Overexpression of maize MYB-IF35 increases chilling tolerance in Arabidopsis

Chen Meng, Na Sui

Abstract

Chilling stress is a critical environmental factor that limits plant growth, yield and distribution. Maize (Zea mays L.) is an important food and forage crop, and industrial raw material, in China. Low temperatures can decrease maize production, especially in early spring. The R2R3-MYB transcription factor ZmMYB-IF35 was isolated from maize cDNA. The open reading frame of ZmMYB-IF35 is 1038 bp, encoding 345 amino acids with a molecular mass of 37.9 kDa. ZmMYB-IF35 localized in the nucleus. Low temperatures induced the expression of ZmMYB-IF35 in maize, and the relative expression level reached its maximum after 4 h of chilling stress. The overexpression of ZmMYB-IF35 under the control of the CaMV35S promoter in Arabidopsis conferred tolerance to chilling stress compared with the wild-type plants by maintaining the maximal photochemical efficiency of photosystem II. Furthermore, under chilling stress, the ZmMYB-IF35 transgenic plants showed greater antioxidant enzyme activity levels, lower reactive oxygen species contents and lower ion leakage levels than those of wild-type plants. Thus, the overexpression of ZmMYB-IF35 may enhance resistance to chilling and oxidative stresses in transgenic Arabidopsis and alleviates PSI photoinhibition.

1. Introduction

Chilling stress is an abiotic factor that limits plant growth, development, distribution and yield. During chilling stress, a number of major biological processes, such as photosynthesis, protein synthesis, lipid metabolism and secondary metabolism, are affected. The first component damaged by chilling stress is the chloroplast membrane. Chilling tolerance is related to the composition and structure of plant membrane lipids and their ability to transition from a liquid-crystalline phase to a gel phase. Tolerance to chilling stress is closely connected with the unsaturated fatty acid content of plant membrane lipids.

Low temperatures can affect all aspects of plant physiological metabolism. Photosynthesis is highly susceptible to low temperatures (Powles, 1984). Low temperature stress can affect many aspects of photosynthesis, including stomatal opening, photosynthetic electron transport rate, carbon assimilation and other processes. When the light energy absorbed by the photosynthetic mechanism exceeds the range that can be used, the photochemical efficiency of the system is reduced and photoinhibition is induced, which results in the decrease in the photosynthetic rate and photosystem II (PSII) photochemical efficiency (Fv/Fm). Under low temperature conditions, the decrease in the photosynthetic rate in plant leaves is related to the damage to the PSII reaction center and chloroplasts. Under long-term low-temperature stress conditions, membranes often suffer severe damage, resulting in a large accumulation of reactive oxygen species (ROS), severe PSI photoinhibition, and even light-related damage, which, to some extent, reduces the damage to photosystem I.

Under abiotic stress conditions, if excess light cannot be dissipated in time, or the carbon assimilation process is blocked, the accumulation of ROS, such as superoxide (O$_2^-$), singlet oxygen, hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical is induced (Foyer and Shigeoka, 2011). The excessive accumulation of ROS causes a variety of serious injuries to plants (Singh and Sinhal, 1999; Stepien and Klobus, 2005; Flowers and Colmer, 2008), such as damage to the proteins, carbohydrates, lipids and DNA, and even results in cell death (Mittler et al., 2004; Foyer and Noctor, 2005). Fortunately, plants have developed some protective mechanisms, including enzymatic and non-enzymatic antioxidant defense systems, to detoxify ROS and to protect themselves from oxidants. The non-enzymatic antioxidants include ascorbic acid, carotenoids, glutathione, tocopherols and flavonoids. Superoxide dismutase (SOD) is a major scavenger that defends against oxidative stress induced by superoxide in plant cells, and it plays a key role in scavenging O$_2^-$ to form H$_2$O$_2$. Ascorbate peroxidase (APX) plays important roles in protecting the component of chloroplasts and other cells from damage by...
H$_2$O$_2$ and the hydroxyl radicals. It converts H$_2$O$_2$ to water with ascorbic acid (AsA) as a specific electron donor and is involved in the most important pathway in H$_2$O$_2$ detoxification in chloroplasts (Foyer and Halliwell, 1976; Noctor and Foyer, 1998; Asada, 1999). Because more than 50% of APX in the chloroplasts is thylakoid bound, the membrane lipid transition from the liquid crystalline phase to gel phase during chilling stress can affect the activity level of thylakoid-bound APX and consequently affect ROS scavenging (Sonoike and Terashima, 1994).

Maize (Zea mays L.) is an important food and crop, as well as an industrial raw material, in China, playing important roles in agriculture and in the national economy. The accumulated temperature of the whole growth period is more than 10 °C. The optimum growth temperature is between 25 °C and 28 °C. When the temperature is lower than 12 °C, the plants are vulnerable to damage by chilling stress. Low temperatures can decrease maize production by affecting seed germination and seedling growth, especially during early spring. However, the mechanism of chilling tolerance in maize is still unknown. In this study, the important ZmMYB-IF35 gene, which is involved in chilling tolerance in maize, was screened, and its function during chilling stress was investigated. The results provide valuable information to increase our understanding of the mechanisms of chilling tolerance in maize.

2. Materials and methods

2.1. Plant materials and growth conditions

In the previous experiment, 102 maize inbred lines were used for chilling tolerance determination, and a chilling tolerant line, M54, and a chilling sensitive line, 753F, were selected for RNA-seq analyses. In this experiment, seeds of maize inbred line M54 (chilling tolerant) were used. Dry seeds were stored in a refrigerator at 4 °C before use. Maize seeds of uniform size were selected and soaked in water for 10 h. Plants were grown at 30/22 °C (day/night), with a 14-/10-h (light/dark) photoperiod and light intensity of ~400 ± 50 µmol m$^{-2}$ s$^{-1}$. The relative humidity levels were 60% and 70% during the day and night, respectively.

Arabidopsis ‘Columbia-0’ was selected as the wild-type (WT) control. Arabidopsis seeds were grown at 22/18 °C (day/night) under a 16-/8-h (light/dark) photoperiod. Two weeks later, the plants were treated with chilling stress (4 °C) for 3, 6, 9 and 12 h to determine the physiological indices.

2.2. Cloning and sequencing of MYB-IF35, and its transformation into Arabidopsis

From the RNA-seq data, the gene of MYB-IF35 which was up-regulated only in M54 was selected in this experiment. Total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions. Gene-specific primers (forward: 5′-ATGGGCCAGGCGCGTGC-3′ and reverse: 5′-GAGATTGCTCCAGGAAGAGG-3′) were used to amplify the full length of MYB-IF35. The full-length open reading frame of MYB-IF35 was inserted into the plant binary vector pROKII to construct pROKII-MYB-IF35. Then, the MYB-IF35 gene controlled by the CaMV35S promoter was transformed into Arabidopsis using the Agrobacterium tumefaciens-mediated transformation method (Zhang et al., 2006; Sui et al., 2017), and the T3 generation was used for further analysis.

2.3. Bioinformatics analysis of MYB-IF35

The BLASTp online tool and DNAMAN software were used to analyze the phylogenetic relationships, based on the amino acid sequences, of ZmMYB-IF35 with MYB-IF35 genes from other plant species.

2.4. Quantitative real-time PCR analysis

The expression profile of ZmMYB-IF35 in maize leaves under chilling stress (4 °C) for 0, 4, 12 and 24 h was investigated using forward (5′-CTTGGCAACAGGTGCTGC-3′) and reverse (5′-GGTGGAGTTCCAGTATGT-3′) primers. ZmMYB-IF35 was also overexpressed in Arabidopsis lines. Amplification of the ACTIN gene was used as an internal control, and the primer pairs used for the amplification were ZmActin-F (5′-GGAGCTCGAGAGATGCCAAGAGG-3′) and ZmActin-R (5′-GACCTCGAGGGAAGAAGCAG-3′) and AtActin2 (5′-AAGCAGGGTTTTATGAGTG-3′) and AtActin2 (5′-TTGTCAACACAAATGCTCTACAT-3′), which were designed according to the ACTIN nucleotide sequences of maize and Arabidopsis, respectively.

2.5. The subcellular localization of MYB-IF35

The full length of ZmMYB-IF35 was generated by PCR and then cloned into the pROKII-GFP vector using forward (5′-CGAGGCTCATTGGGAAGGCCCGTGCG-3′) and reverse (5′-GGGGTGAGTTCCAGTGCGGAAGAAGAGG-3′) primers. The pROKII-GFP-ZmMYB-IF35 transient vector was successfully constructed and transformed into A. tumefaciens ‘EHA105’. Tobacco epidermal cells were then infected, and fluorescence microscopy was used to determine the subcellular localization of the ZmMYB-IF35 protein.

2.6. Determinations of minimal fluorescence (Fo) and maximal photochemical efficiency (Fv/Fm)

Chl fluorescence was determined according to our previous method (Yang et al., 2013; Cheng et al., 2014) using a portable fluorometer (FMS2; Hansatech, King’s Lynn, UK). The Fo with all PSII reaction centers open was determined by modulated light that was low enough not to induce any significant variable fluorescence (Fv). Maximal fluorescence (Fm) with all reaction centers closed was determined by 0.8 s saturating light of 8000 µmol m$^{-2}$ s$^{-1}$ on a dark-adapted leaf (adapted 30 min in darkness). The Fv/Fm of PSII was expressed as: Fv/Fm = (Fm − Fo)/Fm.

2.7. Determinations of the antioxidant enzyme activity levels, and O$_2$− and H$_2$O$_2$ contents

Ascorbate peroxidase (APX) activity was determined according to our previous method (Sui, 2015) by measuring the decreased absorbance at 290 nm. Enzyme activity was calculated per mg of total protein in U, which represents the amount of enzyme needed to oxidize 1 µmol of AsA within 1 min at room temperature.

SOD activity was determined according to
Enzyme activity was calculated as 50% inhibition, expressed in U per mg of total protein, by measuring the absorbance at 560 nm using a UV/Vis spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan).

The \( \text{O}_2^- \) content was analyzed according to the method introduced in our previous study (Liu et al., 2017). The \( \text{O}_2^- \) generated, presented as per g fresh mass of leaves, was determined by measuring the absorbance at 530 nm using the water phase.

Elicitation of \( \text{O}_2^- \) can be \textit{in situ} detected by nitroblue tetrazolium (NBT) according to the method of Buapet and Björk (2016).

The \( \text{H}_2\text{O}_2 \) content was determined according to the method introduced in our previous study (Sui et al., 2018) by measuring the absorbance of the titanium–hydroperoxide complex and a standard curve plotted was used in the determination.

Elicitation of \( \text{H}_2\text{O}_2 \) can be \textit{in situ} detected by 3,3′-diaminobenzidine (DAB) staining according to the method of Thordal-Christensen et al. (1997).
2.8. Determination of relative electronic conductance (REC)

The REC was determined according to our previous method (Zhou et al., 2016). In total, 40 leaf disks (1 cm² each) were placed in a cuvette containing 20 mL distilled water, placed in a vacuum for 30 min, and then subjected to an electrical surge for 4 h to measure the initial electronic conductance (S1). The cuvette was heated in boiling water for 30 min and cooled to room temperature to determine the final electronic conductance (S2). The REC was evaluated as follows: REC = (S1/S2) × 100.

2.9. Statistical analyses

Statistical analyses were performed to ensure the homogeneity of variance. All tests were performed with SPSS Version 19.0 for Windows (SPSS, Chicago, IL, USA). Multiple comparisons between different treatments were performed using Duncan’s multiple range test at the 0.05 significance level.

3. Results

3.1. Sequence analysis of ZmMYB-IF35

The open reading frame of ZmMYB-IF35 of maize is 1038 bp, which encodes 345 amino acids with a molecular mass of 37.9 kDa. To investigate the evolutionary relationships between maize ZmMYB-IF35 and MYB-IF35s from other plants, a phylogenetic tree of the conserved cyclin-box domains was constructed. Maize MYB-IF35 shared the highest identity with MYB-IF35 from Sorghum bicolor (Fig. 1).

3.2. Subcellular localization of ZmMYB-IF35

To check the subcellular localization of ZmMYB-IF35, the recombinant pROKII-GFP-ZmMYB-IF35 fusion vector and the pROKII-GFP vector alone were independently transformed into tobacco epidermal cells through Agrobacterium infection. The GFP alone was expressed throughout the whole cell, while the pROKII-GFP-ZmMYB-IF35 fusion protein accumulated mainly in the nucleus (Fig. 2). This suggests that ZmMYB-IF35 is a nuclear-localized protein.

3.3. Chilling induces the expression of ZmMYB-IF35 in maize

To determine whether the expression of ZmMYB-IF35 was induced by chilling stress, the relative expression level of ZmMYB-IF35 in maize under chilling stress for 0, 4, 12 and 24 h was determined by quantitative real-time PCR (qPCR). As shown in Fig. 3, the relative expression level of ZmMYB-IF35 reached its maximum level after 4 h of chilling stress, and declined slightly thereafter.
high expression levels, were selected (Fig. 4).

transgenic lines (T3-4, T3-29 and T3-33), which exhibited relatively
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3.4. ZmMYB-IF35 overexpressing Arabidopsis lines

To understand the role of ZmMYB-IF35 in the plant abiotic stress
responses, we overexpressed ZmMYB-IF35 in Arabidopsis under the
control of the CaMV 35S promoter. The expression of ZmMYB-IF35 at
the transcriptional level was examined, and three T3 homozygous transgenic
lines (T3-4, T3-29 and T3-33), which exhibited relatively
high expression levels, were selected (Fig. 4).

3.5. Overexpression of ZmMYB-IF35 enhances the chilling tolerance of
Arabidopsis

As shown in Fig. 5, after being treated with chilling stress for 12 and
24 h, the leaves of the WT and Arabidopsis ZmMYB-IF35-overexpression
line T3-33 wilted and yellowed; however, the response was more severe
in WT than in ‘T3-33’ (Fig. 5).

There were no significant differences in Fo and Fv/Fm values among
the WT, T3-4, T3-29 and T3-33 lines (Fig. 6). However, during a low-
temperature treatment, the Fo of the WT, T3-4, T3-29 and T3-33 lines
significantly increased compared with that of the control, whereas the
Fv/Fm decreased under chilling stress (Fig. 6). After being treated at
4 °C for 12 h, the Fo of WT, T3-4, T3-29 and T3-33 lines increased by
197%, 137%, 134% and 153%, respectively; while the Fv/Fm of the
WT, T3-4, T3-29 and T3-33 lines decreased by 42.6%, 26.6%, 22.2%
and 28.1%, respectively.

No differences in the O2− and H2O2 contents were observed between
the WT and transgenic plants under normal conditions. The O2−
and H2O2 contents of WT and transgenic plants initially decreased and
then increased (Fig. 7A and B). During the chilling treatment, the O2−
and H2O2 contents of the WT were greater than those of transgenic
plants. After being treated for 12 h, the O2− contents of the WT, T3-4,
T3-29 and T3-33 lines increased by 73.5%, 35.0%, 23.9% and 35.1%,
respectively; while the H2O2 contents of the WT, T3-4, T3-29 and T3-33
lines increased by 84.7%, 46.2%, 22.4% and 54.5%, respectively. The
degree of NBT and DAB staining was also higher in WT than in trans-
genic plants (Fig. 8).

There were no differences in SOD and APX activity levels between
WT and transgenic plants under normal conditions. However, the SOD
and APX activity levels of the transgenic plants were greater than those
of WT during the chilling treatment (Fig. 7C and D). SOD and APX
activity levels in WT and transgenic plants initially increased and then
decreased under chilling stress. After being treated for 12 h, the SOD
activity of the WT decreased by 60.9%, while the SOD activities of the
T3-4, T3-29 and T3-33 lines of the WT, T3-4, T3-29 and T3-33 lines increased by 84.7%, 46.2%, 22.4% and 54.5%, respectively. The
degree of NBT and DAB staining was also higher in WT than in trans-
genic plants (Fig. 8).

3.6. Expression of chilling stress-related genes in WT and transgenic lines

We determined the expression patterns of some chilling stress-re-
lated genes (AtCBF2, AtCBF3, AtCOR1 and AtCOR2) in WT and trans-
genic lines to evaluate the molecular regulatory mechanism of ZmMYB-
IF35. The expression level of these genes were all greater in the trans-
genic lines than in WT under both control and chilling-treated condition
(Fig. 10). Thus, the overexpression of ZmMYB-IF35 affected the ex-
pression of chilling stress-related genes.

4. Discussion

Low temperature is an important factor that limits the distribution,
yield and quality of plants. Maize is one of the most popular crop
species worldwide, and its optimum growth temperature is between
25 °C and 28 °C. When the temperature is below 12 °C, corn plants are
vulnerable to damage by chilling stress. Thus, it is important to improve
the chilling tolerance of maize using transgenic technology.

In the present study, the cDNA of ZmMYB-IF35, which encodes a
protein of 345 amino acids, was isolated from maize and transformed
into Arabidopsis plants under the control of the 35S-CaMV promoter.
An amino acid sequence analysis showed that ZmMYB-IF35 had the
greatest homology with MYB-IF35 proteins from Sorghum bicolor (Fig. 1). We have determined that ZmMYB-IF35 localizes to the nucleus (Fig. 2). An analysis of ZmMYB-IF35 transcripts in the leaves of maize subjected to a chilling stress for different time periods showed that the greatest transcript level was attained at 4 h of chilling stress (Fig. 3). This suggests that the transcription of maize ZmMYB-IF35 can be induced by a low temperature. We screened and identified Arabidopsis overexpression lines, in which the expression levels of ZmMYB-IF35 were much greater than that in WT (Fig. 4). Three T3 homozygous transgenic lines (T3-4, T3-29 and T3-33) that exhibited relatively high expression levels were selected for further study.

Chilling stress inhibits leaf photosynthesis through PSII-related photoinhibition (Aro et al., 1993; Zhang et al., 2011). PSII is believed to play an important role in plant responses to environmental stresses (Baker, 1991; Sui and Han, 2014). However, the PSII activity level, which is reflected by Fv/Fm, was not affected in WT and transgenic plants under normal conditions (Fig. 6). The Fv/Fm decreased in both WT and transgenic plants during chilling stress, but especially in the former. This suggests that ZmMYB-IF35 overexpression can protect PSII during chilling stress. Changes in the Fo depend on the factors present during energy dissipation and PSII inactivation (Guo et al., 2018). PSII damage or inactivation can result in an increase in Fo (Xu and Wu, 1996). In this study, the Fo increased during chilling stress; however, the increase was smaller in transgenic plants than in WT (Fig. 6), which indicated less damage to the PSII of transgenic plants than that of WT. Thus, the overexpression of maize ZmMYB-IF35 could alleviate damage caused by chilling stress to photosynthetic reaction centers and reduce chilling photoinhibition.

If the excess energy absorbed by plants cannot be dissipated in time, or the CO2 assimilation is blocked, ROS is generated, especially under conditions of environmental stress (Asada, 1992, 1999), and ROS can affect the membrane integrity, aggravate membrane lipid peroxidation and cause plant damage (Smirnoff, 1993; Mehdy, 1994). During evolution, many mechanisms for scavenging ROS were generated in plants, including various nonenzymatic and enzymatic antioxidants, as APX, SOD and catalase (Song et al., 2005). These can scavenge ROS and protect plants from ROS damage (Noctor and Foyer, 1998; Song et al., 2005; Airaki et al., 2012; Liu et al., 2017). Under normal conditions, there were no differences in O2•− and H2O2 contents between the WT and transgenic plants (Fig. 7A and B). However, under chilling stress, the O2•− and H2O2 contents of transgenic plants were lower than those of WT. SOD activity is crucial for the enzymatic scavenging of O2•− to form H2O2. APX is an important enzyme for eliminating toxic H2O2 in plants, and it can scavenge peroxides by converting AsA to dehydroascorbate (Foyer et al., 1994). Under chilling stress, SOD and APX activity levels initially increased and then decreased. In transgenic plants, the SOD and APX activities were greater than those of WT under chilling stress (Fig. 7C and D). The greater SOD and APX activity levels in transgenic plants allows more scavenging of O2•− and H2O2. Thus, the overexpression of maize ZmMYB-IF35 could increase the activity levels of SOD and APX, which could effectively reduce O2•− and H2O2 contents, and reduce ROS damage to plants.

Chilling stress can affect plasma membrane permeability, which is the rate of passive diffusion of molecules through the membrane (Wang et al., 2014). The REC reflects the degree of membrane permeability. A greater REC value indicates an increase in permeability. In the present study, the REC increased in WT and transgenic plants under chilling stress (Fig. 9). However, it was greater in WT than in transgenic plants. Thus, the membranes may be more severely damaged in WT Arabidopsis than in transgenic plants. The overexpression of maize ZmMYB-IF35 delays the REC increase.

In summary, the overexpression of maize ZmMYB-IF35 alleviated the PSII photoinhibition, increased plant tolerance to chilling stress and increased the activity levels of SOD and APX, which can effectively scavenge ROS, reduce ion leakage and protect the photosynthetic apparatus from chilling damage. ZmMYB-IF35 can also regulate the expression of stress-related genes in response to chilling stress. This study provides useful information that increases our understanding of the regulatory mechanisms of MYB transcription factors.
CRediT authorship contribution statement

Chen Meng: Writing – original draft, Investigation, Formal analysis, Writing – original draft. Na Sui: Conceptualization, Supervision, Writing – review & editing.

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