer sequences. It should be noted that high content of relatively weak bands of A + T can facilitate transcription and increase the expression level. GR isoforms (cytosolic and chloroplastic) are generated by different genes or by post-translational modifications of the protein (Creissen and Mullineaux, 1995; Edwards et al., 1994). Alternative splicing of GR nascent mRNAs has been observed in tobacco GR cDNAs (Creissen and Mullineaux, 1995). In peas, a cDNA encoding the predominant isoform of GR has been cloned (Edwards et al., 1994). Differential splicing of nascent GR mRNAs generating different forms of GRs has been reported by Edwards et al. (1994). Since GR transcript codes for a putative plastidial targeting sequence (Creissen et al., 1992), the processing during import into organelles may generate isoforms which are specific to some compartments. Post-translational modifications, such as phosphorylation, glycosylation, and different oxidation processes may be occur (Edwards et al., 1994). However, our results confirmed that GR isoforms are encoded by separate genes rather than post-translational or alternative splicing.

Subcellular location & Transit peptide

Multiple alignment of GR sequence isoforms revealed a targeting signal peptide in the beginning of chloroplastic isoforms which are presented in Fig. 2 a. The protein products of these nuclear-encoded plastid genes are targeted to the chloroplast organelle. Transit peptide sequences located in the N-terminus of these proteins facilitate the transfer from the cytosol, where they are synthesized, to the chloroplast organelle. We analyzed the targeting signal of chloroplastic isoforms that showed this region is rich in Ser (24%) and Leu (11%) but relatively low in Try (0.3%) and Asp (0.6%) which could be due to the folding potential of α – helices (Fig. 2 a). Also, isoelectric point in this section was high (11.44) which may have a pivotal role in intra cellular isoforms. Both cytosolic and chloroplastic isoforms carry the active site, whereas specific cytosolic domain can solely be found in cytosolic isoforms.

Alignment and Molecular Phylogenies of Plant GR Proteins

The alignment of all cytosolic and chloroplastic GRs protein sequences (experimentally verified sequences) retrieved from the NCBI database revealed that the general features of GRs include the important residues in binding GSSG, the redox-active disulphide bridge domain, and the conserved Arg residues required for NADP binding. The amino acid sequence alignment showed that two Cys of the GR redox center are separated by four amino acid residues in the highly conserved motif GGTCV[I/L]RGCVPKK[I/L] LVY (Fig. 2 b). The two redox active cysteines are involved in the transfer of reducing equivalent from FAD to glutathione. A signature was found which clearly identifies higher plant cytosolic isoforms (Fig. 2 b). This signature has 5 residues next to the Glutathione binding site ([ILP]-D-G-[TS]- K) (Fig. 2 b). Although no particular function could be attributed to these signatures, they may affect the biochemical properties of the different GR isoforms. In this study, MEME web tool was used to identify the conserved motifs in GR; we found a total of 15 conserved motifs (Table1). Among those, motifs 1, 2, 3, 4, 5 (except CAA06835), 6, 7, 8 (except CAA06835), 9, 10, 11 and 12 were shared within all isoforms (Fig. 3). To investigate the relationships between motifs and subcellular localization of GR proteins, we further analyzed the subcellular-specific conserved motifs (Fig. 3). We found that motifs 14 and 15 are present in the cytosolic isoforms. Motif 13 was observed only in the chloroplastic isoforms.

Table 1. Motif distribution in GR.

Motif number	Length (aa)	Consensus sequence
Motif 1	50	WAVGDVTNRINLTPVALMEGTCFAKTVFQNQPTKPDYRNVPCAVFCQPPI
Motif 2	41	MCGPDAPEIMQGIAIALKCGLTKQQFDSTVGIHPSAAEEFV
Motif 3	50	YIALEFASIWNGMKCEVHVFYRQEKPLRGFDEEMRDFVAEQMELRGINFH
Motif 4	29	YTAKHIMFATGRRPNMPNIPLEEHGIDMD
Motif 5	41	ICELPFHPISSDWLGGHGGTCVIRGCVPKKILVYGSKYSHE
Motif 6	41	FEDSHNFGWEYNTDPNHNWKKLIQNKNQEIQRLNGIYKNIL
Motif 7	41	LTEEQAIEQYGDVDVYTSNFNPMKNTLSGRPDRVFMKLIVC
Motif 8	25	RHYDYDLFTIGAGSGGVRASRFAAN
Motif 9	21	NAGVKMIEGRGKIVDPHTVEV
Motif 10	15	AIKVDEYSRTSVPSI
Motif 11	15	LDLPSKPKRIAILGG
Motif 12	15	TMRTPTRKVRKNSKP
Motif 13	21	EQSPQAITKSNDGSFSLKTNK
Motif 14	21	PQTNLTELSKTDDGIKVITDH
Motif 15	10	MARKMLKDGE

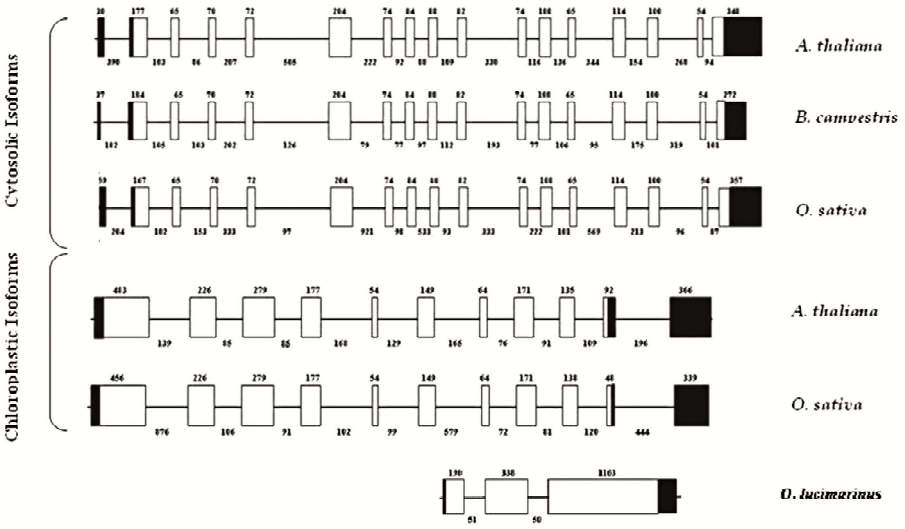

Fig 1. Organization of plant GR genes. The position and size of the intron/exons was predicted using FGENESH and GENSCAN. Exons and non-translated regions are represented by white and black boxes, respectively. Numbers indicate intron/exon size (bp).

Characteristics of functional motifs were predicted by secondary structures of two molecules: AAN13086 and CAB77586 representing cytosolic and chloroplastic isoforms, respectively (Fig. 4). The motif 13 in CAB77586 consisted of loop-α-helix-loop. The motif 14 in AAN13086 was comprised of loop-α-helix-loop-β-sheet-loop. A single β-sheet constituted the motif 15 of AAN13086. Therefore, motifs 14 and 15 were cytosolic-spesific, while motif 11 was chloroplastic-spesific. Phylogenetic analysis were carried out with GR amino acid sequences from species of green plants (Fig. 5). The phylogenetic tree structure clearly revealed that these GRs are split into two main clusters. The first one encompasses chloroplastic isoforms. The second group contains cytosolic isoforms. In addition, we built a phylogenetic tree of nucleotide sequences that conformed result of the above protein sequences (Fig. 6). There is no mixture of members between these two distinct lineages, suggesting that they segregated early in the phylogenetic history. We suggest that an initial duplication event generated the ancestral genes encoding the chloroplastic and the cytosolic isoforms. In addition, the presence of monocot and dicot sequences in both cytosolic and chloroplastic groups suggests that the GR isoforms were generated by duplication events of a single GR ancestral gene that this occurred before the divergence of monocot and dicot species. Our results also demonstrate that none of the monocot specific GR clustered with any group of dicots, implicating that, after the radiation of monocots, GR evolved independently in both monocots and dicots.

Statistical analysis of protein sequence properties

Statistical results showed highly significant differences ($p<0.0001$) in frequencies of Asn, Glu, Pro and Trp and significant differences ($p<0.05$) in frequencies of Gly, Leu, Ser and Thr of cytosolic and chloroplastic GR (Table2). Table 3 presents significant differences ($p<0.05$) on the following features between cytosolic and chloroplastic GRs: isoelectric point, weight, Reduced Cysteines Extinction coefficient Absorption at 280nm 0.001(=1g/l), Reduced Cysteines Extinction coefficient Absorption at 280nm 0.001(=1g/l) and negatively charged. In contrast, no

Table 2. Effect of intracellular location of GR protein sequences on amino acids frequency.

Amino acid	Frequencies mean		P-Value*
	Cytosolic	Chloroplast	
Ala	0.084	0.083	0.887
Arg	0.047	0.047	0.871
Asn	0.044	0.031	0.000
Asp	0.047	0.051	0.149
Cys	0.017	0.015	0.354
Glu	0.071	0.059	0.000
Gln	0.023	0.023	0.735
Gly	0.104	0.090	0.001
His	0.016	0.019	0.209
Ile	0.065	0.053	0.001
Leu	0.074	0.080	0.025
Lys	0.064	0.062	0.337
Met	0.017	0.019	0.337
Phe	0.039	0.043	0.135
Pro	0.040	0.049	0.000
Ser	0.062	0.087	0.002
Thr	0.058	0.068	0.002
Trp	0.012	0.005	0.000
Tyr	0.020	0.021	0.374
Val	0.084	0.084	0.965

*No significant ($P \geq 0.05$)

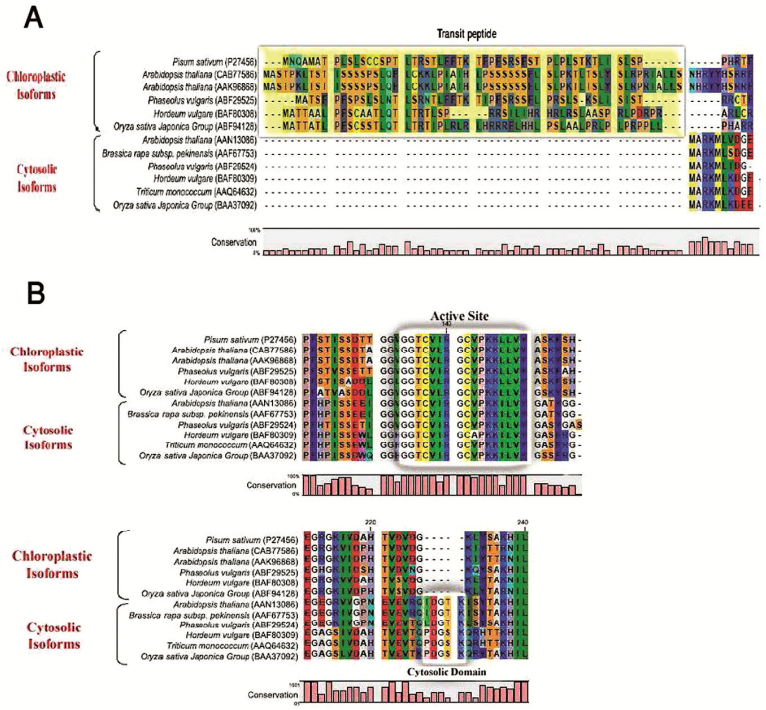

Fig 2. Multiple alignment of isoforms GR sequences. (A) The targeting signal peptide for chloroplastic isoforms; (B) The active site and new cytosolic domain are also indicated. Consensus sequence is appeared bottom of the alignment with graph. The bar shows the conservation of all sequence positions.

significant differences were found between other primary protein structures of GR proteins studied here. Intracellular proteins are relatively poor in cysteine. It has been suggested that this would facilitate or simplify the folding process for intracellular proteins, whereas disulphide bond formation would complicate the process within the cytoplasm (Nakashima and Nishikawa, 1994). No significant difference ($p>0.05$) found between hydrophobic and hydrophilic features of cytosolic and chloroplastic GR and the spectra being mainly negative thus confirming their solubility, respectively, in the cytosol and in the stroma of chloroplast or matrix of mitochondria (Contour-Ansel et al., 2006). Significant difference ($p<0.05$) found in negatively charged residues of GR isoforms which may be important in protein targeting since it has been suggested that this factor plays a role in the recognition phenomenon for the uptake of a particular isoenzyme into an organelle (Contour-Ansel et al., 2006).

Table 3. Effect of intracellular location of GR protein sequences on different protein features.

Feature	Cytosolic	Chloroplast	P-Value*
	mean ± SD	mean ± SD	
Length	395±162	483±137	0.058
Weight (kDa)	35.1 ± 21.2	49.0 ± 18.9	0.027
Aliphatic index	85.3±16.1	85.49±3.83	0.73
Sulphur (frequency)	0.00206±0.000354	0.0024 ±0.000892	0.124
Carbon (frequency)	0.31488±0.00273	0.31394±0.00243	0.236
Nitrogen (frequency)	0.08678±0.00134	0.08619±0.00111	0.112
Oxygen (frequency)	0.095375 ± 0.000751	0.09613±0.00150	0.076
Hydrogen (frequency)	0.50091±0.00324	0.50181± 0.00160	0.201
Isoelectric point	6.621± 0.714	7.61± 1.26	0.009
Non-reduced Cysteines Extinction Coefficient at 280nm	10288± 33895	8989 ± 29118	0.108
Non-reduced Cysteines Extinction Coefficient Absorption at 280nm 0.001(=1g/l)	0.928± 0.409	0.5636± 0.076	0.000
Reduced Cysteines Extinction Coefficient at 280nm Reduced Cysteines	33520±10161	28732 ± 8915	0.104
Negatively Charged	0.119± 0.008	0.110 ± 0.013	0.023
Positively Charged	0.112± 0.006	0.111 ± 0.004	0.299
Hydrophobic	0.525± 0.011	0.513 ± 0.024	0.064
Hydrophilic	0.227± 0.016	0.248± 0.038	0.053

*No significant (P ≥ 0.05)

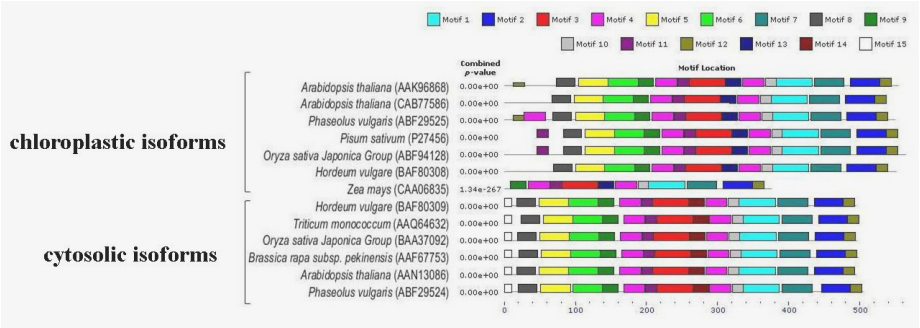

Fig 3. Motif distribution of GR. Motifs of GR were investigated using the MEME web server.

Expression analyses

Statistical analysis showed EST expression of cytosolic GR was significantly ($p<0.05$) higher (6 times more) in stress conditions compared to non-stressed conditions; while no differences ($p>0.05$) found in chloroplastic GRs. These findings were in line with previous studies on progressive drought stress, desiccation and abscisic acid treatment (Contour-Ansel et al., 2006), including progressive drought stress in pea (Bhatnagar-Mathur et al., 2009), high light stress in Arabidopsis (Giacomelli et al., 2007), and drought and ABA treatment in rice (Kaminaka et al., 1998); although the extent of expression in various plants was dependent to the type of stress. It has been argued that gene expression of the cytosolic GRs is regulated under environmental stresses via an ABA-mediated signal transduction pathway (Contour-Ansel et al., 2006). Different subcellular forms of GR may have different expression level under stress conditions. It has been suggested that plastidial/mitochondrial GR may be non-inducible when the organelles are subject to constant oxidative stress through ROS production associated with photosynthesis and respiration (Tarrago et al., 2009). Consequently, proteins involved in prevention of oxidative stress in these organelles may have acquired an essentially house-keeping function (Wu et al., 2006). The results of this study confirmed that ESTs analysis is an effective approach to study of specific GR gene expression in response to different kinds of environmental stresses. For the first time, we suggest that and amino acid in line with cellular location of a protein strongly affect on expression.

Materials and methods

Retrieval of GR Sequences

All proteins encoding GRs in green plants were retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and Expasy (http://expasy.org/) data banks. Then, redundant sequences were removed, and final non-redundant list (including 67 sequences) was used in this work (supplementary 1).

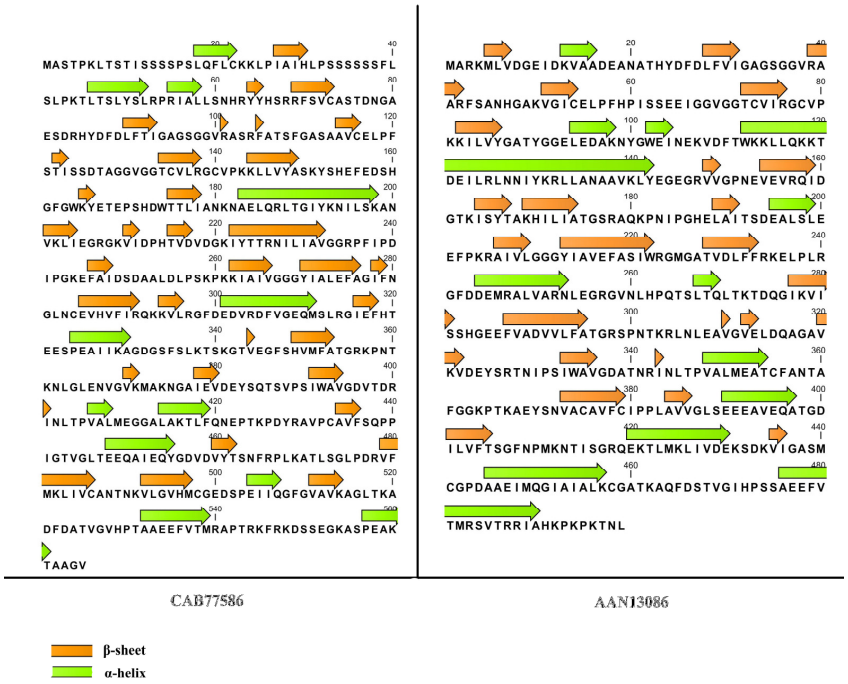

Fig 4. Secondary structure prediction of GR. Two GR from AAN13086 and CAB77586 which represent cytosolic and chloroplastic isoforms, respectively, were selected to illustrate differential motifs.

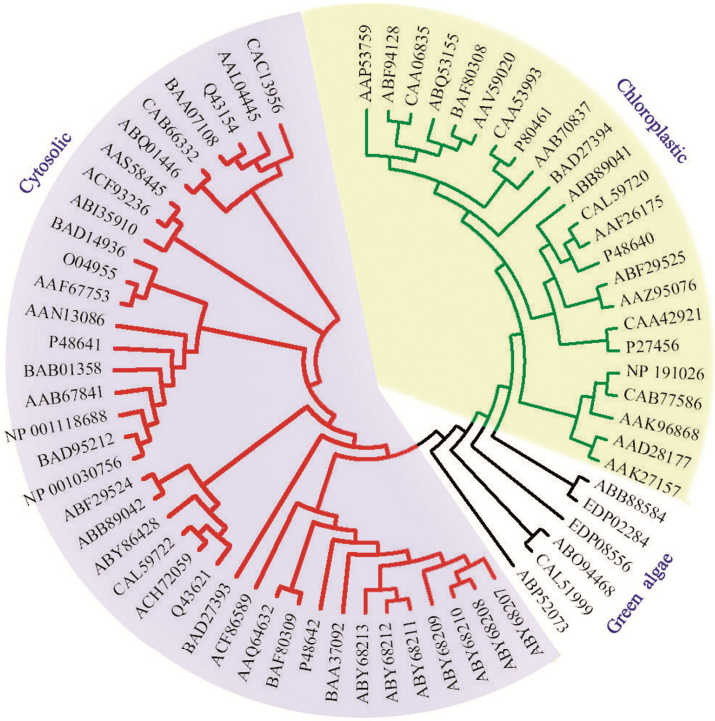

Fig 5. Phylogenetic tree of GR proteins from different organisms. Phylogenetic analysis were conducted using the MEGA version 2.1 software. Protein sequences are identified by their accession numbers in the NCBI database.

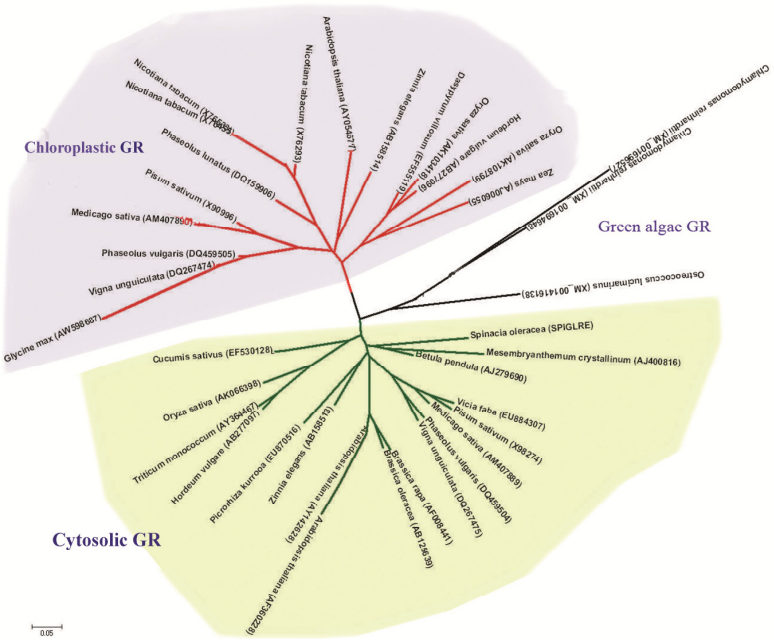

Fig 6. Phylogenetic tree of GR nucleotide sequences. Phylogenetic analysis were conducted using the software MEGA Version 2.1. nucleotide sequences are identified by their accession numbers in the NCBI database.

Nucleotide sequences and cDNA sequences were obtained from the NCBI and TIGR (http://www.tigr.org) databanks.

Gene structure

To investigate the relationships between the GR isoforms, the structural organization of GRs genes was considered by comparison of genomic and cDNAs/ESTs sequences, where available. The structural organization of GR genes were determined by aligning the genomic DNA and cDNAs/ESTs sequences. Genomic sequences were also analyzed in the FGENESH gene structure prediction program (http://www.softberry.com) and GeneMark program (http://opal.biology.gatech.edu/GeneMark).

Sequence Alignments

Multiple sequence alignments of GR sequences were constructed using CLCbio (CLC Main Workbench 5.8) software with the following parameters: gap opening penalty = 10.0, gap extension penalty = 0.05 and BLOSUM protein weight matrices.

Motif identification

MEME program (Bailey et al., 2009) was used for finding motifs in GR. MEME was run from the web server (http://meme.sdsc.edu/ meme4_6_1/cgi-bin/meme.cgi) with the following parameters for each motif, the minimum motif width, 6aa; maximum width, 50 aa; maximum motif number, 15. The consensus motifs were obtained using the MAST. The Secondary structures were predicted using CLC protein workbench tool (www.clcbio.com/protein) based on Makov model (Rost, 2001).

Expression assay

To compare expressions of GR isoforms in stress and non-stress conditions, barley (*Hordeum vulgare*) was selected as a sample and ESTs of 167 cDNA libraries were downloaded from the Gene Index Project of Harvard University (http://compbio.dfci.harvard.edu/tgi). This data bank provides access to the ESTs of various plants and enzymes through metabolic pathways in different conditions. ESTs were divided into cytosolic and chloroplastic groups, and in each group two subgroups of stress and non-stress were considered. From 167 libraries of barely in the mentioned database, ESTs of GRs were found in 44 libraries; 4 chloroplastic and 40 cytosolic. Two of chloroplastic ESTs libraries were from stressed and the others from non-stressed conditions. In cytosolic libraries, 12, 27 and 1 were from stressed, non-stressed and unknown libraries. Statistical differences between expressed genes of GR in stress and non-stress conditions of both chloroplastic and cytosolic isoforms were determined by Mann-Whitney test. The *P* values less than 0.05 were considered statistically significant. Data were analyzed by Minitab 14 (www.minitab.com).

Phylogenetic tree constructions

The molecular evolutionary and phylogenetic analyses of 67 GR sequences in green plants were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software (Version 4.0.). The molecular distances of the aligned sequences were calculated according to the parameter of *p*-distance, and the phylogenetic trees were constructed using the Neighbor-Joining method with pairwise deletion. Interior Branch Test with 1000 Replications was used as phylogenic test (Felsenstein, 1985).

Protein sequence analyses

Protein sorting and subcellular localization predictions were performed according to ProtComp program Version 5 (http://www.softberry.com/) and PSORT software Version 6.4 (http://psort.nibb.ac.jp/). The signal peptide was identified using the SignalP version 4.0. (http://www.cbs.dtu.dk/services/SignalP/). The protein features (molecular weight and length, Amino acid frequency, frequency of atoms, electrical charge, aliphatic index, isoelectric point, extinction coefficient) calculated by using CLC protein workbench tool (www.clcbio.com/protein) (Roy et al., 2011).

Statistical analysis

The effect of protein sequence subcellular localization on molecular weight and length, amino-acid-frequency, frequency of atoms, electrical charge, aliphatic index, isoelectric point, extinction coefficient was tested by comparing protein sequences of chloroplastic versus cytosolic. Differences were statistically evaluated by T-test method (Tahrokh et al., 2011; Ebrahimie et al., 2011). Data were analyzed by Minitab 14 statistical software.

Conclusion

In this investigation, a high degree of structural gene conservation was found for GR isoforms localized in the same subcellular compartment. The cytosolic GR genes showed more genomic comprehensive structures than their chloroplastic counterparts. In addition, the expression analysis realized a strong correlation between the subcellular location of GR isoform and response to different stress conditions. Uncovering the some important structural characteristics of GR sequences in different cell compartments in this study provides the required knowledge for future modification and engineering of these proteins and prediction of subcellular target of GR sequences.

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Supplementary Data

Species and accession numbers of GR protein sequences retrieved from Genbank, and used in the current work.

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