INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0615/2019/22–5–1023–1029
DOI: 10.17957/IJAB/15.1164
http://www.fspublishers.org

Full Length Article

Identification for *GmCDPK3* Gene Family Effect on Symbiosis Based on the Genetic Mapping in Soybean (*Glycine max*)

Lin Chen†, Yan Shi†, Shiyu Huang†, Huilin Chang†, Jianyi Li†, Xueying Liu, Shuping Li, Jinhui Wang, Jianan Zou, Jieqi Wang, Qingying Li, Jingyi Zhu, Yongxu Zhu, Miaoxin Guo, Lu Han, Hanxi Liu, Zhijun Sun, Yingnan Wen, Hongwei Jiang, Zhenbang Hu, Xiaoxia Wu, Zhanguo Zhang, Rongsheng Zhu, Zhaoming Qi, Chunyan Liu*, Qingshan Chen* and Dawei Xin*

[1]*Key Laboratory of Soybean Biology of Chinese Ministry of Education, Key Laboratory of Soybean Biology and Breeding/Genetics of Chinese Agriculture Ministry, College of Science, Northeast Agricultural University, Harbin, Heilongjiang Province, People's Republic of China*

*For correspondence: xdawei@163.com; qshchen@126.com; cyliucn@126.com

†These authors contributed equally to this work

Abstract

Nitrogen is an essential element for normal growth and development of plants. Scientific research evidence on biological nitrogen fixation (BNF) can not only effectively solve the problem of low nitrogen utilization rate and pollution, and conducive to the development of friendly agriculture. Although BNF plays an important role in economic and environmental, there have been few reports on quantitative trait loci (QTL) controlling BNF traits in soybeans. This study focuses on identify QTLs for the total nodule number (TNN) in the soybean population field of 'Suinong 14' × 'ZYD00006' (S×Z, n=160) chromosome segment substitution line (CSSLs). Field collected samples were used to collect phenotypic data of TNN. QTL underlying TNN was analyzed, four QTLs were identified in CSSLs and these QTLs were located on (LGs) D1b, A1, A2 and K, respectively. Gene annotation on these QTLs, indicated that genes encoding proteins were related to signaling transduction. Among them, the gene *Glyma.05g213200* which located on chromosome 5 belongs to Ca^{2+} dependent protein kinases3 (CDPK3) family, which regulated Ca^{2+} signaling transduction. We also analysis another two genes belong to CDPK3 family. Making systematic analysis of this family, to lay the foundation for exploring the relationship between CDPK and nodulation. And the qRT-PCR result support that *CPK-1*(*Glyma.05g213200*) and *CPK-3* (*Glyma.01g223200)* are the two main genes induced by rhizobium. These results provide important candidate genes for exploring the symbiotic relationship between soybean and rhizobium. CPK-1 plays a more important role in inducing nodulation, and *CPK-1* could be used for further research.

Keywords: Soybean; QTL; Total nodule number; CSSL

Introduction

Soybean [*Glycine max* (L.) Merr.], originated in China is accepted, which has been cultivated for more than 5000 years as one of the important food crops (Zhuang *et al.*, 1995; Li *et al.*, 2008). Now, it cultivated throughout China and widely cultivated throughout the world (Lee, 1988; Hyten *et al.*, 2004). Nitrogen is one of the essential elements for normal growth and development of soybean, and plays an important role in the whole growth of soybean. However, nitrogen utilization rate in soil is low, thus, in today's process of agricultural production appear a case: the industrial nitrogen fertilizer used in great quantities. Environmental issues have always been valued, and research in the natural sciences should be carried out around environmental protection. Reducing the use of industrial nitrogen can reduce the damage to the balance of soil nutrients and save agricultural production costs. Therefore, the full use of BNF mode is of great significance to improve the utilization of nitrogen in soil. Rhizobium can establish symbiotic nitrogen fixation system with leguminous plants (Tang *et al.*, 2016), nodules are the appearance of symbiotic relationship between legumes and rhizobia, plants convert free nitrogen into ammonium salt by nodules that are absorbed and fully utilized by the plant body. The establishment of this symbiotic relationship has great significance to the sustainable development of agriculture which can reduce the waste of resources, save production cost and protect the environment.

Symbiotic nitrogen fixation is an important way for plants and microorganisms to establish interaction, the establishment of this symbiotic relationship relies on the

To cite this paper: Chen, L., Y. Shi, S. Huang, H. Chang, J. Li, X. Liu, S. Li, J. Wang, J. Zou, J. Wang, Q. Li, J. Zhu, Y. Zhu, M. Guo, L. Han, H. Liu, Z. Sun, Y. Wen, H. Jiang, Z. Hu, X. Wu, Z. Zhang, R. Zhu, Z. Qi, C. Liu, Q. Chen and D. Xin, 2019. Identification for *GmCDPK3* Gene family effect on symbiosis based on the genetic mapping in soybean (*Glycine max*). *Intl. J. Agric. Biol.*, 22: 1023–1029

conduction of signaling pathways, in which the Ca signal plays an important role. Ca^{2+} is the second messenger of signal transduction in eukaryotic cells (Cheng *et al.,* 2002), changes in Ca^{2+} concentration are the primary manifestations of many reactions in plants, it has been widely studied as one of the early signals produced by foreign bacteria invading plants (Ma *et al.,* 2009; Wurzinger *et al.,* 2011). When foreign bacteria invade cells, the extracellular free Ca^{2+} rapidly enters the cells, inhibiting Ca^{2+} channels and preventing plants from corresponding response. Besides that, when the intracellular and extracellular free concentrations change instantaneously, intracellular free Ca^{2+} will directly or indirectly regulate cell physiological and biochemical processes through different signal transduction pathways. Almost all different extracellular abiotic and biological stimuli can cause rapid changes in intracellular free Ca^{2+} concentration (Rudd and Franklintong, 2001), and some kinases are involved in the conduction process. The establishment of a symbiotic nitrogen fixation system between rhizobia and legumes requires corresponding molecular mechanisms, which also involve Ca^{2+} signal transduction. When rhizobia invaded, flavonoids secreted by legumes roots induced nodule gene expression, which encoding proteins involved in Nod factors synthesis and secretion. Nod factors, a bacterial lipochitooligosaccharide signaling molecules stimulates the formation of leguminous nodules. When nodules form, calcium spiking in root hair cells is caused by Nod factor, and the DMI3 protein is required for calcium spiking downstream signal transduction (Catoira *et al.,* 2000; Mitra *et al.,* 2004). In research on the reactive oxygen species (ROS) found that ROS plays an important role in plant signal transduction (Sun *et al.,* 2015), its production depends on RBOH (respiratory burst oxidase homolog). The research about ROS and Ca signal had been found that ROS can regulates Ca^{2+} channels in broad bean (*Vicia faba*) guard cells (Pei *et al.,* 2000). Limited evidence suggests that ROS changes occur in the early stages of establishing a symbiotic nitrogen fixation relationship between rhizobium and legumes (Luis and Carmen, 2008), and in the process of signal transmission, RBOHD relies on the regulation of Ca^{2+} to produce ROS (Kadota *et al.,* 2015). The symbiotic relationship between rhizobium and legumes requires corresponding molecular mechanisms. These studies have proved that Ca^{2+} plays a very important role in signal transduction and the establishment of symbiotic nitrogen fixation and in the soybean about this aspect research needs to go further.

With the continuous development of molecular breeding, the research of a gene which controls a trait has been deeper and deeper. The first application of QTL is that using molecular markers to map QTLs in tomatoes (Paterson *et al.,* 1988). In recent years, QTL mapping is widely used. And in crops, more QTLs are related to agronomic traits. In soybean, several genes related to nodulation have been found and identified by QTL mapping since the 1950s, which includes *rj1, rj2, rj3, rj4, rj5, rj6, rj7* and *rj8* (Williams and Lynch, 1954; Caldwell, 1966; Harper and Nickell, 1995; Vuong *et al.,* 1996). The first gene associated with nodule control in soybean was cloned by Searle *et al.* (2003). Using the *rj7* and *rj8* super nodulation mutants. Nodulation of *rj1* soybean depended on the T3SS (Okazaki *et al.,* 2010). Analyzing of soybean lines near isogenic for *rj4* indicated that it has a positive effect on resisting the rhizobium that is non-symbiotic with the host plant, which leads to ineffective nodulation (Devine and Kuykendall, 1996). These findings provide important clues for further exploring the molecular mechanism of symbiosis between rhizobium and host. There are also related QTL studies on the nodulation phenotype of nodule mass, nodule dry weight and individual plant weight. By crossing between two Brazilian cultivars, Embrapa 20 × BRS 133, 160 individuals were selected from the F_2 population for gene mapping. 45 SSR markers were selected from 252 SSR markers of the parental genotype to plot the number of nodules, the dry weight of the nodules and the dry weight of the plants (Nicolás *et al.,* 2006). Except soybeans, related QTLs for BNF have also been studied in other legumes, such as peas (Bourion *et al.,* 2010). However, the QTL research on soybean biological nitrogen fixation (BNF) still needs to be further studied. In this study, through a systematic analysis of the candidate genes in QTL loci which located by wild soybean chromosome segment substitution lines (CSSLs) and the relationship between gene family and symbiotic nodulation, lays the foundation for delve into the symbiotic relationship between rhizobium and soybean.

Materials and Methods

Experimental Soybean Population

The experimental chromosome segment substitution line population (CSSLs) used in study consisted of 194 lines (Xin *et al.,* 2016). Among these lines, different chromosomal segments of the Chinese cultivar Suinong 14 were substituted into the genetic background of wild soybean (*Glycine soja* Sieb & Zucc) ZYD00006. Based on CSSLs, using 121 simple sequence repeat (SSR) markers generated a genetic map which covere whole genome (Xin *et al.,* 2016).

Experimental Material Planting and Phenotypic Determination

CSSLs and their parents were grown in Xiangyang crop breeding base of Harbin City, Heilongjiang Province Northeast Agricultural University (45.75°N, 126.53°E) in 2017. Seeds were planted with rows 5 m long, 0.65 m row spacing and with a space of 5 cm between two plants.

Two months after germination of soybean (V10-V13 stage), the total of nodule number (TNN) in CSSLs and

their parents were determined. We have dug five single plants with the same growth trend to calculate TNN, and in this process, try to avoid damage the roots in the soil. Washing the plant and separating the nodule from the root hairs, then we counted up TNN of each plant and calculated the average of five replicates for further data analysis.

QTL Analysis

QTL mapping was performed using the Composite Interval Mapping (CIM) of the WinQTL Cartographer (Wang *et al.*, 2005). The number of control marks is 5 cM, window size is 10 cM, and speed of operation is 0.5 cM. From 1000 permutations of each genotype marker, the experimental threshold level (LOD value) of the chain was calculated. In this study, LOD score peaks greater than 2.5 were considered to be potent QTLs. The identification of genes within the QTL mapping region have been researched (Qi *et al.*, 2014; Xin *et al.*, 2016). From the reported high-quality genetic map (Qi *et al.*, 2014) can be known that each QTL is limited by two SSRs. Using QTL mapping results to further QTL analysis.

Gene Annoation and Enrichment Analysis

We used all kinds of databases for annotating genes (Table 1) and used agriGO (http://bioinfo.cau.edu.cn/agriGO/) for enrichment analyzing. Developers (Du *et al.*, 2010) have enhanced the function of agriGO, it is a web-based toolkit. The agriGO worked out the problem of most GO enrichment tools, which can support specific attention to agricultural species.

Homologous Sequence Alignment and Protein Domain Prediction

The CDS-encoded protein sequence was download from the phytozome, using BLASTP for homologous sequence search on the NCBI. Selecting the homologous gene sequences of different species 56 which more than 85% homology and then using MEGA5 software to build Maximum Likelihood Tree. Using SMART website (http://smart.embl-heidelberg.de/) for protein domain prediction.

qRT-PCR Analyses of Candidate genes

To identify whether the candidate genes involve in the nodulation process of soybean, the qRT-PCR was completed to measure the relative transcript levels of these genes. The root samples of SN14 were collected at 12, 24, 36, 72 and 96 h after inoculations with *S. fredii* HH103. Using the liquid nitrogen to grind the samples into powder and extracting total RNA from the roots with EasyPure® Plant RNA Kit (Transgene Biotech Co.), the extracted RNA was used as the template for a cDNA synthesis with the HiScript® II qRT SuperMix (Vazyme Biotech Co.). Reaction mixtures contained 1 μL of cDNA as template, 0.4 μL of each primer, 10 μL of 2× TransStart Top Green qPCR SuperMix and ddH_2O in a final volume of 20 μL. The program for all qRT-PCR reactions was hold at 95°C for 15 s; 40 cycles of 10 s at 95°C, 20 s at 62°C and 30 s at 72°C; 1 cycles of 5 s at 95°C, 1 min at 65°C and Cool at 4°C.

Results

Phenotypic Analyze

To identify the means and standard errors for TNN and draw a frequency distribution map (Fig. 1), we found that the TNN of CSSLs all lines mainly distributed in the second group to the fourth group. There were fewer lines with high nodules. The TNN of Suinong14 is twice as much as ZYD00006, and there are obvious differences between parents and extreme phenotypes. The minimum TNN is 0, the maximum TNN are 314. The TNN of parents distributing in the third group and the fourth group, and the maximum TNN is 6 times than Suinong14 and 14 times than ZYD00006. It's worth noting that the difference between the extreme materials is very large.

Gene Analysis Based on QTL Mapping Results

CSSLs mapping showed that the common QTL intervals delineated using mapping data ranged between 1.1 and 9.4 cM. Sequence analysis revealed that these regions contain many genes involved in signal recognition and signal transduction. In soybean CSSLs population, there had four QTLs for TNN were identified on different chromosomes (Table 2). These QTL have LOD scores ranging from 4 to 5.9. Based on location results, a QTL was located on chromosome 5. The LOD is 5.9.

A total of 353 genes obtained by us, including 183 genes have GO number. Among them, there are four genes with the function of calcium ion binding (Table 3). The gene *Glyma.05G198300* belongs to CDPK11 family, *Glyma.05g213200* belongs to CDPK3 family. The gene *Glyma.05G199200* and *Glyma.05G199400* were not classified as a family, but we are known about their function. The function of gene *Glyma.05G199200* are calreticulin and calnexin, the function of gene *Glyma.05G199400* is calcium-binding transporter-like protein. For the comparison and analysis of these four genes, we have selected the gene *Glyma.05g213200*. It is related to Ca^{2+} conduction. We marked the genes which contained in the physical section (Block3268-Block3273) and the transcript of the gene which was commented on (Fig. 2). Aim at this gene and its family, we did further research.

Evolution and Analysis of the CDPK3 Gene Family

Against the family of this gene, I did a systematic analysis.

Table 1: All kinds of databases for annotating genes

Web	Website
KEGG	https://www.kegg.jp/
QuickGO	https://www.ebi.ac.uk/QuickGO/
phytozome	www. phytozome. net/soybean
NCBI	http://www.ncbi.nlm.nih.gov/

Table 2: QTL of nodule trait in soybean: total of nodule number (TNN) in the soybean S × Z CSSL population grown at Harbin in 2017

	Trait	Gm	Marker/interval	Position(cM)	LOD	R^2
CSSLs	TNN	Gm02	Block1180-Block1189	63.4-80.2	4	0.143
		Gm05	Block3263-Block3273	91.9-94.2	5.9	0.009
		Gm08	Block5148-Block5151	48.0-73.2	4.1	1.04
		Gm09	Block5479-Block5484	73.7-78.5	5.5	1.816

Table 3: The predicted genes that can combining with calcium ions

Gene ID	Function annotation
Glyma.05G198300	calcium-dependent protein kinase 4
Glyma.05G199200	Calnexin 1
Glyma.05G199400	Mitochondrial substrate carrier family protein
Glyma.05G213200	calcium-dependent protein kinase 3

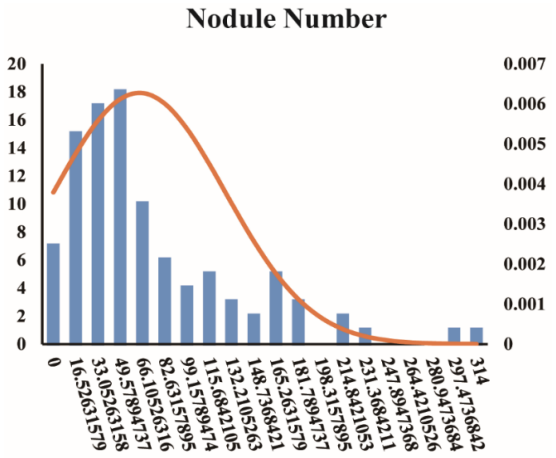

Fig. 1: Frequency distribution of phenotypic values of the nodule number in CSSL

The CDPK3 family includes three genes, respectively located on chromosome 1, chromosome 5 and chromosome 11. These three genes are *Glyma.05g213200* (*CPK-1*), *Glyma.11g020200* (*CPK-2*) and *Glyma.01g223200* (*CPK-3*). The three CDPKs predicted amino acid sequence similarity was higher than 88% (Fig. 3A). Through the SMART to predict the domain displayed that CDPK3 carry two kinds of key domains (Fig. 3B), Ser/Thr kinase domain and EF-hand type calcium-binding domain. And except CPK-3, CPK-1 and CPK-2 contain the low complexity region. Ser/Thr kinase domain are related to protein phosphorylation, which plays a key role in most cellular activities, is a reversible process mediated by protein kinases and phosphoprotein phosphatases.

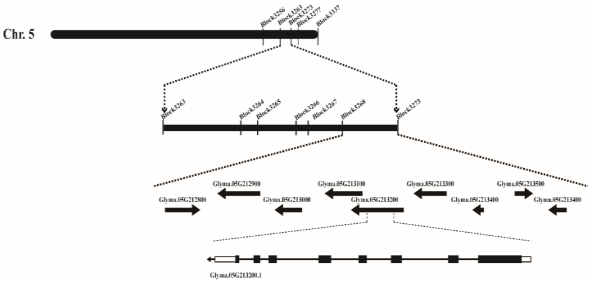

Fig. 2: according to the marker to divide the chromosome physical section. The physical section of chromosome 5 was analyzed in depth and list all the genes involved in the section and the transcript of the CDPK gene

To better understand the amino acid sequence similarity between CDPK3 and other plants, a phylogenetic tree was constructed using multiple sequence alignments from various plant species (Fig. 4). The sequences are as follows: seven genes from *Medicago truncatula*, two genes from *Gossypium hirsutum*, three genes from *Vigna angularis*, two genes from *Populus euphratica*, two genes from *Cicer arietinum*, three genes from *Gossypium arboretum*, two genes from *Nicotiana tabacum*, three genes from *Arabidopsis thaliana Columbia*, and another 22 CDPK3s identified from a variety of species. Including *Theobroma cacao*, *Camellia sinensis*, *Solanum tuberosum*, *Vigna radiate*, *etc*. In this tree, the specie genes that are closely related to each other are marked in the same color. From the Fig. 4, we can see that *CPK-2* and *CPK-3* have the highest homology, up to 90%. And they are closest to *Vigna angularis*. While *CPK-1* has the closest relationship with *Cajanus cajan* and *Vigna angularis*.

The Expression Analysis of Candidate Genes

To detect the variation trend of CDPK3 family expression in soybean root after rhizobium infection, I did qRT-PCR. Designing primers for these three genes were verified by qRT-PCR and using $2^{-\triangle\triangle Ct}$ method for data processing. Based on qRT-PCR analysis (Fig. 5), *CPK-1* was up regulated at 12 to 24 h, down regulated at 24 to 72 h, and up-regulated at 72 to 96 h. *CPK-2* was down regulated at 12 to 24 h, up regulated at 24 to 96 h. *CPK-3* was up regulated at 12 to 48 h, down regulated at 48 to 72 h, and up regulated at 72 to 96 h. The transcript abundance of these three genes have different variation trend, and for their respective functions need to be further studied.

Discussion

In the process of establishing symbiotic nitrogen fixing system between rhizobium and legumes, the expression of specific genes is the basis of effective nodule formation. QTL mapping results can provide a very powerful basis for gene mining and marker-assisted selection (MAS) (Price,

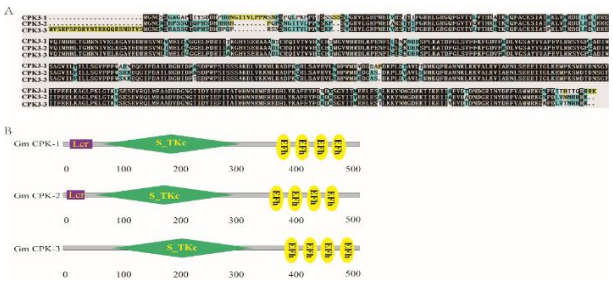

Fig. 3: Sequence comparison between CDPK family. The domain is predicted through the SMART website

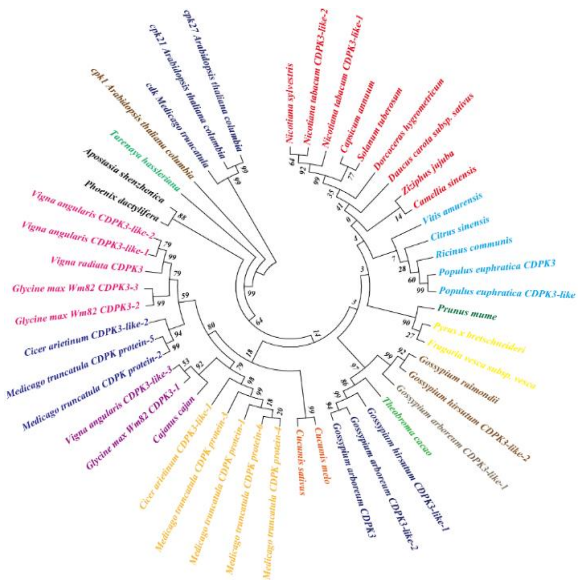

Fig. 4: Phylogenetic tree of plant CDPK3 and CDPK3-like protein sequences. 49 genes of 31 species including the soybean CDPK3 family are analyzed by Maximum Likelihood (ML) using MEGA 5. Genes labeled with the same color were closely related

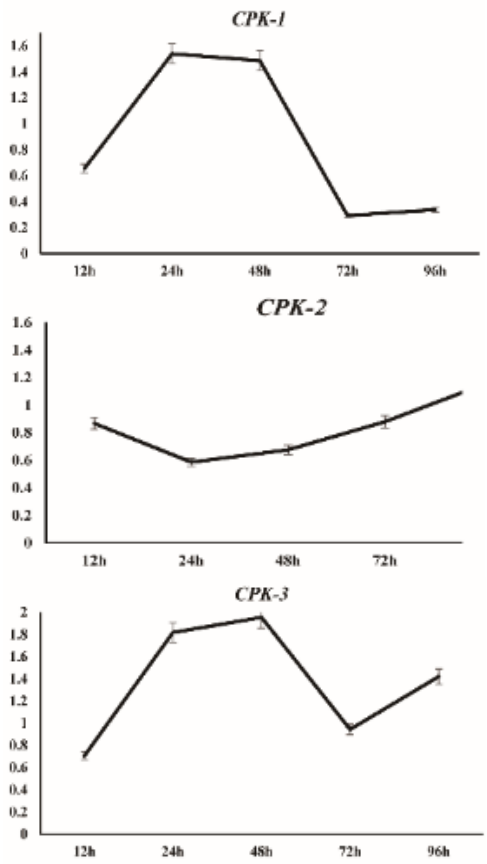

Fig. 5: Quantitative real-time PCR analysis of CPKs transcript abundance in root

2006). In this study, QTL analysis was performed on the traits of total nodule number in the CSSL (n = 160) population. Using the single nucleotide polymorphism map and WinQTL, four QTLs were detected and mapped on 4 different soybean genome LGs. There was a QTL related to the TNN on Gm05 closed to the marker Sat_267, which is consistent with previous research (Hwang *et al.*, 2014). Hwang et al used RIL population to locate QTLs for traits such as nodule number, nodule weight, module size and individual nodule weight in soybean population under field conditions. CIM analysis using the LS average value of RILs for many years to identify two QTLs, these two QTLs located on Gm01 and Gm05 associated with individual nodule weight close to the marker BARC-064293-18611 and BARC-050849-09931. In addition, two QTLs located on Gm01 and Gm05 associated with nodule size close to the marker BARC-011057-00831 and Sat_267 were also be identified. A QTL located on Gm01 associated with nodule weight close to the marker BARC-007726-00090, it had the highest R^2 (20%). A QTL located on Gm11 associated with nodule number had been found by Santos (Santos *et al.*, 2013), this area is related to BNF. The regions that controlling BNF had been reported by Nicolás *et al*. (2006), in which included Gm11 and Gm19. Through the above analysis, we found that Gm01, Gm05 and Gm11 have QTLs controlling BNF traits, and the CDPK3 family genes are near these QTLs. This finding provides a strong basis for proving that CDPK3 have a significant impact on BNF.

CDPKs are a kind of serine/threonine protein kinases (Tai *et al.*, 2009), which are unique to some plants and protists. It is the main receptor of Ca^{2+} signal and important sensor (Reddy *et al.*, 2010). Studies have shown that CDPK plays a role in the signal transduction of the innate immune response. Through the analysis of the protein structure of CDPK we can see that it contains four domains, from N-terminus to C-terminus in turn a variable domain, a protein kinase domain, a linker domain and a regulatory domain, respectively to perform different functions. CDPK is widely found in roots, stems, leaves, flowers and fruits (Trewavas and Malho, 1997; Tai *et al.*, 2009; Wurzinger *et al.*, 2011). CDPKs are involved in multiple signaling pathways in

plants, previous studies on *Medicago* found that in the ROS production pathway, CDPK directly phosphorylates RBOH to participate in the immune response of plants triggered by the invasion of foreign pathogens demonstrating that the CDPK-RBOH complex-mediated immune response regulates the release of rhizobium strains through the infection line, and colonization of rhizobium after entering the host cells (Yu *et al.*, 2018). In addition, previous studies have shown that MtCPK3 is present in the root of the *Medicago truncatula*, and its expression is regulated during the nodulation process (Gargantini *et al.*, 2010). After the infection of rhizobium, the CDPK activity in *Medicago truncatula* and its expression were changed. The expression of MtCPK3 was correlated with time. We can assume that CDPK may play a role in the process of rhizobium infecting plant roots. In *Medicago truncatula*, another CDPK gene *MtCDPK1* which belongs to the distant relationship had been shown to be involved in root development (Davleova *et al.*, 2001), it is likely to participate in the regulation of BNF by regulating root development. To analyze the gene *Glyma.05g213200* and its family in soybean by qRT-PCR found that they were related to rhizobium bacteria.

Through the phylogenetic tree analysis, we found 30 species closely related to CDPK3. Among them, there are three CDPKs in *A. thaliana*, seven CDPKs in *Medicago*. The phylogenetic tree showed that *CPK-2* and *CPK-3* have the highest similarity, and these three genes have the same domain, namely Ser/Thr kinase domain and EF-hand type calcium-binding domain. It is worth noting, the expression levels of the three genes are different in the 96 h period of rhizobia inoculation, and *CPK-2* significantly different from them is that the gene expression level is up-regulated from 24 h to 96 h. Ehrhardt *et al.* (1996) reported that after added the host-specific nodulation factor around the root hair of *Medicago*, there was a calcium oscillation in the proximal nucleus of root hair cells. The calcium spiking starts about ten min after the host-specific nodulation factor invades the root and an average of one spike per min. In the near-nuclear region, the propagation of this oscillation is two-way. However, no such calcium oscillations were observed in a non-nodulated *Medicago* mutant. This pattern of reaction is very similar to that described in peas (Walker *et al.*, 2000), they believe that the formation of calcium spiking may be a part of the signal transduction chain in nodulation. From which it can be assumed that CDPK is likely to be involved in the signal transduction process. Previous studies have speculated that Ca^{2+} might be involved in intracellular information transmission, gene expression or protein synthesis, and calcium spiking production is one of the representations which occurs in the early stages of nodulation (Schultze and Kondorosi, 1998; Felle *et al.*, 1999; Walker *et al.*, 2000).

Based on the result, we speculate that the reason for the different expression trends of these three genes within 96 h of rhizobia inoculation due to these three genes play different roles in Ca signal conduction pathways after rhizobium invaded the root of host soybean. The expression levels of *CPK-1* and *CPK-3* are floating within 96 h, we speculate that these two genes may be related to Ca influx. After 24 h of rhizobium invasion, the expression level of *CPK-2* increased all the time and did not decrease, we speculate that it might be related to Ca spiking, because Ca spiking is sustained in the short term, which may depend on continuous conduction of *CPK-2*. Since we measured it in hours and the time interval was more than 10 h, it may not be able to accurately capture more information, but I think it is necessary to further study the result that the expression level of *CPK-2* continues to increase after 24 h. Overall, this work paves the way for detailed analyze the role of CDPK in the process of rhizobium invading soybean root.

Various experimental studies on *CPK-1* and its gene family are conducive to the exploration of genetic diversity. It is the main characteristic of molecular markers to study identifiable genetic markers based on molecular level (Wang *et al.*, 2019), which plays a very important role in the study of genetic diversity. By directional selection of genes through molecular markers, the major genes of phenotypic traits can be screened, which is beneficial to improve breeding selection efficiency and effectively improve traditional breeding methods. At the same time, it can save a lot of manpower and material resources consumed in the breeding process.

Conclusion

In this study found four QTLs in total, we identified gene *Glyma.05g213200* and systematically analyzed *Glyma.05g213200* and two other genes in its family. We preliminarily determine that CDPK3 family might be related to symbiosis in soybean. Future work could be deeply investigated the relationship between CDPK3 and soybean nodules.

Acknowledgments

Financial support was received from the Ministry of Science and Technology of the People's Republic of China Project (Grant number: 2017YFE0111000), the National Natural Science Foundation of China (Grant numbers: 31400074, 31471516, 31271747, and 30971809), the Natural Science Foundation of Heilongjiang Province of China (Grant number: ZD201213), the Heilongjiang Postdoctoral Science Foundation (Grant number: LBH-Q16014), the Harbin Science Technology Project (Grant numbers: 2013RFQXJ005 and 2014RFXXJ012), the National Key R & D Program of China (Grant numbers: 2016YFD0100500, 2016YFD0100300, and 2016YFD0100201-21), and the Foundation for University Key Teachers from the Education Department of Heilongjiang Province in China (Grant number: 1254G011). University Project of Young Scientist (UNPYSCT-2015011). Conflicts of Interest: The authors declare that they have no conflict of interest.

References

Bourion, V., S.M.H. Rizvi, S. Fournier, H.D. Larambergue, F. Galmiche, P. Marget, G. Duc and J. Burstin, 2010. Genetic dissection of nitrogen nutrition in pea through a QTL approach of root, nodule, and shoot variability. *Theor. Appl. Genet.*, 121: 71–86

Caldwell, B.E., 1966. Inheritance of a Strain-Specific Ineffective Nodulation in Soybeans. *Crop Sci.*, 6: 427–428

Catoira, R., C. Galera, B.F.D. Penmetsa, R.V. Journet, E.P. Maillet, F. Rosenberg, C. Cook and D.C. Gough, 2000. Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. *Plant Cell*, 12: 1647–1666

Cheng, S.H., M.R. Willmann, H.C. Chen and J. Sheen, 2002. Calcium, signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. *Plant Physiol.*, 129: 469–485

Davleova, S., T.P. Meszaros and A. Oberschall, 2001. Auxin and heat shock activation of a novel member of the calmodulin like domain protein kinase gene family in cultured alfalfa cells. *J. Exp. Bot.*, 52: 215–221

Devine, T.E. and L.D. Kuykendall, 1996. Host genetic control of symbiosis in soybean (*Glycine max* L.) *Plant Soil*, 186: 173–187

Du, Z., X. Zhou, Y. Ling, Z. Zhang and Z. Su, 2010. agriGO: a GO analysis toolkit for the agricultural community. *Nucl. Acids Res.*, 38: 64–70

Ehrhardt, D.W., R. Wais and S.R. Long, 1996. calcium spiking in plant root hairs responding to rhizobium nodulation signals. *Cell*, 85: 673–681

Felle, H.H., É. Kondorosi, Á. Kondorosi and M. Schultze, 1999. Nod factors modulate the concentration of cytosolic free calcium differently in growing and non-growing root hairs of *Medicago sativa* L. *Planta*, 209: 207–212

Gargantini, P.R., G.R. Silvina, C. Delphine, R. Marcela, V. Giammaria, R.M. Ulloa, F. Frugier and M.D. Crespi, 2010. A CDPK isoform participates in the regulation of nodule number in *Medicago truncatula*. *Plant J.*, 48: 843–856

Hyten, D.L., V.R. Pantalone, C.E. Sams, A.M. Saxton, D. Landau-Ellis, T.R. Stefaniak and M.E. Schmidt, 2004. Seed quality QTL in a prominent soybean population. *Theor. Appl. Genet.*, 109: 552–561

Harper, J.E. and C.D. Nickell, 1995. Genetic analysis of nonnodulating soybean mutants in a hypernodulated background. *Soybean Genet. Newslett.*, 22: 185–190

Hwang, S., J.D. Ray, P.B. Cregan, C.A. King, M.K. Davies and L.C. Purcell, 2014. Genetics and mapping of quantitative traits for nodule number, weight, and size in soybean (*Glycine max* L. [Merr.]). *Euphytica*, 195: 419–434

Kadota, Y., K. Shirasu and C. Zipfel, 2015. Regulation of the NADPH oxidase RBOHD during plant immunity. *Plant Cell Physiol.*, 56: 1472–1480

Lee, S.W., 1988. The study on the origin of soybean cultivation. *J. Kor. Soc. Food Cult.*, 3: 1–5

Li, Y., R. Guan, Z. Liu, Y. Ma, L. Wang, L. Li, F. Lin, W. Luan, P. Chen, Z. Yan, Y. Guan, L. Zhu, X. Ning, J. Marinus, W. Li, R. Piao, Y. Cui, Z. Yu, M. Guan, R. Chang, A. Hou, A. Shi, B. Zhang, S. Zhu and L. Qiu, 2008. Genetic structure and diversity of cultivated soybean (*Glycine max* (L.) Merr.) landraces in China. *Theor. Appl. Genet.*, 117: 857–871

Luis, C. and Q. Carmen, 2008. Reactive oxygen species (ROS) as early signals in root hair cells responding to rhizobial nodulation factors. *Plant Signal. Behav.*, 3: 1101–1102

Ma, W., Z. Qi, A. Smigel, R.K. Walker, R. Verma and G.A. Berkowitz, 2009. Ca^{2+}, cAMP, and transduction of non-self perception during plant immune responses. *Proc. Natl. Acad. Sci. USA*, 106: 20995–21000

Mitra, R.M., C.A. Gleason, A. Edwards, J. Hadfield, J.A. Downie, G.E.D. Oldroyd and S.R. Long, 2004. A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. *Proc. Natl. Acad. Sci. USA*, 101: 4701–4705

Nicolás, M.F., M. Hungria and A. Caa, 2006. Identification of quantitative trait loci controlling nodulation and shoot mass in progenies from two Brazilian soybean cultivars. *Field Crops Res.*, 95: 355–366

Okazaki, S., S. Zehner, J. Hempel and K. Lang, 2010. Genetic organization and functional analysis of the type III secretion system of *Bradyrhizobium elkanii*. *FEMS Microbiol. Lett.*, 295: 88–95

Paterson, A.H., E.S. Lander, J.D. Hewitt, S. Peterson, S.E. Lincoln and S.D. Tanksley, 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature*, 335: 721–726

Pei, Z.M., Y. Murata, G. Benning, S. Thomine, B. Klüsener, G.J. Allen, E. Grill and J.I. Schroeder, 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature*, 406: 731–734

Price, A.H., 2006. Believe it or not, QTLs are accurate! *Trends Plant Sci.*, 11: 213–216

Qi, Z., L. Huang, S. Zhu, D. Xin, C. Liu, X. Han, H. Jiang, W. Hong, G. Hu, H. Zheng and Q. Chen, 2014. A high-density genetic map for soybean based on specific length amplified fragment sequencing. *Plos One*, 9: e104871

Reddy, A.S.N., G.S. Ali, H. Celesnik and I.S. Day, 2010. Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell*, 23: 2010–2032

Rudd, J.J. and V.E. Franklintong, 2001. Unravelling response-specificity in Ca^{2+} signaling pathways in plant cells. *New Phytol.*, 151: 7–33

Santos, M.A., I. Geraldi, N. Bortolatto and A.A. Garcia, 2013. Mapping of QTLs associated with biological nitrogen fixation traits in soybean. *Hereditas*, 150: 17–25

Schultze, M. and A. Kondorosi, 1998. Regulation of symbiotic root nodule development. *Annu. Rev. Genet.*, 32: 33–57

Searle, I.R., A.E. Men, T.S. Laniya, D.M. Buzas, I. Iturbe-Ormaetxe, B.J. Carroll and P.M. Gresshoff, 2003. Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. *Science*, 299: 109–112

Sun, X.D., H.U. Xiang-Yang and Y.P. Yang, 2015. *Molecular and Functional Comparisons of Reactive Burst Oxygen Species Gene Family in Arabidopsis*. Plant Diversity Resources

Tai, S.S., G.S. Liu and Y.H. Sun, 2009. Cloning and expression of Calcium-Dependent Protein Kinase (CDPK) gene family in common tobacco. *J. Integr. Agric.*, 8: 1448–1457

Tang, F., S. Yang, J. Liu and H. Zhu, 2016. *Rj4*, a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. *Plant Physiol.*, 170: 26–32

Trewavas, A.J. and R. Malho, 1997. Signal perception and transduction: The origin of the phenotype. *Plant Cell*, 9: 1181–1195

Vuong, T.D., C.D. Nickell and J.E. Harper, 1996. Genetic and allelism analyses of hypernodulation soybean mutants from two genetic backgrounds. *Crop Sci.*, 36: 1153–1158

Walker, S.A., V. Viprey and J.A. Downie, 2000. Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by nod factors and chitin oligomers. *Proc. Natl. Acad. Sci. U.S.A.*, 97: 13413–13418

Wang, S., C. Basten and Z. Zeng, 2005. *Windows QTL Cartographer 2.5. Department of Statistics*. NCSU, Raleigh, North Carolina, USA

Wang, Z., H. Ji and E. Wang, 2019. Application and Prospect of Molecular Technology in Peony Research. *Anhui Agric.*, 25: 10–13

Williams, L.F. and D.L. Lynch, 1954. Inheritance of a non-nodulating character in the soybean. *Agron. J.*, 46: 28–29

Wurzinger, B., A. Mair, B. Pfister and M. Teige, 2011. Cross-talk of calcium-dependent protein kinase and MAP kinase signaling. *Plant Signal. Behav.*, 6: 8–12

Xin, D., Z. Qi, H. Jiang, Z. Hu, R. Zhu, J. Hu, H. Han, G. Hu, C. Liu and Q. Chen, 2016. QTL location and epistatic effect analysis of 100-seed weight using wild soybean (*Glycine soja* Sieb. & Zucc.) chromosome segment substitution lines. *PLoS One*, 11: 1–18

Yu, H., A. Xiao, R. Dong, Y. Fan, X. Zhang, C. Liu, C. Wang, H. Zhu, D. Duanmu, Y. Cao and Z. Zhang, 2018. Suppression of innate immunity mediated by the CDPK-Rboh Complex is Required for Rhizobial Colonization in Medicago Truncatula Nodules. New Phytol.,

Zhuang, B., D. Hui, Y. Wang, J. Gu, B. Xu and S. Chen, 1995. Comparison of RAPD profiles of different soybeans in China. *Chin. Sci. Bull.*, 40: 1129–1129

[Received 11 Apr 2019; Accepted 12 Jun 2019; Published (online) 10 Nov 2019]