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Research article

Antisense-mediated depletion of tomato chloroplast glutathione reductase enhances susceptibility to chilling stress

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ABSTRACT

A tomato (*Lycopersicon esculentum* Mill.) chloroplast glutathione reductase gene (*LeGR*) was isolated and antisense transgenic tomato lines were obtained. Under chilling stress, transgenic plants accumulated more H_2O_2, leaked more electrolyte and showed lower net photosynthetic rate (Pn), maximal photochemical efficiency of PSII (Fv/Fm) and oxidizable P700 compared with wild-type (WT) plants. Transgenic seedlings were more suppressed in fresh-weight growth and lost more cotyledon chlorophyll. The decrease in the activity of ascorbate peroxidase (APX) was implied to be potentially relevant to the greater accumulation of H_2O_2 in transgenic plants. Chilling treatment induced more decrease in the level of reducted glutathione (GSH) and redox ratio of glutathione in transgenic plants than in WT plants, but aroused more increase in GSSG in transgenic plants than in WT plants. Total glutathione displayed no change. Besides, chilling stress resulted in greater decreases in the level of reducted ascorbate (AsA), total ascorbate and redox ratio of ascorbate in transgenic plants than in WT plants, but led to equivalent degree of dehydroascorbate (DHA) increase in WT and transgenic plants. These assessments of glutathione–ascorbate cycle revealed that the decrease of glutathione reductase activity in transgenic plants affected glutathione regeneration, and consequently affected ascorbate regeneration and total ascorbate content. This resulted in a greater accumulation of H_2O_2 and an enhanced sensitivity to chilling stress in transgenic plants. Moreover, a putative concept model of ecophysiological reaction was discussed.

1. Introduction

Chilling stress is a major factor limiting the geographical distribution and productivity of chilling-sensitive plant species, including important vegetable crops such as tomato, cucumber and sweet pepper. Chilling stress constrains the activity of enzymes in Calvin cycle and consequently reduces the utilization of absorbed light energy for CO_2 assimilation [1]. The photosystem reaction centers are thus overreducted, which leads to accumulation of reactive oxygen species (ROS) in the cell [2]. The presence of ROS in cells is an inescapable feature of plants which have been exposed in an oxygen atmosphere throughout their life [3]. Under normal growth conditions, ROS are continuously produced in the chloroplasts, mitochondria and peroxisomes as byproducts of aerobic metabolic processes like photosynthesis, respiration and photorespiration. The chloroplast appears to be a main site of ROS generation [4]. Furthermore, the production of ROS increases significantly during unfavorable conditions like drought, salinity, ozone stress, high light and chilling stresses [5,6]. These excess ROS cause damage to proteins, lipids, carbohydrates and DNA, and ultimately result in cell death [7,8].

Plants have evolved antioxidative systems to keep ROS under control. Glutathione is one of the most important antioxidants which detoxify ROS and protect plants from oxidative damage [9,10]. Glutathione exists in two different forms: the reduced form (GSH) and the oxidized disulphide form (GSSG). When acting as an antioxidant, GSH is oxidized to GSSG and the antioxidant function of glutathione is mainly attributed to its reduced form [9]. Thus, it is necessary for plants to maintain a high proportion of reduced

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; Fv/Fm, the maximal photochemical efficiency of PSII; EDTA, ethylenediaminetetraacetic acid; GFP, green fluorescence protein; GR, glutathione reductase; GSH, reducted glutathione; GSSG, oxidated glutathione; MDA, monodehydroascorbate radical; MDAR, monodehydroascorbate reductase; MV, methyl viologen; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate; NEM, N-ethylmaleimide; ORF, open reading frame; PFD, photon flux density; Pn, the net photosynthetic rate; RACE, rapid amplification of cDNA ends; REC, relative electronic conductance; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; WT, wild type.

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glutathione (expressed in the ratio of GSH/GSSG) [10,11]. GSH can be synthesized in both the chloroplast and the cytosol of plant leaves. In addition, glutathione reductase (GR, EC 1.6.4.2), with the NADPH as electron donor, can catalyze the reduction of GSSG to GSH, which takes place in the ascorbate–glutathione cycle [12,13]. The ascorbate–glutathione cycle is considered as the main pathway of ROS detoxification in the chloroplast [13]. In addition to GSH, there is another significant antioxidant ascorbate in this cycle. Ascorbate provides electron to ascorbate peroxidase (APX) for detoxification of H_2O_2. Moreover, it has been shown that ascorbate can donate electrons to PSII in thylakoids to protect PSII from photoinhibition when electron donation from water is disturbed [14]. Ascorbate exists as the reduced form (AsA) and the oxidized disulphide form (DHA) as well. A high proportion of AsA (expressed in the ratio of AsA/DHA) is also important for plants. GR is considered essential for plants to maintain the high proportion of GSH and AsA.

GR plays an important role in response to oxidative stresses in plants [11,15], which has been universally advocated by numerous existing reports based on high activities of GR in stress-tolerant plants [16] and in transgenic plants with overexpression of GR [1,17]. Besides, Aono et al. [18] and Ding et al. [19] reported that the suppression to chloroplast GR activity enhanced the sensitivity to paraquat and methyl viologen (MV), respectively, in transgenic tobacco. However, the mechanism of GR in determining plant tolerance to stresses remains not clear. As far as chilling stress in particular, it had been reported that PSI, rather than PSII, was the basic site of photoinhibition under chilling stress with low irradiance [20]. A positive correlation between resistance to chilling-induced photoinhibition and high GR activities has been shown in rice [21], maize [22] and tomato [23]. However, the role of GR in protection to PSI against photoinhibition still needs to be estimated. How GR influences plant tolerance to chilling stress remains unclear.

In order to investigate the mechanisms of GR to determine plant tolerance to chilling stress, we obtained antisense transgenic plants with 60% decrease in GR activity and measured the changes of their parameters relating to photosynthesis, growth and oxidative damage, which indicated the enhanced sensitivity to chilling stress in transgenic plants relative to WT plants. The decrease in the activity of ascorbate peroxidase (APX) implied to be potentially relevant to the greater accumulation of H_2O_2 in transgenic plants. Furthermore, the assessment of glutathione–ascorbate cycle revealed that the decrease of glutathione reductase activity in transgenic plants affected glutathione regeneration, and consequently affected ascorbate regeneration and total ascorbate content. This led to a greater accumulation of H_2O_2 and an enhanced sensitivity to chilling stress in transgenic plants.

2. Materials and methods

2.1. Plant growth and treatments

Tomato seeds of wild-type cultivar (*Lycopersicon esculentum* cv. Zhongshu 4) and T_1 generation of antisense GR transgenic plants lines were allowed to germinate on moistened filter paper at 25 °C for 3 days. Sprouted seedlings were transplanted into sterilized soil and grew at 25/20 °C (day/night) with a 16/8 h photoperiod, 300–400 μmol m^{-2} s^{-1} photon flux density (PFD), and 50–60% relative humidity in greenhouse. The 7-week-old WT and transgenic plants were used for subsequent abiotic stress assays. For temperature treatment, the whole plants were exposed to low temperatures (4 °C) for 24 h in an illuminated incubation chamber (GXZ-260C) at PFD of 100 μmol m^{-2} s^{-1}. All the physiological and biochemical measurements were carried out on the youngest fully expanded leaves.

2.2. Isolation and subcellular localization of LeGR

Total RNA isolated from tomato leaves using the total RNA isolation system (Promega Corporation, Madison, WI) was used for reverse transcription-polymerase chain reaction (RT-PCR) to produce cDNA. To isolate the chloroplast GR gene, a 338 bp fragment was amplified from cDNA prepared. Primers GR1: 5′-AT(A/C/T)TGGGC(A/T)GTGGG(A/G/T)GATG-3′ and GR2: 5′-GGTGCATC(A/T)(G/T)(G/C)(A/T)CCACACAT-3′, which clamp conserved sequence, were designed based on the homology of chloroplast GR gene between tobacco (*Nicotiana tabacum*, X76293), soybean (*Glycine max*, L11632), mustard (*Brassica juncea*, AF109694) and zinnia (*Zinnia violacea*, AB158514). The 5′-rapid amplification of cDNA ends (RACE) PCR was carried out by using the gene-specific primer GR3: 5′-ATGGCCTCTTCTTCTGTCAGG-3′ and an abridged universal amplification primer AAP according to the manufacturer instructions (GIBCO-BRL Kit; Invitrogen, Carlsbad, CA). The 3′-RACE PCR was carried out by using the gene-specific primer GR4: 5′-GGTAGTATGTGCGAAGTCTAG-3′ and B26. The putative full length of LeGR was amplified from the cDNA using 5′- and 3′-specific primer pair: GR5: 5′-CATGGCTACATCTTTGAGCT-3′ and GR6: 5′-AAGCAATCCGCTTAACTGCT-3′. All the primers were synthesized from Bioasia Bio-engineering Limited Company, Shanghai, China. Nucleotide and putative amino acid sequences were analyzed with DNAMAN version 5.2 (Lynnon Biosoft, Vaudreuil-Dorion, Canada). Sequence data from this article have been deposited at GenBank under accession number EU285581.

Two DNA plasmids (p35S-GFP and p35S-LeGR-GFP) were used to investigate the intracellular targeting of LeGR by observation on transient expression of GFP in Arabidopsis mesophyll protoplasts. Plasmid p35S-GFP was obtained from the Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences (Beijing, China). The complete coding region of LeGR was integrated into the p35S-GFP vector between the Hind III and Sal I sites, with the green fluorescence protein (GFP) coding region upstream and in frame. Arabidopsis mesophyll protoplasts were isolated, transfected with the two constructions and examined by dual channel confocal microscopy (LSM510 META, Zeiss, Germany). The bright field image, GFP fluorescence and the red autofluorescence of chloroplast from protoplast expression were recorded simultaneously and compared. The potential colocalization of GFP fluorescence and chloroplast autofluorescence was further analyzed by checking the presence of yellow signals in the superimposed images.

2.3. Plasmid construction and Agrobacterium mediated transformation of tomato plants

Full-length LeGR cDNA was subcloned into the pBI121 with caulifower mosaic virus 35S promoter (35S-CaMV) promoter upstream to form antisense constructs (pBI-RVLeGR). The antisense 35S-CaMV:LeGR recombinant was introduced into *Agrobacterium tumefaciens* LBA4404 via freezing transformation method. Leaf disk transformation was performed as described by Horsch et al. [24]. Leaf disks infected with *A. tumefaciens* were cultured on the medium to induce shoots. Four to eight weeks later, regenerated shoots were transferred to the medium to induce roots. Both of the media above contained cefotaxime sodium (250 μg mL^{-1}) to suppress *A. tumefaciens* and kanamycin (50 μg mL^{-1}) as selection pressure. As a consequence, from tissue culture 28 individual kanamycin-resistant lines were obtained and 18 lines were screened out as T_0 generation of transgenic plants by PCR