Title: A Na$_2$CO$_3$-responsive chitinase gene from Chinese wildrye improve pathogen resistance and saline-alkali stress tolerance in transgenic tobacco and maize

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Highlight: Overexpression of the *LcCHI2* increased chitinase activity and enhanced pathogen resistance and alkaline salt stress tolerance in both dicotyledonous and monocotyledonous plants.
Abstract
Salinity and microbial pathogens are the major limiting factors for crop production.
Although the manipulation of many genes could improve plant performance under either
of these stresses, few genes have reported that could improve both pathogen resistance and
saline-alkali stress tolerance. In this study, we identified a new chitinase gene CHITINASE
2 (LcCHI2) that encodes a class II chitinase from a Chinese wildrye (Leymus Chinensis),
which grows naturally on alkaline-sodic soil. Overexpression of LcCHI2 increased
chitinase activity in transgenic plants. The transgenic tobacco and maize exhibited
improved pathogen resistance and enhanced both neutral salt and alkaline salt stress
tolerance. Overexpression of LcCHI2 reduced sodium (Na⁺) accumulation,
malondialdehyde content and relative electrical conductivity in transgenic tobacco under
salt stress. In addition, the transgenic tobacco showed diminished lesion against bacterial
and fungal pathogen challenge, suggesting an improved disease resistance. Similar
improved performance was also observed in LcCHI2-overexpressed maize under both
pathogen and salt stresses. It is worth noting that this genetic manipulation does not impair
the growth and yield of transgenic tobacco and maize under normal cultivation condition.
Apparently, application of LcCHI2 provides a new train of thought for genetically
engineering saline-alkali and pathogen resistant crops of both dicots and monocots.

Keywords
Biotic stress, Chinese wildrye, Chitinase, Maize, Overexpression, Saline-alkali stress,
Tobacco
Introduction

The biotic and abiotic stresses, such as pathogens and salinity, severely affected crop growth and agricultural productivity worldwide. According to incomplete statistics of UNESCO and FAO, 950 million ha (6.4%) of the world’s land area has saline-alkali soil, and about 10% of this area is found in China. The alkaline soils of China contain high levels of Na$_2$CO$_3$ and NaHCO$_3$ (Wang et al., 1993). Only few species of plants could grow in such soils and they are sparsely distributed. Chinese wildrye (Leymus Chinensis), a perennial grass of family gramineae, grows naturally on alkaline soil, suggesting the existence of such a mechanism. Apparently, identification of functional genes specifically responsible for stress tolerance would be a prerequisite of crop improvement through biotech breeding.

Chitinase is one of the important enzyme family that has been found to involve in such a mechanism of divergent biological functions. Chitinases (EC 3.2.1.14) are a family of glycosyl hydrolases (GHs) responsible for the hydrolysis of the chitin polymer (a β-1,4-linked N-acetylglucosamine), a structural component found in the cell walls of fungi, insects, a variety of crustaceans and nematode eggs (Kesari et al., 2015). Interestingly, although the chitin is not present in plants, different subgroups of chitinase genes were identified in plants (Kesari et al., 2015). Plant chitinase proteins are generally divided into six classes, I to VI (Patil et al., 2000). The classes III and V belong to the GH18 family, whereas class I, II, IV and VI belong to the GH19 family (Patil et al., 2000). Both families exhibit diversity in their nucleic acid sequences, protein structures, substrate specificities, sensitivity to inhibitors and mechanisms of catalysis. Along with active chitinases, the plant genome also consists of a large number of sequences encoding catalytically inactive chitinases that are referred to as chitinase-like (CTL) proteins (Kesari et al., 2015). A few studies suggested that the likely substrates of plant CTL proteins may be arabinogalactan protein, chitooligosaccharides, N-acetylchitooligosaccharides and other GlcNAc-containing glycoproteins or glycolipids, but the substrates of most plant CTL proteins remain uncertain (Sanchez-Rodriguez et al., 2012; Zhong et al., 2002; van Hengel et al., 2001). The intrinsic diversities of plant chitinase imply that the chitinase/CTL genes likely have a broad function.

Because chitin is the major component of fungal cell walls, earlier studies on the role of chitinase genes focused extensively on its involvement in plant defense responses to fungal pathogen infection (Verburg and Huynh, 1991; Brogue et al., 1991). Chitinase gene
expression in plant tissues is strongly induced by fungal pathogens and chitin oligosaccharides (Seo et al., 2008; Hong and Hwang, 2002). Interestingly, the expression of chitinase gene responded also to infection of viruses, bacteria and oomycetes, which do not have chitin or related structures in their cell wall (Hong and Hwang, 2006; van Loon et al., 2006). Accordingly, a number of plant chitinases are defined as classic pathogenesis-related proteins (PRs) including PR-3, -4, -8 and -11 families (van Loon et al., 2006). Overexpression of these PR proteins conferred resistance to plant disease in different plant species (Brogue et al., 1991; Yamamoto et al., 2000; Tabaeizadeh et al., 1999; Nishizawa et al., 1999; Shin et al., 2008; Takahashi et al., 2005).

Plants chitinase and CTL genes play a role not only in defense related processes but also in abiotic stress tolerance. In Arabidopsis thaliana, the AtPR3 gene which encodes a class II chitinase was induced by high salt (Seo et al., 2008). The pepper class II basic chitinase gene CaChi2 was induced by salt, drought and osmotic stresses (Hong and Hwang, 2002, 2006). The expression of pitch28 from Lycopersicon chilense plants induced by osmotic and abscisic acid (Chen et al., 1994). In addition to salt, osmotic and drought stresses, expression of the class II chitinase gene from Bermuda grass was reported to be up-regulated by cold stress and function as an antifreeze protein (Nakamura et al., 2008). Similarly, in winter rye (Secale cereal) and highbush blueberry, cold stress induced the chitinase like proteins, which showed antifreeze activity in vitro (Yeh et al., 2000; Kikuchi and Masuda, 2009). Interestingly, most of the reported chitinase genes that responsive to both biotic and abiotic stresses belonged to the class II family and homologue to PR3 proteins (Figure S1). Furthermore, overexpression of these PR3 genes, such as AtPR3 and CaChi2, significantly increased the salt resistance of the transgenic plants (Seo et al., 2008; Hong and Hwang, 2006). In contrast, mutation of the AtCTL1 resulted in oversensitive to salt and drought stresses (Kwon et al., 2007).

Wildly grown plants have developed through evolution into adaptive mechanisms against the various environmental threatening. Apparently, identification of functional genes specifically responsible for stress tolerance would be a prerequisite of crop improvement through biotech breeding. In a previous report, a chitinase like EST sequence was isolated from Chinese wildrye which was treated with Na2CO3 (Jin et al., 2006). Accordingly, we cloned a unique chitinase gene and designated it as LcCHI2, which belongs to class II chitinase gene family. In the cultivated wildrye, expression of LcCHI2 was induced by salt stress leading to an increase in chitinase activity.
Overexpression of *LcCHI2* increased pathogen and salt stress resistance in both tobacco and Maize. This study provides a novel train of thought in improvement of both pathogen resistance and salt stress tolerance, in particular alkaline salt stress, through overexpression of a single gene in dicots and monocots.

**Material and methods**

**Cloning and expression analysis of *LcCHI2***

Total RNA was extracted from 4-week-old seedlings of Chinese wildrye treated with 100 mM Na$_2$CO$_3$ using RNAiso plus (TaKaRa, Japan) according to the instruction manual. Full-length cDNA of *LcCHI2* sequence was obtained using a SMART™ RACE cDNA Amplification Kit (Clontech, USA) for 3’ RACE with the primers 5’-CCGACCAGTTCCAATGGGGCT-3’ and 5’-GGCCACGCCTGACTAGTACTTTTTTTTTTTT-3’.

For Expression analysis of *LcCHI2*, total RNA was extracted from 4-week-old Chinese wildrye harvested at different time points under various stress treatments. A 771 bp fragment of *LcCHI2* cDNA was amplified in 30 cycles with the primers 5’-ATGGCGAGGTTTGCTGCCCTCG-3’ and 5’-CTAGCTAGCGAAGTTTCGCTGGGTG-3’.

**Agrobacterium-mediated tobacco and maize transformation**

The *LcCHI2* gene was amplified with the primers 5’-ccggaattcATGGCGAGGT TTGCTGCCCTCG-3’ and 5’-cgeggatccCTAGCTAGCGAAGTTTCGCTGGGTG-3’. The PCR products were digested with EcoRI / BamHI and cloned into the pUC119-35s-NOS vector to generate pUC119-35s::*LcCHI2*-NOS. The fragment of 35s::*LcCHI2*-NOS was collected by Hind III digestion and cloned into pBI121 vector. After sequencing the positive clones, the final construct pBI121-35s::*LcCHI2*-NOS were transformed into *Agrobacterium* strain EHA105. Transgenic tobacco plants were produced by using...
Agrobacterium-mediated transformation as described by the previously reported method (Hoekema et al., 1983). Transgenic plants were selected on MS medium containing 50 mg/ml kanamycin and 500 mg/ml carbenicillin.

For maize transformation, the sequence of *LeCHI2* from pBI121-35s::*LeCHI2-NOS* was digested with SpeI / BamH I and cloned into the pTF101-ubi-NOS vector to generate pTF101-ubi::*LeCHI2-NOS*. Immature zygotic embryos of HiII (B73 × A188) were infected with *Agrobacterium* strain EHA105 harboring the binary vector pTF101-ubi::*LeCHI2-NOS*. Transgenic plants were selected and produced by the previously reported method (Frame et al., 2002).

**Chitinase activity assay**

Chitinase activity was measured in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM 4-methylumbelliferyl-β-D-N, N'-diacetylchitobiose (Sigma, Germany) as a substrate by the previously reported method (O'Brien and Colwell, 1987). One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μmol of 4-methylumbelliferone per minute.

**Measurement of Malondialdehyde (MDA) contents and ion leakage ratio**

Fresh leaves (0.3 g) were ground properly in 5 ml of 5 % trichloroacetic acid solution and centrifuged for 10 min at 3000 rpm. 2 ml of the supernatant was reacted with 2 ml 0.67% thiobarbituric acid and then it was heated for 30 min, at 100°C in a water bath and then immediately cooled on ice. After centrifugation for 10 min, at 3000 rpm, the absorbance of the supernatant was read at 450, 532 and 600 nm. The contents of MDA were calculated using the formula: MDA concentration (μmol/L) = 6.45 × (A532-A600) - 0.56 × A450.

Ion leakage ratio was measured as relative electrical conductivity parameter. 0.1 g leaves were sampled from different plants, rinsed briefly with deionized water and immediately placed into a tube with 10 mL of deionized water. Conductivity (I1) was measured using an electroconductivity meter (model 1054, VWR Scientific, Phoenix) after the tubes were placed at 22°C overnight. Then, the samples were heated at 100°C for 30 min and conductivity (I2) was measured again. Relative electrical conductivity was expressed as (I1/I2) × 100%.

**Sodium content**
Seedings of wild type (WT) and transgenic tobacco plants were cultured in nutrient solution for one month and then treated with 0 or 200 mM NaCl. After 6 days of treatment, the shoots and roots were oven dried, digested with H$_2$SO$_4$-H$_2$O$_2$. The digest was dissolved in deionised water and sodium content was estimated by flame spectrophotometer (M410, Waters Corporation, America).

Salt stress treatments

The plant seedlings of Chinese wildrye (Leymus Chinensis) were grown in vermiculite with a light/dark cycle of 16 h/8 h at 25°C. They were irrigated with one-half-diluted Murashige and Skoog (MS) medium every 3 days. 4-week-old seedlings of Chinese wildrye were treated with 400 mM NaCl and 100 mM Na$_2$CO$_3$, respectively. Leaves of treated plants were harvested up to 0, 6, 12, and 24 h, respectively, by rapid freezing in liquid nitrogen and kept at -80°C for further analysis.

To determine abiotic stresses tolerance of the transgenic tobacco (Nicotiana tabacum) on disc, seeds of wild-type and transgenic tobacco were sowed on MS medium containing 200 mM NaCl, 30 mM Na$_2$CO$_3$ or 500 mM sorbitol, respectively. For soil experiment, tobaccoseeds germinated in soil cultured with a light/dark cycle of 16 h/8 h at 25°C. Thirty plants for each line were used for salt and drought stress treatment, respectively. For salt stress, nearly 3-week-old plants were irrigated for 25 days with either tap water or tap water containing 400 mM NaCl or 100 mM Na$_2$CO$_3$.

To determine abiotic stresses tolerance of the transgenic maize, T2 generation transgenic event (CHI-Ox2, CHI-Ox5 and CHI-Ox7) and WT plants were grown in pots (10×10 cm) containing peat: vermiculite (5:1, v/v) medium in a greenhouse with a light/dark cycle of 16 h/8 h at 30°C. PCR analysis was used to screen for positive transformants, and 2-week-old seedlings were water with either tap water or tap water containing 200 mM NaCl or 50 mM Na$_2$CO$_3$ for 6 days. Photograph was recorded after each treatment by a camera.

Pathogen response assays of transgenic tobacco and Maize

For bacterial infection analysis, Pseudomonas tabaci (Wolf et Foster) Stevens was grown in NBY medium and bacterial cells were collected, washed and resuspended in 10 mM MgSO$_4$. The density of bacterial cells was determined by measuring absorbance at OD$_{600}$. Bacterial cells in suspension were infiltrated into fully expanded 6-week-old tobacco
leaves using a 1 ml plastic syringe without a needle. After 5 days, the average lesion area for each independent transgenic line was calculated and compared with that of wild-type tobaccos.

For fungal resistance analysis, Alternaria alternata (Fries) Keissler was cultured on potato dextrose agar medium at 28°C. When the mycelia reached the edge of the plate, 0.5 cm diameter agar discs were excised from the edge of growing colonies using a cork borer and inverted onto the detached leaves from wild-type and transgenic tobaccos. All leaves were placed on wet filter paper in Petri dishes and incubated at 28°C to permit normal disease development under high humidity. After 7 days, the average lesion area for each independent transgenic line was calculated and compared with that of wild-type tobaccos.

Maize pathogen Exserohilum turcicum and curvularia lunata were provided by Institute of plant protection, Jilin academy of agricultural sciences. Exserohilum turcicum and curvularia lunata were cultured on potato dextrose agar medium at 28°C. When the mycelia reached the edge of the plate, 0.6 cm diameter agar discs were excised from the edge of growing colonies using a cork borer and inverted onto the detached leaves from transgenic and null control maize plants. All leaves were placed on wet filter paper in Petri dishes and incubated at 28°C to permit normal disease development under high humidity. After 3 days, the average lesion area for each independent transgenic line was calculated.

Nucleotide and protein sequences accession numbers

The nucleotide and protein sequences reported in this paper have been deposited in the Genbank nucleotide database and protein database under the accession number GQ397277 and ACV20870.

Results

Cloning and characterization of LcCHI2 from Chinese wildrye

According to the EST sequence information from literature (Jin et al., 2006), we cloned a full-length cDNA of the target chitinase gene by 3‘RACE technology from Chinese wildrye, and was designated as LcCHI2 (GenBank accession number GQ397277). Sequence analysis revealed that LcCHI2 contains a 771 bp open reading frame encoding a polypeptide of 256 amino acids. Pfam scan showed that the deduced protein is a class II chitinase belonging to the GH19 family (PF00182).
To obtain more information of the LcCHI2 protein, a phylogenetic tree was constructed including LcCHI2 orthologues and all the class II chitinases of Arabidopsis. LcCHI2 belonged to a cluster including three pathogenesis-related protein 3 (HvPR3, TaPR3 and AtPR3) and two chitinases (ScCHI46, CaCHI2) (Figure S1). LcCHI2 showed more than 95% homology to HvPR3, TaPR3 and ScCHI46 and 71% homology to CaCHI2 (Figure S2A). Multiple protein alignment revealed two conserved chitinase family 19 signature domains in all the five orthologues (Figure S2B). However, an N-terminal secretive signal peptide only found in LcCHI2 and its monocot orthologues (Figure S2B).

**Sodium stress induced the expression of LcCHI2 and chitinase activity in Chinese wildrye**

As the EST of LcCHI2 was isolated from alkaline-sodic stressed tissues, we measured the expression of LcCHI2 in Chinese wildrye under different abiotic conditions. A nearly 8-fold induction of LcCHI2 transcripts was observed under 400 mM NaCl after 24 hours treatment comparing with the control plant (Figure 1A). Similarly, the expression of LcCHI2 increased more than 2-fold under 100 mM Na2CO3 after 12 and 24 hours treatment compared with the control plant (Figure 1A). Subsequently, the chitinase activities were determined under NaCl and Na2CO3 conditions after 24 hours treatment. The results showed that chitinase activities increased by 4.3-fold and 2.6-fold after 24 hours exposure to 400 mM NaCl and 100 mM Na2CO3, respectively (Figure 1B). In contrast, the expression of LcCHI2 and chitinase activity were not changed in Chinese wildrye under 20% PEG condition in 24 hours (data not show).

**Overexpression of LcCHI2 in tobacco enhanced plant tolerance to salt stress**

To further investigate the function of LcCHI2, transgenic tobacco plants that constitutively express the LcCHI2 gene under the control of the CaMV 35S promoter were developed by agrobacterium-mediated transformation. Three independent positive transgenic lines were selected by kanamycin resistance and used for further analysis. The expression of LcCHI2 could be detected in all the three transgenic lines other than wild-type (WT) plants (Figure 2A). The results showed that chitinase activity increased by 1.5 - 1.7 folds over WT plants in the transgenic lines (Figure 2B). Comparing with the WT plants, the LcCHI2-overexpression plants exhibited no obvious phenotypic difference from the WTs.
under normal growth conditions (Figure S3).

As the expression of \(LcCHI2\) is induced by salt stress, phenotypes of the WT and \(LcCHI2\)-overexpression lines were analyzed on MS medium containing 200 mM NaCl, 30 mM Na\(_2\)CO\(_3\) and 500 mM sorbitol, respectively. No difference was observed between WT and \(LcCHI2\)-overexpression plants growth on normal MS medium. However, the \(LcCHI2\)-overexpression plants survived well under salt stress (NaCl and Na\(_2\)CO\(_3\)) in comparison with WT plants, especially the C10 and C15 lines (Figure 3A). In contrast, the \(LcCHI2\)-overexpression plants only exhibited a minor effect for osmotic stress (Figure 3A). To further evaluate the abiotic resistance of \(LcCHI2\)-overexpression plants, soil growth plants were growth under salt and drought stress conditions. There were no observed differences in phenotypes between \(LcCHI2\)-overexpression and WT plants, when they were growth under normal and drought stress conditions (Figure S4). However, 6 days after watering 400 mM NaCl or 100 mM Na\(_2\)CO\(_3\), the wild-type seedlings showed wilting phenotypes and ultimately died, whereas the transgenic tobaccos continued to grow (Figure S4).

Because the overexpression of \(LcCHI2\) increased salt stress tolerance other than osmotic or drought stresses, sodium contents were determined in the roots and shoots of WT and transgenic plants under normal and NaCl treatment conditions. As expected, NaCl treatment significantly increased Na\(^+\) content in the shoots and roots of WT and transgenic plants (Figure 3B, 3C). The overexpression of \(LcCHI2\) decreased the Na\(^+\) content from 10% to 33% in the shoots and from 11% to 22% in the roots of transgenic lines compared with the corresponding WTs under NaCl treatment condition (Figure 3B, 3C). Meanwhile, the Na\(^+\) content showed a significantly decrease only in transgenic lines C10 and C15 other than line C6, which is coincident with the strong resistance to sodium stress of the transgenic lines C10 and C15 (Figure 3A).

Malondialdehyde (MDA) content and relative electrical conductivity are widely used as indicators for lipid peroxidation and the degree of plant cell injury under stress treatment, respectively. There are no obvious differences in MDA content and relative electrical conductivity between WT and \(LcCHI2\) overexpression plants under normal growth condition. Although 24 hours salt treatment significantly increased the MDA content and relative electrical conductivity in both WT and \(LcCHI2\) overexpression plants, the transgenic lines showed a lower MDA content and relative electrical conductivity compared with WT under salt stress condition (Figure S5).
Overexpression of \textit{LcCHI2} conferred pathogen tolerance in tobacco

As a homologue of PR3 proteins, \textit{LcCHI2} is presumed to be involved in pathogen resistance too. Thus, the \textit{LcCHI2}-overexpression plants were inoculated with bacterial and fungal pathogens, respectively. Leaves of WT and \textit{LcCHI2}-overexpression tobaccos were inject-inoculated with spore suspensions of the bacterial pathogen \textit{Pseudomonas tabaci} (Wolf et Foster) Stevens and symptom development was subsequently monitored for 5 days. The examined disease symptoms included chlorosis and necrosis expansion surrounding the primary infection sites. As shown in Fig. 4, WT tobaccos were more sensitive to bacterial infection than \textit{LcCHI2}-overexpression tobaccos in detached leaves, in which pathogen infection resulted in significantly reduced disease symptoms. The leaf lesion size of WT tobaccos was 5- to 10-fold larger than that of \textit{LcCHI2}-overexpression tobaccos (Figure. 4A, 4B). Resistance to the fungal pathogen \textit{Alternaria alternata} (Fries) Keissler was also identified in \textit{LcCHI2}-overexpression tobacco plants by a detached leaf inoculation test after 7 days inoculation. WT tobaccos showed typical necrosis symptoms surrounded by chlorotic halos and extensive pathogen sporulation, while transgenic lines had significantly smaller lesion sizes than wild-type tobacco (Figure. 4C, 4D). The enhanced resistance to \textit{Pseudomonas tabaci} and \textit{Alternaria alternata} in transgenic tobacco demonstrated that disease resistance conferred by \textit{LcCHI2} overexpression is effective against both bacterial and fungal pathogen.

Overexpression of \textit{LcCHI2} in Maize enhanced tolerance to salt and pathogen stresses

To determine whether the overexpression of the \textit{LcCHI2} gene could phenocopy the stress tolerances of transgenic tobacco in monocotyledons crops, we produced transgenic maize plants harboring the \textit{pTF101.1-ubi::LcCHI2} construct. DNAs were extracted from leaves of T\textsubscript{0} generation transgenic plants and use to detect the integration of target sequence into genomes of transgenic plants by PCR. The results showed that six transgenic events harbored both the selection marker and \textit{LcCHI2} genes (Figure S6). Reverse-transcriptional PCR (RT-PCR) and chitinase activity assay indicated that three representative transgenic lines accumulated abundance \textit{LcCHI2} transcripts and enhanced chitinase activities compared to null plants with the absence of \textit{LcCHI2} (Figure 5A, 5B). Then these transgenic plants were grown in normal and alkaline soils in the greenhouse (Figure S7). There are no significant different between the growth of transgenic (Ox) and their
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respective non-transgenic plants in normal soil (null). However, the transgenic plants were healthier than the corresponding null plants in alkaline soil. A significant increased plant height and SPAD value were observed in transgenic plants compared their null plants (Figure S7B, S7C). To further evaluate the effects of divergent salt stress on the transgenic maize, two week-old plants were watered with 200 mM NaCl and 50 mM Na$_2$CO$_3$, respectively. As expected, no significant differences were observed between the growths of transgenic (Ox) and their respective null plants after six-day treatment with distilled water (Figure 6A, 6B). In contrast, the overexpression plants showed resistance to NaCl or Na$_2$CO$_3$ treatments other than the null plants (Figure 6C, 6D). Although NaCl or Na$_2$CO$_3$ treatments increased the relative electrical conductivity and MDA content in both overexpression and null plants, the magnitudes of the overexpression plants were significantly lower than null plants (Figure 6E, 6F).

Biotic resistances of the transgenic plants were also measured by infection the maize with two major fungal pathogens *Exserohilum turcicum* and *curvularia lunata*, respectively. Typical necrosis symptoms were observed in the null plants while transgenic lines had significantly smaller lesion area after infection by *Exserohilum turcicum* or *curvularia lunata* (Figure. 7A, 7B, 7C). These results indicated that the overexpression of LcCHI2 in maize significantly inhibited the growth of fungal pathogens.

To evaluate the effects of LcCHI2 overexpression in agronomic traits, the gene in the transgenic events with HiII background (CHI-Ox2, CHI-Ox5 and CHI-Ox7) were backcross to local commercial inbred line X923-1. The genetically segregated positive and negative BC5F2 plants were crossed respectively with Y822 to produce hybrid seeds. Agronomic traits were measured from the hybrid lines in two replicated experiments. The results (Table S1) showed that all transgenic events exhibited statistically no negative impact such as cob weight, cob diameter, ear kernel weight and ear diameter, indicating that overexpression of LcCHI2 has no penalty on the growth and yield in the field conditions comparing with the controls.

**Discussion**

In this study, the full-length cDNA of a chitinase-like gene was cloned and named as LcCHI2, which was first identified from an EST library in *Leymus Chinensis* under Na$_2$CO$_3$ stress (Jin et al., 2006). The cDNA-deduced protein sequence analysis indicated that the LcCHI2 contains two conserved domains including the signature 1 (PS00773) and signature 2 (PS00774) of glycosyl hydrolase 19 family (Figure S2B). Protein cluster
analysis indicates that LcCHI2 belongs to class II chitinase (Figure S1). Several members of the class II chitinase proteins, such as AtPR3 and pcht28, were reported to have the typical chitinase activity and overexpression of those chitinase genes could increase chitinase activity in transgenic plants (Verburg and Huynh, 1991; Tabaeizadeh et al., 1999; Chen et al., 1994). In this study, a significantly increased chitinase activity was positively correlated with the up-regulation of LcCHI2 in Leymus Chinensis under both NaCl and Na2CO3 stress condition. Moreover, the transgenic tobacco and maize plants that overexpressed LcCHI2 also showed higher chitinase activity than control plants. Interestingly, protein alignment indicated that LcCHI2 and its homologues contain an N terminal secretary signal peptide (Figure S2B). These results supported that LcCHI2 is a secreted active chitinase in Leymus Chinensis.

Different types of chitinases were induced by differential pathogen attack and have been characterized as pathogenesis-related (PR) proteins (Kesari et al., 2015; van Loon et al., 2006). Phylogenetic analysis showed that LcCHI2 is a homologue of several reported PR3 proteins, including TaPR3, HvPR3, CaCHI2, pcht28 and AtPR3 (Figure S2) (Verburg and Huynh, 1991; Hong and Hwang, 2002; Tabaeizadeh et al., 1999; Shin et al., 2008; Scheler et al., 2016). These PR3 proteins were proposed to be involved in plant defense responses. Plant chitinases were thought to degrade the major structural polysaccharide of fungal cell walls in the intercellular space to limit fungal growth (Brogue et al., 1991). The purified AtPR3 protein showed antifungal chitinase activity and inhibited the growth of Trichoderma reesei in vitro (Verburg and Huynh, 1991). The overexpression of chitinase resulted in enhanced resistance in tomato, rice, Italian ryegrass and grapevine to Verticillium dahlia, Magnaporthe grisea, Puccinia coronata and Uncinula necator, respectively (Yamamoto et al., 2000; Tabaeizadeh et al., 1999; Nishizawa et al., 1999; Takahashi et al., 2005). Moreover, expression of a barley PR3 chitinase gene in transgenic wheat resulted in enhanced resistance to infection by Erysiphe graminis, Blumeria graminis, Puccinia recondita and Fusarium graminearum (Shin et al., 2008; Oldach et al., 2001). To elucidate the defense response of LcCHI2, we investigated the biological functions of the LcCHI2 in transgenic tobacco and maize. The overexpression of LcCHI2 significantly enhanced resistance to multiple pathogens in tobacco and maize. The transgenic tobacco plants also showed resistance to a bacterial pathogen Pseudomonas tabaci Stevens, which do not have chitin or related structure in their cell wall. A similar phenomenon was observed in the transgenic Arabidopsis when overexpressing CaCHI2.
gene (Hong and Hwang, 2006). In fact, chitinase expression in plant tissues is strongly induced by infection with viruses and bacteria in addition to fungal pathogen (van Loon et al., 2006). Therefore, the PR3 type protein may inhibit the pathogen invasion by an unknown mechanism other than directly degrading the chitin oligomers.

In addition to biotic stress, a number of cluster II chitinases were reported to be involved in developmental and various abiotic responses. The ScCHT46 showed 92% protein identity with LcCHI2 and encode a chitinase-antifreeze protein in winter rye (Yeh et al., 2000). The expression of ScCHT46 and its homologs in winter wheat, HvPR3, are responsive to cold and drought (Yeh et al., 2000). Moreover, it is reported that NaCl, drought and Mannitol induces the expression of several class II chitinases, including CaCHI2, pcht28 and ArPR3 (Seo et al., 2008; Hong and Hwang, 2002, 2006; Chen et al., 1994). The overexpression of CaCHI2 increased the osmotic stress in Arabidopsis (Hong and Hwang, 2006). In contrast, a mutation of AtPR3 affected the seeds germinating under high salt (Seo et al., 2008). Similarly, our results showed that the expression of LcCHI2 was up-regulated by Na\textsuperscript{+} stress conditions including treatment with NaCl and Na\textsubscript{2}CO\textsubscript{3}. Moreover, the activity of chitinase was increased under NaCl and Na\textsubscript{2}CO\textsubscript{3} stresses in Chinese wildrye. Taking together, these results suggested that LcCHI2 play an important role in abiotic stress tolerance similar to its homologues in other plants.

Interestingly, the overexpression of LcCHI2 showed a significant tolerance to NaCl and Na\textsubscript{2}CO\textsubscript{3} treatments in both tobacco and maize but not to the osmotic or drought stresses. These results implicated that the overexpression of LcCHI2 might assist to reduce Na\textsuperscript{+} ionic toxicity other than osmotic stress or improving water-use-efficiency stress. Coincidence with this assumption, the Na\textsuperscript{+} contents were decreased in the transgenic tobacco under NaCl treatment. Recently, it is proposed that the amount of the carboxyl groups on de-methesterified pectin could bind cations such as Na\textsuperscript{+} and sequester more Na\textsuperscript{+} in the cell wall, which may contribute to salt stress resistance in plants (de Lima et al., 2014; Byrt et al., 2018; An et al., 2014). However, we did not detect any significant difference in the pectin content between transgenic and WT plants (data not show). Instead, increased cellulose and decreased hemicelluloses were observed in the overexpression lines compared with WT plants under salt treatment condition (Fig S8). This phenomenon is reminiscent of the Na\textsuperscript{+} sensitive mutant Atctl1, which affects the contents of cellulose and extractability of hemicelluloses (Sanchez-Rodriguez et al., 2012; Hermans et al., 2011). It is reported that mutation of AtCTL1 increased the Na\textsuperscript{+} influx rather than defects.
in Na\(^+\) efflux activity (Kwon et al., 2007). Therefore, the overexpression of the \(LcCHI2\), a homologue of \(AtCTL1\) (Fig S2), may have an opposite effect and inhibit the Na\(^+\) influx, which could explain the deceased Na\(^+\) content in \(LcCHI2\) transgenic plants.

Although cell wall metabolism is rationally correlated with salt stress, the exact mechanism is still largely unknown (Le Gall et al., 2015). Mutation of \(AtCES8\) (cellulose synthase 8) or \(AtCSLD5\) (cellulose synthase-like 5) resulted tolerance or hypersensitive to salt stress, respectively (Zhu et al., 2010; Chen et al., 2005). Moreover, mutation the chitinase-like gene \(AtCTL1\) reduced the cellulose content and led to salt-sensitive of Arabidopsis (Sanchez-Rodriguez et al., 2012; Kwon et al., 2007). Compared with dicot, there has been no report on the modification of cell wall compound contributing to salt tolerance in grass. In this study, the overexpression of \(LiCHI2\) in both tobacco and maize enhanced the salt and pathogen stress tolerances, suggesting similar roles of the chitinase in dicot and grass. Although the overall architectures of plant cell walls are similar in that they both consist of a network of cellulose fibers surrounded by a matrix of non-cellulosic polysaccharides, the types and abundance of non-cellulosic polysaccharides are significant different between grass and dicot cell walls (Vogel, 2008). It seems that cellulose represents the common component in plant cell walls and plays a pivotal role in regulating the salt stress. The changed cellulose content may regulate cross-linking and rigidity of the cell wall, which acts as a barrier for salt entrance or pathogen invasion.

Although a large number of alkali-responsive genes were reported from different plants, a few have been cloned and characterized (Zhao et al., 2016). Overexpression of the alkali-stress-inducable \(RMtATP6\) and \(NADP-ME2\) in rice and Arabidopsis enhanced the tolerance against osmotic, \(NaCl\) and \(Na_2CO_3\) stresses (Liu et al., 2007; Zhang et al., 2006). Transgenic rice with \(PtNHA1\) and \(PutNHX\) genes, which were cloned from an alkaline soil plant, increased tolerance of shoots to \(NaCl\) and roots to \(NaHCO_3\), respectively (Kobayashi et al., 2012). It is noted that all these studies were conducted in nutrient solution or agar plate in laboratory. In contrast, we presented the performance of the transgenic maize with natural alkaline soil or treated soil (Figure S7). Moreover, the transgenic maize showed no penalty on growth and yield in field condition (Table S1). Therefore, we proposed that overexpression of \(LcCHI2\) represent a potential strategy for engineering biotic and alkaline resistance plants.

**Supplementary data**
Fig. S1 Phylogenetic tree constructed with class II chitinase sequences retrieved by BLAST in the NCBI database.

Fig. S2 (A) Protein similarity of LcCHI2 with its homologues. (B) Alignment of deduced amino acid sequences of LcCHI2 with its homologues from other plant species.

Fig. S3 Morphology of the *LeCHI2*-overexpressing transgenic tobacco plants under normal growth condition.

Fig. S4 Drought and salt stress tolerance assays of wild-type and LcCHI2 overexpression plants grow in soil.

Fig. S5 MDA contents and relative electrical conductivity of wild-type and *LeCHI2*-transgenic tobaccos under 200 mM NaCl treatment.

Fig. S6 PCR detection of selection marker (a) and *LeCHI2* genes (b) in transgenic maize plants.

Fig. S7 Growth assessment of transgenic maize in normal and alkaline soils.

Fig. S8 Cellulose and hemicelluloses contents in wild-type and *LeCHI2*-transgenic tobaccos under 200 mM NaCl treatment.

Table S1. Agronomic traits of transgenic maize grow in the field condition.

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Figures legends

Figure 1 Regulation of LcCHI2 and chitinase activity under salt stress conditions in Chinese wildrye. (A) Expression analysis of the LcCHI2 after treatment with 400 mM NaCl and 100 mM Na2CO3 for the indicated times. Total RNA were extracted from 4-week-old seedlings of Chinese wildrye following treatments as indicated and reverse transcribed. The cDNA were used as templates for RT-PCR and the ACTIN gene was amplified as an internal control. The PCR products were examined by electrophoresis in 1% (w/v) agarose gel. (B) Chitinase activity assay under salt stress conditions in Chinese wildrye. Enzyme activity assays were carried out with the leaves of Chinese wildrye after treatment with 400 mM NaCl and 100 mM Na2CO3 for 24 hours. Values are the mean ± SE obtained from three biological replicates. ** one-way ANOVA; P < 0.01.

Figure 2 (A) Northern blot analysis of LcCHI2 transcripts in wild-type (WT) and transgenic tobacco plants (C6, C10, C15). A DIG-labeled LcCHI2 probe was used for hybridization. (B) Chitinase activity of WT and transgenic tobacco plants. Values are the mean ± SE obtained from three biological replicates.

Figure 3 Abiotic stress tolerance assays of wild-type and LcCHI2-overexpressed plants in tobacco. (A) The WT and LcCHI2 overexpression (C6, C10 and C15) plants were grown on MS agar plates containing 200 mM NaCl, 30 mM Na2CO3 and 500 mM sorbitol for 30 days. (B) and (C) Na+ content of wild-type and LcCHI2 overexpression plants in shoots and roots. Values are the mean ± SE obtained from three biological replicates.

Figure 4 Pathogenesis analysis of LcCHI2-transgenic tobacco. (A) and (B) Resistance of transgenic tobacco to the bacterial pathogen Pseudomonas tabaci (Wolf and Foster) Stevens. Fully expanded leaves of tobaccos were syringe-infiltrated with 0.1 ml solution of P. tabaci. Arrows in the upper line represent the sites inoculated with bacteria and arrows in the lower line represent the sites with mock-inoculated. The average lesion area of each independent transgenic line was calculated and their relative lesion areas are shown in columns after comparison with the average lesion area on wild-type tobacco. The photograph was taken 5 days after inoculation. (C) and (D) Responses of transgenic tobacco to the fungal pathogen Alternaria alternata (Fries) Keissler. Detached leaves
were challenged with mycelia of *A. alternata*. The average lesion area of each independent transgenic line was calculated and their relative lesion areas are shown in columns after comparison with the average lesion area on wild-type tobacco. The photograph was taken 7 days after inoculation. Values are the mean ± SE obtained from three biological replicates.

Figure 5 (A) RT-PCR analysis of *LcCHI2* transcript in wild-type (WT) and transgenic Maize plants (Ox2, Ox5, Ox7). Total RNA were extracted from 2-week-old seedlings of positive and null transgenic plants. The cDNA were used as templates for RT-PCR and the actin gene was amplified as an internal control. The PCR products were examined by electrophoresis in 1% (w/v) agarose gel. (B) Chitinase activity of null and transgenic maize plants. Values are the mean ± SE obtained from three biological replicates.

Figure 6 Salt stress tolerance assays of null and *LcCHI2* overexpression plants in maize. (A), (B), (C) and (D) Soil growth null and *LcCHI2* overexpression plants were watered with tap water, 200 mM NaCl and 50 mM Na$_2$CO$_3$ for 6 days. (E) and (F) MDA contents and relative electrical conductivity of null and *LcCHI2* transgenic maize were measured every day. Values are the mean ± SE obtained from three biological replicates. * one-way ANOVA; P < 0.05.

Figure 7 Pathogenesis analysis of null and *LcCHI2*-overexpressed maize. (A) Resistance of transgenic maize to fungal pathogen *Exserohilum turcicum*. (B) Responses of transgenic maize to the fungal pathogen *Curvularia lunata*. (C) The average lesion area of each independent transgenic line was calculated and their relative lesion areas are shown in columns after comparison with the average lesion area on wild-type tobacco. Values are the mean ± SE obtained from three biological replicates.
Figure 1

A

Intensity

LcCHI2

ACTIN

0 h 6 h 12 h 24 h

400 mM NaCl

0 h 6 h 12 h 24 h

100 mM Na2CO3

B

Chitinase activity (U/g)

NaCl

Na2CO3

Control

Treatment

**
Figure 2

A

LcCHI2

rRNA

B

Chitinase activity (U/g)

CK  C6  C10  C15
Figure 3

(A) Comparison of plant growth under different treatments.

(B) Graph showing Na⁺ content in shoots.

(C) Graph showing Na⁺ content in roots.

The images demonstrate the effects of various treatments on plant growth, with comparisons between wild type (WT) and transgenic lines (C6, C10, C15). The Na⁺ content in both shoots and roots is measured under control (CK), 200 mM NaCl, 3 mM Na₂CO₃, and 500 mM Sorbitol conditions.
Figure 5

A

\[ LcCHI2 \]

\[ ZmGAPDH \]

B

Chitinase activity (U/g)

Null2   Ox2   Null5  Ox5  Null7  Ox7
Figure 6

A. CK-0d

Null Ox2

B. CK-6d

Null Ox2

C. 200 mM NaCl

Null Ox2

D. 50 mM Na$_2$CO$_3$

Null Ox2

E. Electrical conductivity

F. Content of MDA (µmol/g FW)
Figure 7

A B
Null Ox2 Null Ox2

C

Relative lesion areas (%)

Null Ox  Null Ox
Exserohilum turcicum Curvularia lunata