Characterization of Peanut Germin-Like Proteins, *AhGLPs* in Plant Development and Defense

Tong Wang1,2,3, Xiaoping Chen1, Fanghe Zhu1, Haifen Li1, Ling Li3, Qingli Yang2, Xiaoyuan Chi2, Shanlin Yu2, Xuanqiang Liang1*

1 Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, People’s Republic of China, 2 Shandong Peanut Research Institute, Qingdao, People’s Republic of China, 3 College of Life Science, South China Normal University, Guangzhou, People’s Republic of China

Abstract

**Background:** Germin-like superfamily members are ubiquitously expressed in various plant species and play important roles in plant development and defense. Although several GLPs have been identified in peanut (*Arachis hypogaea* L.), their roles in development and defense remain unknown. In this research, we study the spatiotemporal expression of *AhGLPs* in peanut and their functions in plant defense.

**Results:** We have identified three new *AhGLP* members (*AhGLP*3b, *AhGLP*5b and *AhGLP*7b) that have distinct but very closely related DNA sequences. The spatial and temporal expression profiles revealed that each peanut GLP gene has its distinct expression pattern in various tissues and developmental stages. This suggests that these genes all have their distinct roles in peanut development. Subcellular location analysis demonstrated that *AhGLP*2 and 5 undergo a protein transport process after synthesis. The expression of all *AhGLPs* increased in responding to *Aspergillus flavus* infection, suggesting *AhGLPs’* ubiquitous roles in defense to *A. flavus*. Each *AhGLP* gene had its unique response to various abiotic stresses (including salt, H2O2 stress and wound), biotic stresses (including leaf spot, mosaic and rust) and plant hormone stimulations (including SA and ABA treatments). These results indicate that *AhGLPs* have their distinct roles in plant defense. Moreover, in vivo study of *AhGLP* transgenic *Arabidopsis* showed that both *AhGLP*2 and 3 had salt tolerance, which made transgenic *Arabidopsis* grow well under 100 mM NaCl stress.

**Conclusions:** For the first time, our study analyzes the *AhGLP* gene expression profiles in peanut and reveals their roles under various stresses. These results provide an insight into the developmental and defensive roles of GLP gene family in peanut.

Introduction

Peanut (*Arachis hypogaea* L.) is one of the major worldwide oil crops. Peanut has very high nutritional and commercial value. However, the increase in its production is hampered by pathogens such as fungi, bacteria, viruses, insect pests and physiological stresses caused by chemicals and salt. It is estimated that yield losses of peanut are up to about 30% due to various disease and adverse physiological conditions [1]. So it is an urgent task to identify and characterized resistant genes in peanut development and defense. An insight into functions and usage of resistant genes will make a great progress in peanut cultivation.

Germins and germin-like proteins (GLPs) are plant exclusive cupin subfamily water-soluble glycoproteins. Germin was first identified during wheat germination [2] and later was found to be oxalate oxidases (OXOs) [3]. Germins and germin-like protein subfamily are characterized by the presence of germin boxes (PHIHPRATEI) and a conserved cupin superfamily derived-motif [4,5]. This motif is a conserved beta-barrel protein with a metal ion binding ability [6]. According to their sequence similarities and other characters, Germins and the GLP gene family are divided into two distinct group proteins. The first group named “the true germins” is only identified in “true cereals”, which contain barley, corn, oat, rice, rye and wheat. Members in this group have relatively homogeneous protein sequences [7] and always carry OXO enzyme activity. The second group is designated as germin-like proteins (GLPs), whose members show relatively high sequence divergence. Their amino acid sequence similarity to wheat germin varies from 30% to 70%. The second group contains more numerous members than the first group and only few of the second group members possess OXO activity.

GLPs are a large gene family and have a wide range of distribution among plants. Expressed sequence tags (ESTs) or genomic sequencing have identified more than 100 GLPs in...
higher plant *Arabidopsis thaliana* genome, 27 GLP genes have been discovered [8,9]. Also, 14 GLP genes in barley and 8 GLP genes in rice have been identified [10,11]. In lower plants, Nakata et al. have identified 77 EST clones of GLPs from *Physcomitrella patens* [12]. GLPs also have a wide range of expression in various plant organs and developmental stages. GLPs have been identified to express in a variety of tissues such as roots, leaves and flowers [13–15]. The ubiquitous distribution of GLPs implies the GLPs’ fundamental and indispensable functions in plants. And their expression in various organs suggests that GLPs may execute roles in the development of various plant organs.

GLPs play critical roles not only in plant development but also in plant defense responses. Several evidences have suggested the functions of GLPs in plant defense [16]. One is the observation of increasing expression of certain GLPs in various plants under stresses like fungal, bacteria, and virus infections [5,17–20], parasite attacks, insect invasions [21], chemical toxicities, salt pressures [22,23] and drought stresses [24]. The other evidence of GLPs’ roles in plant defense is the enhanced resistance of transgenic GLP plants to various stresses.

For example, transformation of a wheat GLP into soybean, sunflower and tobacco provided them the resistance to *Sclerotinia sclerotiorum*. Transient overexpression of GLPs in barley resulted in enhanced plant resistance to the powdery mildew fungus [5]. It is proposed that the mechanism by which GLPs function plant defense responses is associated to their enzyme activity of OXO and superoxide dismutase (SOD), which can generate H$_2$O$_2$ to influence plant defense. Additional enzyme activities of GLPs that may function in plant defense include ADP glucose pyrophosphatase/phosphodiesterase (AGPPase) [25] and serine protease inhibitors [26].

Expressed sequence tags (ESTs) have identified 8 *Arachis hypogaea* L. germ-like proteins (AhGLPs) in peanut [27]. The previous studies in our lab have revealed that the expression level of *AhGLP1*, originally named oxalate oxidase (OXO), significantly increased in *Aspergillus*- resistant peanut seeds after both drought and *A. flavus* treatments [28]. Due to GLPs’ key functions in plant development and defense, we determined the expression of AhGLPs during peanut development and characterized the functions of *AhGLP* both in biotic and abiotic stresses. Our results in this study suggest that *AhGLPs* express in various organs during peanut development and play important roles in plant defense. AhGLPs are valuable resistant genes that can be used in improving peanut pathogen resistance, thus increasing the yield in peanut cultivation.

### Results

**Identification of new AhGLP homologue genes**

Through homology matrix, we have identified three new homologous members in the *AhGLP* family. According to their sequence similarity, we termed them as *AhGLP3a*, *AhGLP5a* and *AhGLP7a*. Sequence alignments revealed that these three new members all share more than 97% nucleotide homologies with other corresponding members in the family (Figure S1). The genetic polymorphism between *AhGLP3a* and *AhGLP3b* includes two point mutations and one deletion (Table 1), which lead to 3 amino acid changes. Seven nucleotide variations between *AhGLP5a* and *AhGLP5b*, and ten nucleotide variations between *AhGLP7a* and *AhGLP7b* were identified, respectively. The genetic variations relative to *AhGLP5a* and *AhGLP7a* are all point mutations. Among the seven point mutations between *AhGLP5a*

### Table 1. Difference of closely related multiple AhGLPs.

| Genes | Nom. | Position | Type | Base Mutation | Protein Mutation |
|-------|------|----------|------|---------------|-----------------|
| AhGLP3a vs AhGLP3b | 1 | 107–108 | Codon | AGC→A | Ser→Glut |
| | 2 | 124 | Point | G→A | Val→Met |
| | 3 | 562 | Point | T→G | Phe→Val |
| AhGLP5a vs AhGLP5b | 1 | 31 | Point | G→A | Val→Ile |
| | 2 | 90 | Point | T→C | Tyr (Silent) |
| | 3 | 370 | Point | A→G | Ile124→Val124 (Silent) |
| | 4 | 381 | Point | T→C | Asp127 (Silent) |
| | 5 | 429 | Point | C→T | Tyr143 (Silent) |
| | 6 | 553 | Point | C→T | Pro185→Ser185 |
| | 7 | 597 | Point | N→G | Ser199→Glu199 |
| AhGLP7a vs AhGLP7b | 1 | 29 | Point | T→A | Ile→Ala |
| | 2 | 73 | Point | G→A | Ala→Thr |
| | 3 | 150 | Point | T→C | Cys (Silent) |
| | 4 | 208 | Point | G→A | Ala→Thr |
| | 5 | 241 | Point | G→A | Ala→Thr |
| | 6 | 295 | Point | A→G | Thr→Ala |
| | 7 | 307 | Point | C→T | Arg→Lys (Silent) |
| | 8 | 363 | Point | A→G | Pro→Ser |
| | 9 | 643 | Point | G→A | Glu→Lys |
| | 10 | 651 | Point | N→T | Ser217→Ile217 |

*aAhGLP3a* (GU457419.1), *AhGLP5a* (GU457421.1) and *AhGLP7a* (GU457423.1) were the known genes as reported by Chen et al (2011) in NCBI genebank, three newfound genes were designated *AhGLP3b*, *AhGLP5b* and *AhGLP7b* distinguished from *AhGLP3a*, *AhGLP5a* and *AhGLP7a* respectively. *Un-know bases in the nucleotide sequence of AhGLP5a and AhGLP7a with indefinable amino acids (*).*
and AhGLP5b, three were silent mutations, the other four ones result in amino acid variations. Similarly, among the ten point mutations between AhGLP7a and AhGLP7b, three were silent mutations, the other seven ones produce amino acid variations.

Mapping of the developmental expression pattern of AhGLP mRNA in peanut

Expression pattern of the AhGLP family genes was first time examined in a variety of peanut tissues and various peanut developmental stages (Figure 1). During pods development (1–35d), AhGLP1 mRNA level was the highest 1 day after gynophore penetrated into under-soil, and decreased afterward. The mRNA of AhGLP5 increased gradually during pods development. AhGLP2, AhGLP3 and AhGLP8 had similar expression pattern, which have the lowest level in the middle stages. Other three genes (AhGLP4, AhGLP6 and AhGLP7) expressed at a very low level in these stages. During peanut seed germination, the expression level of all the AhGLP genes reached the highest at day 3, except that of AhGLP3 which was the highest at day 5.

The mRNA level of the AhGLP family genes was determined in roots, stems, leaves, flower buds, and flowers. In the stems, AhGLP1 had the highest mRNA level while AhGLP4 and AhGLP7 had the lowest expression level. In the leaves, AhGLP5, AhGLP4 and AhGLP5 were all highly expressed, while both AhGLP6 and AhGLP7 had a very low expression level. In the roots, AhGLP1 also had the highest mRNA level. On the contrary, the mRNA level of AhGLP5 and AhGLP6 was very low. All the AhGLP family genes had a high mRNA level in old roots. The mRNA in flower buds decreased in the sequence of AhGLP8, AhGLP4, AhGLP5, AhGLP1, AhGLP3, AhGLP2 and AhGLP6, AhGLP7. In peanut flowers, only AhGLP2, AhGLP3 and AhGLP4 had a medium level expression. The various expression patterns of different AhGLP family genes suggest their various functions in different tissues and during various development stages.

Determination of the subcellular localization of AhGLP proteins

The protein sequence bioinformation analysis showed that most AhGLPs contain putative extracellular localization signal peptides in their N-terminal. The prediction suggests that AhGLP proteins potentially target to the cell membrane or cell wall [27]. To confirm this possibility, the coding region of each AhGLP gene was respectively fused to the N-terminus of soluble modified GFP (smGFP) gene, whose expression was under the control of the constitutive CaMV35S promoter (AhGLPs::smGFP). Onion epidermal cell is a convenient system and widely used for analyzing protein subcellular localization [29]. The obtained constructs, together with non-AhGLP control vector were then introduced into onion epidermal cells by Agrobacterium-mediated transient transformation. Subcellular localization of AhGLP proteins in onion (Allium cepa) epidermal cells, indicated by the GFP signal was observed under fluorescence microscopy. The results showed that compared to the control vector (35S-GFP), AhGLP1, 3 and 7 had similar subcellular location pattern being distributed both in cytoplasm and plasma membrane or cell wall whereas AhGLP4 was only localized in cytoplasm (Figure 2). On the contrary, AhGLP2 and 5 were only localized to the plasma membrane or the cell wall. The results suggest that most of AhGLPs can locate to the cell membrane or cell wall in consistence with AhGLPs having putative extracellular localization signal peptides in their N-terminal.

Examination of the expression of AhGLPs in responding to A. flavus infection

Aspergillus flavus is a crop saprophyte that causes severe aflatoxin contamination to peanut seeds. To understand the possible roles of AhGLPs during A. flavus infection, we profiled the expression patterns of AhGLP genes in pre- and post-harvested peanut seeds after A. flavus infection. Compared to untreated control and
drought-stresses treated pre-harvested seeds, according to T-test statistical analysis the expression level of \textit{AbGLP1}, 2, 3, 4 and 5 were significantly up-regulated after \textit{A. flavus} infections (Figure 3A). Conversely, the expression level of \textit{AbGLP6}, 7 and 8 did not change much after \textit{A. flavus} infections. In addition, according to T-test statistical analysis drought treatments significantly decreased the expression of \textit{AbGLP6}. This suggests that \textit{AbGLP6} is the major gene responding to drought stresses.

For a further confirmation, the expression of \textit{AbGLPs} in response to \textit{A. flavus} infection was tested in post-harvested peanut seeds with 20\% relative humidity (RH). Compared to untreated control, the expression level of all \textit{AbGLPs} except \textit{AbGLP5} was up-regulated 9 days after \textit{A. flavus} infections (Figure 3B). And according to T-test statistical analysis, \textit{AbGLP5} also significantly increased in day 3 after the infection. This suggests that \textit{AbGLP5} is a quick responding gene in \textit{A. flavus} infections. The data demonstrated that all \textit{AbGLPs} are \textit{A. flavus} responding genes and may have functions in peanut defense of \textit{A. flavus} infections.

**Profiling of the expression of \textit{AbGLPs} under various biotic, abiotic and hormone stresses**

In order to determine the roles of \textit{AbGLPs} in plant defense, we examined the expression of \textit{AbGLPs} responding to a variety of biotic, abiotic and hormone stress treatments. 7-day-old peanut seedlings were treated with various abiotic stresses including \textit{H}_2\textit{O}_2, salt and wound. Expression of \textit{AbGLPs} in leaves and roots was then determined by quantitative RT-PCR analysis. Compared to mock-treated control, according to T-test statistical analysis \textit{H}_2\textit{O}_2 treatments significantly increased the expression of \textit{AbGLP2}, 3, 4, 5, 7 and 8 and decreased \textit{AbGLP6} expression in roots after 12 and/or 24 hours (Figure 4A). According to T-test statistical analysis, NaCl treatments significantly increased the expression of \textit{AbGLP3}, 5, 7 and 8 and decreased \textit{AbGLP6} expression in root in 24 hours (Figure 4A). In the leaves, the expression of \textit{AbGLP2}, 6 and 7 was significantly up-regulated while \textit{AbGLP1} expression significantly down-regulated 12 and/or 24 hours after \textit{H}_2\textit{O}_2 treatments according to T-test statistical analysis (Figure 4B). According to T-test statistical analysis, NaCl treatments significantly increased \textit{AbGLP2} level in leaves. The expression of \textit{AbGLP4} and 5 in leaves was up-regulated while the expression of \textit{AbGLP1}, 3, 6 and 7 in leaves was down-regulated after 24 hours under NaCl treatments (Figure 4B). The level of \textit{AbGLP1}, 2, 5, 6 and 8 was up-regulated, while the level of \textit{AbGLP3} and 7 was down-regulated 12 and/or 24 hours after wound treatments on the leaves (Figure 4B).

To demonstrate the functions of \textit{AbGLP} in plant disease defense, 80-day-old peanut plants were treated with a serial of biotic stresses including leaf spot, mosaic and rust. Then, the expression of \textit{AbGLP} mRNA in leaves was analyzed by qRT-PCR. According to T-test statistical analysis, leaf spot treatment significantly increased the expression of \textit{AbGLP1}, 2, 5, 6, 7 and 8 (Figure 4C). According to T-test statistical analysis, mosaic treatment significantly increased the expression of all \textit{AbGLPs} except \textit{AbGLP6} (Figure 4C). The level of \textit{AbGLP2}, 7 and 8 was significantly up-regulated under rust treatments according to T-test statistical analysis (Figure 4C).

In order to examine the responses of \textit{AbGLP} to hormone stimulations, their mRNA level was determined in the leaves of 7-day-old peanut seedlings after the stimulations of hormones including salicylic acid (SA) and abscisic acid (ABA). According to T-test statistical analysis, the expression of \textit{AbGLP2}, 3, 4, 5, 7 and 8 was all significantly up-regulated 5 h after SA stimulation (Figure 4D). Among them, only \textit{AbGLP3} expression kept going up, while the level of all others went down 10 hours after SA treatment. The level of \textit{AbGLP6} kept going down after SA treatments. The expression of all \textit{AbGLPs} except \textit{AbGLP6} was up-regulated 1 h after ABA stimulation (Figure 4D). Except \textit{AbGLP7} and 8, the level of all other \textit{AbGLPs} went down 5 hours after the treatments. The level of \textit{AbGLP7} and 8 also went down in 10 h, while the expression of \textit{AbGLP4} and 6 increased again 10 h after ABA treatments.

The results showed that various \textit{AbGLP} gene expressions were induced in responding to different stresses stimulations. Their expressions induced were different and their expression had unique time windows under the stresses. This suggests that each \textit{AbGLP} has their unique functions responding to various stresses in the plant defense. To have a comprehensive knowledge of how \textit{AbGLPs} responding to various stimulations including hormone (including SA and ABA treatments), abiotic stress (including salt, \textit{H}_2\textit{O}_2 stress and wound) and biotic stress (including leaf spot, mosaic and rust), the overlap analysis of \textit{AbGLP} genes response was performed (Figure 5). Most of the \textit{AbGLP} genes including \textit{AbGLP1}, 2, 3, 4, 5, 7 and 8 had increasing expression in responding to all of these three stimulations. Whereas, \textit{AbGLP6} expression was down-regulated in responding to most of the stimulations except wound and leaf spot treatments. Additionally, the expression of \textit{AbGLP1}, 3 and 7 was down-regulated under abiotic stresses. It is also interesting to note that all peanut GLP family genes primarily had

---

Figure 2. Subcellular localization of \textit{AhGLPs}-GFP proteins in onion epidermal cells. Localization of \textit{AhGLPs}-GFP fusion protein. Control: fluorescence of onion epidermal cells under empty vector. 35S-smGF: onion epidermal cells expressing the GFP gene only driven by the 35S promoter. GFP fluorescence and differential interference contrast images and Visible/GFP merged images are shown from left to right.

doi:10.1371/journal.pone.0061722.g002
Figure 3. Expression of AhGLPs in response to A. flavus infection in pre- and post-harvested peanut seeds. A: Con: control; Dt: drought stress; Ad: A. flavus infection under drought stress condition; B: Changes of the expression of AhGLP family genes in damp-dry peanut seed with 20% RH (relative humidity) under A. flavus infection. Con: control; A. f: A. flavus infection. doi:10.1371/journal.pone.0061722.g003

Figure 4. Differential expression of peanut AhGLP genes in response to various abiotic, biotic and hormone treatment conditions. AhGLPs transcript abundance was detected by qRT-PCR analysis. Con1 and Con2: control sample of seedling leaf and root to abiotic stresses, respectively; Con3: control of 100-day-old peanut plant leaf to biotic stresses; Con4: control of seedling leaf to hormone treatments; Wnd: wound treatment; Numbers below bars correspond to duration of treatments (h); SA: salicylic acid; ABA: abscisic acid. doi:10.1371/journal.pone.0061722.g004
A great deal of GLP family members have been identified and divided into 5 subfamilies including bryophyte GLP subfamily, gymnosperm GLP subfamily, “ture gemin subfamily”, GLP subfamily 1, GLP subfamily 2 and GLP subfamily 3. Guo et al [27] reported the presence of eight GLP members in peanut based on the analysis of EST database and divided them into three classes including GLP subfamily I (AhGLP3a), GLP subfamily II (AhGLP2, 6 and 8) and GLP subfamily III (AhGLP1, 4, 5a and 7a) according their protein sequence feature. The sequence identities of these subfamily members ranged from 31.3% to 72.0%. In this study, we have identified three new AhGLP homologues named as AhGLP3b, AhGLP5b and AhGLP7b. They all showed highly homology compared with the corresponding AhGLP homologues like AhGLP3a (99.1%), AhGLP5a (98.6%) and AhGLP7a (97.7%) (Additional File 3). It has been found that the closely related GLPs including AhGLP2a/b (97.7% identity) and AhGLP3a/b (99.5% identity) in Arabidopsis had very similar functions [8]. So, we deduced that these closely related AhGLPs could also have the similar functions in peanut.

Although the spatiotemporal expression of germins and GLPs in different plant species has been characterized [30–33], their pattern in peanut remained unclear. In this study, we surveyed the transcript accumulation of the eight AhGLP genes across a wide range of tissues/organs and developmental stages of peanut through qRT-PCR analysis. Our results for the basal expression patterns of each of the peanut GLP genes (Figure 1) suggest that each AhGLP has its distinct expression pattern in different tissues and stages, indicating that the spatiotemporal regulation of their expression is distinct. Furtheremore each AhGLP had its distinct function during peanut development. For example, AhGLP2 and AhGLP4 had a very high level in flower buds and significantly decreased in flowers, suggesting that these two genes may function in peanut flowering. The low expression level of AhGLP6 and AhGLP7 in both flower buds and flowers indicates that the two genes may not function in flowering. Also, the expression pattern of AhGLP1 suggests its potential roles in roots and pods development and seed germination. Besides, AhGLP3, AhGLP4 and AhGLP5 may function in leaf development and AHGLP67 may play a role during seed germination. Moreover, the specificity of AhGLP3 expression paved the way that AhGLP3 can be used as markers of various developmental stages in future.

It has been identified that in wheat about 40% germins are associated with cell wall and critical for development [34]. The N-terminal signal peptide in Germin and GLPs has been proved to help their secretion from the cell [35]. Cellular localization studies also confirmed the association of GLPs with the cell surface [20,32]. In rice, a high abundance of GLPs has been shown to
locate in epidermal cells [36]. Sequence analysis identified a signal peptide in AhGLPs N-terminal region, suggesting the possibility of AhGLP apoplastic or plasma membrane localization [27]. Our results showed that all AhGLPs are distributed at plasma membrane or cell wall (Figure 2). This confirmed their N-terminal signal peptide’s functions. Among these AhGLPs, only AhGLP2 and 5 were excluded from the nucleus. The results suggest that AhGLP2 and 5 may perform a protein translocation process after synthesis.

The expression of GLPs and germin has been shown to be differentially regulated responding to pathogen attack in several plant species such as barley [5], rice [20], grapevine [31] and Arabidopsis [37]. They are expressed in a diverse range of tissues and have inding of the broad spectrum of defensive activities in host-pathogen interaction [35]. It is worth mention that the overexpression of barley OXO gene results in enhanced resistance to the *Sclerotinia* minor in peanut [38]. In contrast, transient silencing of some barley GLP subfamilies increased the susceptibility to powdery mildew fungus [5]. Recently, our previous proteomics study revealed that the level of AhGLP1 protein, originally named as OXO, was positively correlated to *A. flavus* infection in pre-harvested resistant peanut seed [28]. In our study,
the expression of most AhGLP family genes was also induced significantly when attacked by fungal pathogens (leaf spot, *A. flavus*) and bacteria (Rust disease) in peanut seeds (Figure 4B). This suggests that AhGLPs play important roles in pathogen defense. To further understand the molecular function of AhGLP family genes in response to *A. flavus* infection, the expression patterns were analyzed in pre- and post-harvest peanut seeds during *A. flavus* invasion, respectively (Figure 3A and B). The results showed that all AhGLP mRNAs might response to *A. flavus* infection in seeds. Like GLPs in other plant species, our results suggested that AhGLPs are also broad spectrum and effective defense proteins against multiple pathogens.

Plants have a variety of strategies to adapt to unfavourable environmental conditions including various abiotic and biotic stresses [39]. Many studies have indicated that the germin and GLPs play important roles in resistance to various abiotic and/or biotic stresses [35]. In some model crops and plants, such as wheat, barley, rice and *Arabidopsis*, a number of GLP genes have been shown to function in response to stresses mainly thought their SOD [33,40,41], OXO [42] and AGPase enzyme activity [25]. Although some of peanut GLP gene sequences were present in the GenBank database, so far only AhGLP2 has been identified to have SOD enzyme activity [27]. In this study, we analyze the differential expression of AhGLPs between peanut roots treated with different abiotic (NaCl, H$_2$O$_2$, wound) stresses and untreated control (Figure 4A). Same analysis was performed in the leaves. Six barley HvGER family genes [5] appeared to participate in multiple abiotic stress responses in leaf and root. Similarly, this analysis showed that all the peanut GLP genes were significantly differentially expressed under at least one of the abiotic stresses in leaves. Moreover, in roots, only AhGLP2, 3, 4, 5, 7 and 8 changed their expression after NaCl and H$_2$O$_2$ treatments. These results indicated that the expression of peanut GLP genes has tissue-variability and AhGLPs are involved in different regulation pathways in response to abiotic stresses. Phytohormones are important factors that participate in plant gene regulation networks involving abiotic and biotic response and tolerance [43]. The transcript level of some GLP genes is enhanced or suppressed after application of phytohormones including salicylic acid (SA) [21,31,44] and abscisic acid (ABA) [45]. In this study, all the peanut GLP genes significantly changed their expression level under treatment of exogenous SA and ABA (Figure 4C). Among them, AhGLP6 expression was inhibited, the expression of all other seven members was induced. The result suggests that they might play roles in SA/ABA-dependent signaling transduction pathways during abiotic and biotic stress responses. Germin and GLPs are multi-functional proteins, and many expression studies of these genes have shown crosstalk between various stimuli such as biotic, abiotic and hormone [5,20,31]. Our overlap analysis also found relation between tissue-/developmental stage-specific expression pattern and stress responses of peanut GLP genes (Figure 1, 2 and 3). It is noteworthy that AhGLP family genes showed broad spectrum stress responses mostly in peanut leaves. However, only AhGLP6 showed response to specific stimuli (Figure 3). GLP resistance may be broad spectrum and effective against various environmental stimuli [20,37]. These commonly regulated AhGLPs will provide some promising candidate genes for genetic engineering for improving crop resistance to different stresses.

GLPs play important roles in salt resistance. It has been proven that in responding to salt stress GLP expression increased in barley roots [22]. Moreover, salt stress can prolong the expression of GLP in barley [46]. Also, the GLP genes were proved to response to salt stress in soybean [47]. GLP proteins were gradually up-regulated during the period of salt treatment in wheat leaves [48]. Proteome analysis demonstrated that germin-like protein increased significantly in response to salt stress in the tobacco leaves [49]. In respond to salt stress, not only the expression of GLP but also the expression site changes. In salt-stressed wheat embryos, germin mRNA change their location to coleoptile cells instead of its original site, coleorhiza tissue [50]. In a moss, it is proposed that dissociation of GLP protein from the cell wall into the medium in the cells caused the induction of GLP gene by salt stress during the logarithmic phase [51]. Recently, it has been discovered that several other plant GLP genes can enhance the tolerance to salt stresses in transgenic plants [52]. After salt treatments, the expression of *AhGLP2*, 3 and 7 significantly increased in root. However, in the leaves only *AhGLP2* expression increase obviously in response to salt treatment. The results suggest these 4 AhGLPs may function under salt stresses. To confirm this possibility, we engineered AhGLP transgenic *Arabidopsis* plants and challenge the plants with salt stresses. Both AhGLP2- and 3-overexpression *Arabidopsis* seeds displayed significantly higher germination rates than other seeds (Figure 6B). Moreover, the highest germination rates maintained throughout the time points (from day1 to day6) (Figure 5C). After treated with 100 mM NaCl for 15 days, only the seedlings of AhGLP2- and 3-overexpressing *Arabidopsis* still grew very well with green leaves (Figure 6D). These results suggested that both AhGLP2 and 3 genes were involved in the salt stress response and tolerance in plants.

Salt stress could accelerate production of active oxygen species (AOS) and subsequently cause oxidative damage in plants. Antioxidants, therefore, are elements of the salt stress response in a manner similar to general stress responses in plants [53], and various types of SODs are thought to have important roles in controlling oxidative stress [53]. Thus, one of possible explanation for the tolerance function of AhGLP-2 and 3 in *Arabidopsis* could be the SOD enzymatic activity, although only AhGLP2 have been identified to have SOD activity [27]. Moreover, it has been reported that the high level of flavonoid may be related to high salt stress tolerance through scavenging of stress-induced AOS [54]. In this study, *Arabidopsis* plants overexpressing AhGLP2 and 3 enhanced the transcript levels for flavonoid biosynthetic genes including *DEF*, *CHS* and *3GT* than control plants. Furthermore, the expression of PR proteins (AtPR3, 4 and 5) in transgenic *Arabidopsis* with *AhGLP* was enhanced diversely. This result is consistent with the studies by Knecht [37], in which *BrGLP1* overexpression in *Arabidopsis* induced SA- and JA-dependent PR genes transcripts. These transgenic lines could be better protected through activation of the defense genes even in the absence of the pathogens. This strongly suggests that AhGLP2 and 3 may play importantly functional roles in plants by specifically regulating the expression of a set of plant defense-related genes. The function of *AhGLP* under pathogen attack will be further studied.

Conclusions

In general, expression patterns of 8 peanut GLP genes were analyzed in different tissues and stages. Their expression in response to various biotic stresses and abiotic stresses, and plant exogenous hormone treatments was also analyzed through qRT-PCR. The results revealed that expression levels of AhGLP family genes are varied greatly in different tissue-/developmental stage and various stresses. Moreover, six AhGLPs have been isolated and were used for expression in *Arabidopsis* and subcellular localization analysis in onion cells. The results indicated that AhGLP2 and 3 might be salt stress response and tolerance genes and most of the GLP genes located plasma membrane or cell wall.
Materials and Methods

Ethics Statement

No specific permits were required for the described field studies. No specific permissions were required for these locations and activities. The location is not privately-owned or protected in any way and the field studies did not involve endangered or protected species.

Plant material and sampling

A cultivar peanut (Arachis hypogaea, L) YJ-1 with resistance to Aspergillus flavus infection was provided by Crops Research Institute, Guangdong Academy of Agricultural Sciences (GDAAS, China). The seeds were surface sterilized using 70% (v/v) ethanol for 2 min, and then sown in pots of compost soil in a greenhouse under white fluorescent light (16 hr light/8 hr dark) at 30°C and 70% relative humidity. After germination, seedlings and plants were randomly divided into several groups (each containing six samples) and subjected to different stress treatments as explained below. Mature leaves, roots, stems, various stage panicles and seeds were collected. Roots from 7-day-old seedlings were also harvested.

For salt and H2O2 stress treatments, 7-day-old light-grown peanut seedlings were watered with solutions of 100 mM NaCl and 100 mM H2O2, respectively. After 12 and 24 h, the leaves and roots were collected. Likewise, 7-day-old seedlings were incubated with 100 μM ABA-solution and 50 μM salicylic acid (SA)-solution, respectively. And the leaves were collected after 1, 5 and 10 h. For the wound treatment, primary leaves of 7-day-old seedlings were rubbed gently with fine sandpaper and samples were collected after 12 and 24 h. Untreated samples were collected as controls at the same time points.

For peanut leaf disease treatments, 35-day-old plants were sprayed or inoculated with a spore suspension of leaf spot, mosaic and rust [55]. Triplicate samples of control and infected peanut leaves were collected. The post- and pre-harvested peanut seeds of cultivar YJ-1 were challenged with A. flavus according to the method of Liang [56] and Wang [28], respectively. The post-harvest seeds were collected 0, 3, 6 and 9 days after A. flavus infection, and the pre-harvest seeds were sampled 5, 10, 15 and 20 days after treatments with both drought and A. flavus stresses. All plant materials were snap frozen in liquid nitrogen and stored at −80°C.

RNA isolation and purification

Total RNA was isolated from peanut tissues and Arabidopsis leaves (wild-type and AhGLPs-transgenic) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. All samples were collected from three biological replicates [28]. All RNA extracts were treated with RNase-free DNase I (Takara, Dalian, China) then cleaned up with RNeasy Cleanup Kit (Qiagen, Beijing, China). RNA concentration and purity were assessed by Nano Drop ND-100 spectrophotometer (Nano Drop Technologies Inc., Delaware, USA) and electrophoresis on 1% agarose gel. The obtained RNA was stored at −80°C.

Quantitative real time RT-PCR

All qRT-PCR reactions were performed as described previously [28]. 4 μg of total RNA was reverse transcribed to cDNA using PrimeScript II 1st Strand cDNA Synthesis kit (Takara, Dalian, China) according to the manufacturer’s protocols. Quantitative real-time RT-PCR was performed with SYBR® Premix Ex Taq™ II kit (Takara, Dalian, China) in LightCycler 480 instrument (Roche, Germany) equipped with Light-Cycler Soft-ware version 1.5 (Roche, Germany) according to the manufacturer’s instructions [57]. All the primers specific for peanut AhGLPs and Arabidopsis stress-related genes were designed using the Primer version 5.0 (PREMIER BioSoft International) and listed in Table S1. The 18S rRNA and actin gene were used as internal controls for calculating relative transcript abundance in peanut and Arabidopsis, respectively. All real-time PCR reactions were repeated three times. The relative quantification of RNA expression was calibrated using formula 2−ΔΔCt method [58]. The mean of technical replicates was presented in the results. T-test analysis was performed to determine the statistical significance.

Generation of AhGLPs-transgenic Arabidopsis plants

The full-length coding sequence (ATG to TAA) of AhGLP1, 2, 3, 4, 5 and 7 were amplified by PCR with the gene-specific primers (Table S2). These PCR products were inserted into pCAMBIA1301 vector, which expression was under the control of Cauliflower mosaic virus (CaMV) 35S promoter. After sequencing confirmation, the recombinant plasmids were introduced into Agrobacterium tumefaciens GV3101 through the freeze-thaw method and then introduced into wild-type Arabidopsis thaliana var. Columbia by the floral dip method [59]. Transgenic Arabidopsis seeds were selected on solid Murashige and Skoog (MS) medium supplemented with 25 μg/L hygromycin (hyg). Independent hyg-resistant transgenic plants were further confirmed by PCR amplifications of the insertion cDNA.

Germination and tolerance analysis of AhGLPs in transgenic Arabidopsis

For in vivo salt-tolerance arrays, seeds from wild type and AhGLPs transgenic Arabidopsis were surface-sterilized as described by Clough and Bent [59] and sown on MS plates plus 2% sucrose containing 0, 50 and 100 mM NaCl, respectively. After stratification at 4°C for 3 days, plates were transferred in a growth chamber (100 μE m−2 s−1, 16 hr light/8 hr dark, 22°C). Germination (scored based on radicle emergence) was monitored daily for 6 days. After 100 mM NaCl treatment for 15 days, the seedlings were photographed and the tolerance of different transgenic Arabidopsis lines was observed. All the experiments were performed in duplicates. The mean of technical replicates was presented in the results. T-test analysis was performed to determine the statistical significance.

Subcellular localization of AhGLPs::GFP in onion epidermal cells

The coding sequences of AhGLP1, 2, 3, 4, 5 and 7 were amplified by PCR (Additional File 2) and fused to the 3′ region of the GFP gene, respectively. The AhGLP-GFP fusion genes were subcloned into the pCAMBIA1301 vector, for the expression under the control of CaMV35S promoter. These AhGLP-GFP fusion constructs and empty vector control were introduced into onion epidermal cells through the Agrobacterium-mediated system. The obtained cells were cultured on 1/2 MS medium at 26°C in darkness for 24 h. Subcellular localization of AhGLP proteins in onion (Allium cepa) epidermal cells indicated by the GFP signal was observed under fluorescence microscopy (Axio Observer A1, Zeiss, Germany). All transient expression assays were repeated at least three times.

Supporting Information

Figure S1 Homology matrix of predicted amino acid sequences of AhGLP family.
Table S1 Primers used to quantify transcripts from the peanut GLP family and JBS genes by qRT-PCR.

(DOC)

Table S2 Primers used for AhGLPs transgenic and subcellular localization analysis.

(DOC)

References

1. Nelson SC, Simpson CE, Starr JL. (1989) Resistance to Meloidogyne arenaria in Arachis spp. Germplasm. J Nematol 21(4):654–660.

2. Thompson EW, Lane BG. (1980) Relation of protein synthesis in imbibing wheat embryos to the cell-free translational capacities of bulk mRNA from dry and imbibing embryos. J Biol Chem 255(12):5965–5976.

3. Lane BG, Dunwell JM, Ray JA, Schmitt MR, Cuming AC. (1993) Germin-like protein marker of early plant development, is an oxalate oxidase. J Biol Chem 268(17):12239–12244.

4. Dunwell JM, Khuri S, Gane PJ. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. Microbiol Mol Biol Rev 64(1):153–179.

5. Zimmermann G, Baumeil H, Mock HP, Himmelbach A, Schweizer P. (2002) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. Plant Physiol 129(1):181–192.

6. Chakraborty S, Chakraborty N, Jain D, Salunke DM, Bhatta A. (2002) Active site geometry of oxalate decarboxylase from Flammulina velutipes: Role of histidine-coordinated manganese in substrate recognition. Protein Sci 11(9):2138–2147.

7. Lane BG. (2000) Oxalate oxidases and differentiating surface structure in wheat: germins. Biochem J 349(Pt 1):309–321.

8. Rietz S, Bernoull FE, Cai D. (2012) Members of the germin-like protein family in Brassica napus are candidates for the initiation of an oxidative burst that impedes pathogenesis of Sclerotinia sclerotiorum. Journal of Experimental Botany, 63(5):3507–3519.

9. El-Sharkawy I, Mila I, Bouzayen M, Jayasankar S. (2010) Regulation of two花生 mRNA markers in peanut under osmotic and salinity stress. Biochem Biophys Res Commun 397(3):176–177.

10. 21. Lou Y, Baldwin IT. (2006) Silencing of a germin-like gene in Nicotiana attenuata conferring broad-spectrum disease resistance in rice. Plant Physiol 149(1):286–298.

11. 22. Manosalva PM, Davidson RM, Liu B, Zhu X, Hulbert SH, et al. (2009) A gene. Plant Mol Biol 53(2):67–75.

12. 23. Hurkman WJ, Lane BG, Schmitt MR, Cuming AC. (1993) Germin-like protein marker of early plant development, is an oxalate oxidase. J Biol Chem 268(17):12239–12244.

13. 24. Dunwell JM, Khuri S, Gane PJ. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. Microbiol Mol Biol Rev 64(1):153–179.

14. 25. Zimmermann G, Baumeil H, Mock HP, Himmelbach A, Schweizer P. (2002) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. Plant Physiol 129(1):181–192.

15. 26. Lane BG. (2000) Oxalate oxidases and differentiating surface structure in wheat: germins. Biochem J 349(Pt 1):309–321.

16. 27. Guo B, Chen X, Liang X, Dang P, et al. (2009) Analysis of Gene Expression Profiles in Leaf Tissues of Cultivated Peanuts and Development of EST-SSR Markers and Gene Discovery. I J Plant Genomics 2009:716530.

17. 28. Wang T, Zhang F, Chen X, Li L, Liang X. (2010) Identification of seed proteins associated with resistance to pre-harvested aflatoxin contamination in peanut (Arachis hypogaea L.). BMC Plant Biol 10:267.

18. 29. Lou Y, Ma H, Lin WH, Chu ZZ, Mueller-Roemer B, et al. (2006) The highly charged region of plant beta-tubulin/d-anthrinoxin 4-kine is involved in membrane targeting and phospholipid binding. Plant Mol Biol 60(3):729–746.

19. 30. Vallelian-Bindschedler L, Mosinger E, Metraux JP, Schweizer P. (1998) Structure, expression and localization of a germin-like protein in barley (Hordeum vulgare L.) that is insolubilized in stressed leaves. Plant Mol Biol 37(2):297–308.

20. 31. Godfrey D, Able AJ, Dwyer B. (2007) Induction of a grapevine germin-like protein (VvGLPs) gene is closely linked to the site of Erysiphe necator infection: a possible role in defense. Mol Plant Microbe Interact 20(9):1112–1125.

21. 32. Heintzen C, Fischer R, Melzer S, Kappeler S, Apel K, et al. (1994) Circadian oscillations of a transcript encoding a germin-like protein that is associated with cell wall young in leaves of the long-day plant Sinapis alba L. Plant Physiol 106(3):905–915.

22. 33. Kukavica B, Vucinic Z, Vuletic M. (2005) Superoxide dismutase, peroxidase, and germin-like protein activity in plasma membranes and apoplast of maize roots. Protoplasma 226(3–4):191–197.

23. 34. Lane BG, Cuming AC, Carpita NC, Hurkman WJ, et al. (1992) Germin isoforms are discrete temporal markers of wheat development. PNAS 91:268–272.

24. 35. Breen J, Bellgard M (2010) Germin-like proteins (GLPs) in cereal genomes: gene clustering and dynamic roles in plant defence. Funt Integr Genomics 10(4):463–476.

25. 36. Banerjee J, Maiti MK. (2010) Functional role of rice germin protein in regulation of plant height and disease resistance. Biochem Biophys Commun 394(1):178–183.

26. 37. Knoch C, Seyfarth M, Dessel C, Thurai T, Sherameri I, et al. (2010) Expression of BvGLP-1 encoding a germin-like protein from sugar beet cells. Arabidopsis thaliana leads to resistance against phytopathogenic fungi. Mol Plant Microbe Interact 23(4):446–457.

27. 38. Livingstone DM, Hampton JL, Phupps PM, Grabau EA (2005) Enhancing resistance to Sclerotinia in peanut by expressing a barley oxalate oxidase gene. Plant Physiol 137(4):1117–1122.

28. 39. Attia J, de Vos RC, Bones AM, Hall RD (2010) Plant molecular stress responses face climate change. Trends Plant Sci 15(2):66–67.

29. 40. Wooten JM, Goodenough PW, Marvier AC, Pickersgill RW (2000) Germin is a manganese containing homeoxenon with oxalate oxidase and superoxide dismutase activities. Nat Struct Biol 7(11):1036–1040.

30. 41. Yamahara T, Shinjo T, Suzuki T, Tanaka K, Takio S, et al. (1999) Isolation of a germin-like protein with manganese superoxide dismutase activity from cells of a moss, Barbassa unguiculata. J Biol Chem 274(47):33274–33278.

31. 42. Dumas B, Saulain A, Chevret JP, Freysinet G, Pallett K (1995) Identification of barley oxalate oxidase as a germin-like protein. C R Acad Sci III 316(8):795–798.

32. 43. Moeder W, Ung H, Mosher S, Yoshikoa K (2010) SA-ABA antagonism in defense responses. Plant Signal Behav 5(10):1231–1233.

33. 44. Park CJ, An JM, Shin YC, Kim KJ, Lee BJ, et al. (2004) Molecular characterization of pepper germin-like protein as the novel PR-16 family of pathogenesis-related proteins isolated during the resistance response to viral and bacterial infection. Planta 220(3):469–476.

34. 45. Tabuchi T, Kaneda Y, Nemoto S, Yano T (2003) The expression of a germin-like protein with superoxide dismutase activity in the halephyle Atriplex lentiformis is differentially regulated by wounding and ascorbic acid. Physiol Plant 114:391–397.

35. 46. Hurkman WJ, Tanaka CK (1996) Effect of Salt Stress on Germin Gene Expression in Barley Roots. Plant Physiol 110(3):971–977.

36. 47. Lu M, Han YP, Gao JG, Wang XJ, Li WB (2011) Identification and analysis of the germin-like gene family in soybean. BMC Genomics 11:262.

37. 48. Kamal AH, Cho K, Kim DE, Uozumi N, Chung KY, et al. (2012) Changes in the expression of germin-like genes and proteins in tobacco plants. PloS ONE 7(4):e39599.

Author Contributions

Conceived and designed the experiments: XQL YXC. Performed the experiments: TW FHZ HFL. Analyzed the data: TW XPC LL. Contributed reagents/materials/analysis tools: XPC TW SLY QLY. Wrote the paper: TW XQL YXC SLY.
49. Dani V, Simon WJ, Duranti M, Croy RR (2005) Changes in the tobacco leaf apoplastic proteome in response to salt stress. Proteomics 5(3):737–745.
50. Caliskan M (2009) Salt stress causes a shift in the localization pattern of germin gene expression. Genet Mol Res 8(4):1250–1256.
51. Nakata M, Shioso T, Watanabe Y, Satoh T (2002) Salt stress-induced dissociation from cells of a germin-like protein with Mn-SOD activity and an increase in its mRNA in a moss, Barbula unguiculata. Plant Cell Physiol 43(12):1568–1574.
52. Patnaik D, Khurana P (2001) Germins and germin like proteins: an overview. Indian J Exp Biol 39(3):191–200.
53. Gucciardo S, Wisniewski JP, Brewin NJ, Bornemann S (2007) A germin-like protein with superoxide dismutase activity in pea nodules with high protein sequence identity to a putative rhicadhesin receptor. J Exp Bot 58(5):1161–1171.
54. Hayashi M, Takahashi H, Tamura K, Huang J, Yu LH, et al. (2005) Enhanced dihydroflavonol-4-reductase activity and NAD homeostasis leading to cell death tolerance in transgenic rice. Proc Natl Acad Sci U S A 102(19):7020–7025.
55. De Jesus WC, Do Vale FXR, Coelho RR, Hau B, Zambolim L, et al. (2001) Effects of Angular Leaf Spot and Rust on Yield Loss of Phaseolus vulgaris. Phytopathology 91(11):1045–1053.
56. Liang XQ, Hollbrook CC, Lynch RE, Guo BZ (2005) beta-1,3-Glucanase Activity in Peanut Seed (Arachis hypogaea) is Induced by Inoculation with Aspergillus flavus and Copurifies with a Conglutin-Like Protein. Phytopathology 95(5):506–511.
57. Alou E, Roca M, Iglesias DJ, Minguez-Mosquera MI, Damasceno CMB, et al. (2008) An evaluation of the basis and consequences of a stay-green mutation in the navel negra citrus mutant using transcriptomic and proteomic profiling and metabolite analysis. Plant Physiol 147(3):1300–1315.
58. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402–408.
59. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16(6):735–743.