Genome-wide investigation of calcium-dependent protein kinase gene family in pineapple: evolution and expression profiles during development and stress

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Abstract

Background: Calcium-dependent protein kinase (CPK) is one of the main Ca2+ combined protein kinase that play significant roles in plant growth, development and response to multiple stresses. Despite an important member of the stress responsive gene family, little is known about the evolutionary history and expression patterns of CPK genes in pineapple.

Results: Herein, we identified and characterized 17 AcoCPK genes from pineapple genome, which were unevenly distributed across eight chromosomes. Based on the gene structure and phylogenetic tree analyses, AcoCPKs were divided into four groups with conserved domain. Synteny analysis identified 7 segmental duplication events of AcoCPKs and 5 syntenic blocks of CPK genes between pineapple and Arabidopsis, and 8 between pineapple and rice. Expression pattern of different tissues and development stages suggested that several genes are involved in the functional development of plants. Different expression levels under various abiotic stresses also indicated that the CPK family underwent functional divergence during long-term evolution. AcoCPK1, AcoCPK3 and AcoCPK6, which were repressed by the abiotic stresses, were shown to be function in regulating pathogen resistance.

Conclusions: 17 AcoCPK genes from pineapple genome were identified. Our analyses provide an important foundation for understanding the potential roles of AcoCPKs in regulating pineapple response to biotic and abiotic stresses.

Keywords: Pineapple, CPK, Genome-wide, Expression pattern, Stress

Background

In order to survive continual biotic and abiotic stresses occurred in the environment, plants have evolved an effective defense mechanism, including a variety of signal transduction pathways, especially the Calcium (Ca2+), which is a universal second messenger and can induce signal transduction in all eukaryotes. Particularly, plants can sense Ca2+ signaling to regulate growth, development, as well as responses to various biotic and abiotic stimuli [1, 2]. Plants possess several kinds of Ca2+ sensors, and many of them own the EF-hand motif, while the specific helix-loop-helix structure coordinates a single Ca2+ ion, providing direct Ca2+-binding ability to the sensors [3]. When plants were subjected to various stresses, Ca2+ sensors, such as calmodulin-like proteins (CaMLs), calmodulins (CaMs), and calcium-dependent protein kinases (CPKs), can sense and decoded the calcium fluxes concentration changes [4, 5]. In addition,
the protein kinase and calmodulin-like domains of CPKs are located in a single polypeptide, resulting in Ca^{2+}-binding and Ca^{2+}-stimulated kinase activities within an independent protein product, which may arose direct translation of Ca^{2+} into downstream phosphorylation signals [6, 7].

Calcium-dependent protein kinases (CPKs) as a kind of ser/thr protein kinases, have been identified throughout the plant kingdom [6]. CPKs comprise four functional domains, including a serine/threonine kinase domain (STKD), an N-terminal variable domain (ND), an auto-inhibitory junction domain (AID) and a C-terminal regulatory calmodulin-like domain (CaM-LD) [6, 8]. The STKD is highly conserved, containing ATP binding catalytic domain and adjacent to the autoinhibitory junction domain [9]. The N-terminal domain consists myristoylation and palmitoylation sites, which are crucial for subcellular localization and molecular function and the two show the highest sequence divergence among CPK domains [10]. The AID, which may sometimes be part of the CaM-LD [9], contains a pseudosubstrate sequence so as to interact with the active site or inhibit kinase activity. The calmodulin-like domain contains one to four EF-hand structures for Ca^{2+}-binding [8]. Because of these unique features, the CPKs is sensitive to Ca^{2+} and play an important role in regulating the downstream components of calcium signaling pathway.

Recently, genetic evidences indicates that CPK genes are ubiquitously functional in plant growth and developmental process such as flowering [11], pollen tube growth [11], fruit development [12], root development [13], cell division and differentiation, and cell death [14]. CPKs are also involved in abiotic and biotic stress responses. In Arabidopsis, AtCPK4/11/21, as positive regulators in the ABA signaling processes, were involved in resistance to drought and salt stresses [15, 16]. CPK gene from maize, such as ZmCPK4, also has similar functions in the responses to salt and drought stresses [17]. Furthermore, OsCPK12, which is involved in the ABA signaling process, improved salt resistance through a reduction in ROS accumulation [18]. The expression of OsCPK13 can be induced under low temperature [19], however, ZmCPK1 plays as a negative regulator in response to cold stress [20]. Overexpressing the OsCPK7 gene enhanced tolerance of transgenic plants to drought, salt, and cold stresses [21]. The recombinant protein StCPK7, an active Ca^{2+}-dependent protein kinase, functions in plant defense response and can be induced upon infection with Phytophthora infestans in potato [22]. VaCPK20 gene overexpression significantly increased resveratrol content of Vitis amurensis Rupr [23].

The genes encoding CPKs form a multi-gene family and they have been well characterized in many plant species. To date, genome-wide analyses have identified 34 CPK genes in Arabidopsis [8], 31 CPK genes in rice [24], 30 CPK genes in poplar [25], 20 CPK genes in wheat [26], 41 CPK genes in cotton [27], 29 CPK genes in tomato [28], and 19 CPK genes in cucumber [29]. Nevertheless, our knowledge of CPK gene family for many other economically important horticultural crops, such as pineapple (Ananas comosus), is still limited. Like other economical plants, pineapple is often affected by various abiotic and biotic stresses such as salt, drought, pathogens and so on. The decoding of the pineapple genome sequencing provided a chance to reveal the organization, expression and evolutionary characterization of pineapple CPK genes at the genome-wide level [30]. In this study, a total of 17 CPK genes were found and these CPKs were grouped based on their phylogenetic relationships into four subgroups and were located to specific chromosomes. Our study concluded the exon-intron organization, motif compositions, gene duplications, phylogenetic and synteny relationships of pineapple CPKs. Global expression analyses were also performed to identify involvement of specific pineapple CPK genes in different tissues and various stresses. This work provides insights into the evolutionary history and biological functions of pineapple CPK family.

Results
Identification of CPK genes in pineapple genome
A total of 17 putative CPK genes were identified from the pineapple genome, and named from AcoCPK1 to AcoCPK17 (Additional file 4: Table S1). The full-length of 17 CPK proteins varied from 603 (AcoCPK1) to 578 (AcoCPK13) amino acid residues with CDS ranging from 4.91 to 8.25. All of them contain the typical CPK structure, including an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain, and a CaM-like domain. In addition, all the pineapple CPK genes exist four EF-hand motifs in the CaM-like domain by predicting, which can recognize and bind Ca^{2+} molecules (Additional file 4: Table S1, [8, 31]). Among the identified 17 pineapple CPK proteins, 6 CPKs were predicted to contain myristoylation motifs at their N-termini (Additional file 4: Table S1).

Phylogenetic analysis, gene structure of CPK genes and their chromosomal location
To examine the phylogenetic relationship among the CPKs in pineapple, the CPKs of four species, including pineapple, Arabidopsis, grape and rice, were constructed using MEGA5.0. CPK genes were grouped into four subfamilies, including 5, 4, 6 and 2 members in group I, II, III, and IV, respectively (Fig. 1 and Additional file 4: Table S1).
To obtain the possible structural evolution of CPK genes in the pineapple genome, diverse exon-intron organizations of AcoCPKs were compared. As shown in Fig. 2a, all AcoCPK genes possess six to eleven introns (four with six introns, ten with seven introns, one with eight introns, and two with eleven introns). Genes in the same subfamily shared very similar exon-intron structures. All members of group I possessed seven exons. In subfamily II, diverse numbers of exons were found in different members: eight exons were found in AcoCPK4, AcoCPK16 and AcoCPK11, nine exons were found in AcoCPK7. Compared with the Group I members, Group II members have one or two additional exons. In group III, all members had eight exons. The two members in group IV had 12 and 7 exons with 11 introns. The results indicate that CPK genes with higher homogenous sequences tend to have the same numbers of exons. A schematic representing the structure of all AcoCPK proteins was constructed from the MEME motif analysis results. As exhibited in Fig. 2b, a total of 10 distinct conserved motifs were found (Additional file 1: Figure S1), almost all the CPK family members harbor ten motifs, except for AcoCPK6 in group I without motif 7, AcoCPK12 and 17 in group IV without motif 5 and motif 7/2/6/9. In conclusion, group IV may be the most conserved and presented earliest.

All of 17 pineapple AcoCPK genes were mapped onto eight chromosomes (Fig. 3). Some chromosomes have more genes, whereas others have few: the largest numbers of CPK genes (five) were located to chromosome 9; 3 CPK genes were located to chromosome 7, and chromosomes 3, 17 and 23 were found to harbor two CPK genes each. Chromosome 1, 16 and 22 were each found to harbor one CPK gene.

**Synteny analysis of pineapple CPK genes**

To elucidate the expanded mechanism of the CPK gene family in pineapple, gene duplication events, including tandem and segmental duplications, were investigated. A total of 7 duplicated CPK gene pairs, AcoCPK3/
AcoCPK6, AcoCPK8/AcoCPK10, AcoCPK7/AcoCPK11, AcoCPK2/AcoCPK9, AcoCPK14/AcoCPK15, AcoCPK5/AcoCPK13, and AcoCPK12/AcoCPK17, were found in the pineapple genome; all of these were segmental duplicates (Fig. 3a, Additional file 5: Table S2). The results suggested that segmental duplication played an important role in the amplification of CPK gene family members in the pineapple genome.

In order to infer the evolutionary mechanism of pineapple CPK family, we constructed two comparative syntenic maps of pineapple associated with Arabidopsis and rice (Fig. 3b, c). A total of five AcoCPK genes showed syntenic relationship with those in Arabidopsis, eight in rice (Additional file 6: Table S3, Additional file 7: Table S4). Between Arabidopsis and pineapple CPK genes, we could find several kinds of syntenic orthologous gene pairs: one pineapple gene vs multiple Arabidopsis genes, such as AcoCPK6-ATCPK1/2/20, AcoCPK7-ATCPK9/21/33; one Arabidopsis gene vs multiple pineapple genes, such as: ATCPK26-AcoCPK8/10 (Fig. 3b, Additional file 6: Table S3). Between rice and pineapple CPK genes, four pairs of syntenic orthologous genes (one to one) were identified: AcoCPK8-OsCPK5, AcoCPK12-OsCPK18, AcoCPK14-OsCPK20 and AcoCPK10-OsCPK2 (Fig. 3c, Additional file 7: Table S4), indicating that these genes might be derived from the same ancestor of rice and pineapple. We also
found that one pineapple gene corresponds to multiple rice genes, such as AcoCPK1-OsCPK24/28. Interestingly, some orthologous gene pairs mapped between pineapple and rice were not found between pineapple and Arabidopsis, such as AcoCPK5-OsCPK3/16, which may indicate that these orthologous pairs formed after the divergence of dicotyledonous and monocotyledonous plants. For further evolutionary studies, the Ka, Ks and Ka/Ks of the orthologous gene pairs were calculated based on the comparative synteny map (Additional file 6: Table S3, Additional file 7: Table S4). The majority of orthologous CPK gene pairs had Ka/Ks < 1, suggesting that the pineapple CPK gene family might have experienced strong purifying selective pressure during evolution.
Pineapple CPK genes are expressed in different tissues in pineapple plants
To investigate the possible roles of the CPK genes in the pineapple genome, we analyzed the expression profiles of the 17 CPK genes in different tissues and developmental stages using RNA-seq expression data recently published by Ming et al. from MD2 pineapple plants (Additional file 2: Figure S2 and Additional file 8: Table S5, [30]. The results showed that all the CPK genes were expressed in different tissues and developmental stages in pineapple. Some genes showed preferential expression across the detected tissues. Remarkably, AcoCPK16 showed high expression level in flower and leaf while barely any expression in root and different stage fruits, and AcoCPK2, AcoCPK6 and AcoCPK9 also had high expression level in flower and leaf but lower than AcoCPK16, and AcoCPK2, AcoCPK6 and AcoCPK9 showed similar expression pattern. On the contrary, AcoCPK7 displayed high expression level in different development stage of fruit, indicating they might participate in the maturity process of pineapple fruit. Besides, AcoCPK12, AcoCPK4, AcoCPK10 and AcoCPK14 showed similar expression level in different tissues and fruits in different stages, suggesting they might be constitutive expression in pineapple and involved in different development stages. All these data suggest that the members of the CPK gene family might be involved in the growth and development of different tissues or organs of pineapple.

Expression profiles of pineapple CPK genes in response to different treatments
To explore the mechanisms of CPK response to the abiotic stresses, we searched for 15 stress-related cis-elements in the AcoCPK promoters, such as W-box, HSE and MBS, (Additional file 9: Table S6). The results showed that more than one different cis-elements located in the promoters of all 17 CPK genes with the least 4 cis-elements in the promoters of AcoCPK1 and more than 9 cis-elements in the promoter of AcoCPK12 and AcoCPK15. Some elements were detected more than one copy in the promoter regions. For example, the promoter of AcoCPK3 contained 4 copies of MBS sequences and the promoter of AcoCPK12 contained 4 copies of W-box sequences. At least one MBS was present in 94% (16 out of 17) of AcoCPK promoters, indicating that MBS plays a crucial role in response to stress in pineapple.

To further confirm whether the expression of AcoCPK genes were influenced by different abiotic stresses, qRT-PCR experiments were performed to analyze the CPK gene family members expression patterns in response to different treatments, including cold, heat, salt stress, drought stress and (Dysmicoccus brevipes) infection (Figs. 4, 5 and 6, Additional file 10: Table S7, Additional file 11: Table S8, Additional file 12: Table S9, Additional file 13: Table S10 and Additional file 14: Table S11). When response to biotic stress, most members were induced by mealybugs, except for AcoCPK4 and AcoCPK13. Some AcoCPK genes were induced at early stage of infection (24 h) and then downregulated continuously, such as AcoCPK1/3/6; some genes showed highest expression level at 72 h, such as AcoCPK7/9. Overall, we found all family members had responses to all the abiotic treatments, except for AcoCPK13. some AcoCPK genes were induced/repressed by multiple treatments. For instance, AcoCPK2/12 were significantly induced by all tested treatments, while AcoCPK4 was repressed by all tested treatments. Upon these stresses, some genes were suppressed at 2 h and then upregulated continuously, such as AcoCPK1/14/15 response to salt stress, AcoCPK1/11 response to heat stress, so they might be crucial for later stage of stress responses; some genes were upregulated until 6 h after that they were suppressed, such as AcoCPK5/14 response to drought stress, so they might play an important role at early stage of stress responses. Interestingly, some AcoCPK genes showed opposing expression patterns under different treatments. For instance, AcoCPK11 was suppressed at 2 h and then upregulated continuously when response to salt, drought and heat stress, but it showed opposite expression pattern facing cold stress.

Function analysis of AcoCPK1, AcoCPK3 and AcoCPK6
The expression pattern of pineapple CPK family showed that the members of CPK play crucial role in response to different abiotic and biotic stress. In order to further investigate their function, three group members (AcoCPK1/3/6) were selected for further research. To investigate the subcellular location of AcoCPK1, AcoCPK3 and AcoCPK6, the coding regions of these genes were fused with GFP and transiently expressed in Nicotiana benthamiana leaves. The control vector (35S::GFP)-transformed leaves displayed GFP in both cell nuclei and membrane. Interestingly, we found that about 80% GFP signals of AcoCPK1-GFP, AcoCPK3-GFP and AcoCPK6-GFP were predominantly localized at cellular membranes, while some signals (~20%) were co-localized with DAPI-stained cell nuclei in the infiltrated leaf areas (Fig. 7). To determine the role of these three genes in response to abiotic and biotic stresses, we generated AcoCPK1, AcoDCPK3 and AcoCPK6 overexpression transgenic Arabidopsis plants. For each gene, two independent homozygous lines with relative high expression of transgenes were selected for further research (Additional file 3: Figure S3). Under normal condition, all transgenic lines showed no significant phenotypic differences with the wild type (Columbia-0). Under salt and...
drought stress conditions, overexpression lines of AcoCPK1, AcoCPK3 and AcoCPK6 are more sensitive to the salt and D-mannitol. As shown in the Fig. 8, Arabidopsis plants that overexpress AcoCPK1, AcoCPK3 and AcoCPK6 showed much reduced seed germination ratios or green cotyledon under stress conditions (Fig. 8a), and their root length and fresh weight were lower than wild type (Fig. 8b).

Previous studies have shown that the expression of many CPK genes be induced by biotic and abiotic stresses in Arabidopsis and rice [18, 32], so we checked the function of these three CPK genes upon plant disease resistance. The leaf surface of WT, OX-AcoCPK1, OX-AcoCPK3 and OX-AcoCPK6 were infected with S. sclerotiorum. After 24 h inoculation of S. sclerotiorum, AcoCPK gene overexpression lines are more sensitive to S. sclerotiorum, and the lesion areas are bigger than wild type, and they can generate more H2O2 (Fig. 9a, b and c).

As we know, phytohormones play key roles in local and systemic acquired resistance (SAR) to necrotrophic pathogens, such as jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). Disease resistance marker genes, PDF1.2 and LOX4, which are related to JA, have been suggested to be involved in the plant’s defense pathway [33, 34]; ACS6 and ERF, which is related to ET, are function in several necrotrophic fungi resistance [35, 36]; ABI2 and ABI5, which is related to ABA, can response to the plant disease [37]. We checked expression pattern of these marker genes that response to these phytohormones and found that all of them were downregulated significantly in the AcoCPK gene overexpression lines compared with wild type (Fig. 9d), coinciding with the reduced resistance to pathogen in the AcoCPK1, AcoCPK3 or AcoCPK6 overexpression lines.

**Discussion**

Pineapple (Ananas comosus) is a tropical plant and the most economically significant plant in the Bromeliaceae family. CPK genes play important roles in diverse plant developmental and physiological process, as well as various plant biotic and abiotic stress responses. In the current study, a search for CPK genes in the pineapple genome resulted in identification of 17 members, which named from AcoCPK1 to AcoCPK17 on the basis of their chromosomal location, together with an analysis of their structure, evolutionary history and expression diversity with respect to biotic and abiotic stresses.

Evolutionary analysis indicated that CPK genes in pineapple can be divided into four groups, and same evolutionary classification was also found in other species, such as Arabidopsis, grape and rice [8, 10, 24]. In addition, the classification result was further confirmed by gene structure and conserved motif analyses. In pineapple, the number of introns changed from 6 to 11, which is similar with melon and pepper [38, 39], indicating that different species display similar gene structure diversity of CPK genes. According to a previous report, the rate of intron loss is faster than the rate of intron gain after segmental duplication in rice [40]. We also observed that group IV in pineapple own more number of introns, indicating that group IV might contain the original genes, and this conclusion can be further supported by the evidence that motifs in group IV were the most conserved.

Most of the CPK proteins were slightly acidic in terms of biochemical properties, with isoelectric points (pI) ranging 5–7 [38, 39]. However, a few CPK proteins mainly distributed group IV, had basic pIs of 8 or more [6, 41]. In our research, CPK proteins in group I were
slightly acidic with PIs ranging from 4.91 to 5.83. However, the other three groups all had member with PIs more than 7, such as AcoCPK7 in group II, AcoCPK14/15 in group III and AcoCPK12 in group IV (Additional file 5: Table S2). In the previous research, people have found that CPK proteins which own less than four EF-hands, have been found broadly in both monocotyledon and dicotyledon, such as Arabidopsis, soybean, maize and rice [8, 42–44]. According to gene structure investigation, we found that all 17 pineapple CPK genes contain four EF-hands (Additional file 4: Table S1), which is similar to that of barley and pepper [39, 41].

In our study, phylogenetic relationships were investigated to illuminate the evolutionary history of the CPK gene family. Our results demonstrated the origin of the CPK genes before the divergence of eudicot and monocot with the result that all CPK genes from pineapple, Arabidopsis, grape and rice were distributed among all four groups [38, 39, 45]. We compared the number of CPK genes in pineapple with other sequenced eudicot and monocot genomes, including Arabidopsis, soybean, rice and grape, and found that pineapple possesses less number of genes [8, 24, 39, 42, 46]. Compared the genome with the basal angiosperm Amborella, people found that the grasses underwent three whole-genome duplication (WGD) events (τ, σ, and ρ) [30, 47], while pineapple only underwent two genome doublings (τ and σ) [30]. This might the reason that pineapple possesses the smaller
number of CPK genes. Gene duplication events play a vital role in genomic rearrangements and expansions [48] and are defined as either tandem duplications or segmental duplications depending on duplicated genes on the same or different chromosomes [49]. In our research, chromosomal locations and duplication events implied that gene duplication, especially segmental duplication was the major evolutionary mechanism resulting in pineapple CPK expansions (Fig. 3a). We found that the two genes in the same gene pair tended to be clustered into one group, such that segmental pairs AcoCPK3-AcoCPK6, AcoCPK2-AcoCPK9 and AcoCPK12-AcoCPK17 were clustered to group I, III and IV, respectively.

The synteny analysis illuminates the relevance between functional and evolutionary in two species, such as pineapple and Arabidopsis or rice syntenic genes. Many Arabidopsis and rice CPK genes response to biotic and abiotic stresses have been reported. For example, CPK5/CPK6 in Arabidopsis are involved in the wounding-induced ethylene biosynthesis via differential regulation of ACS genes at transcriptional level [50]. As a component of the innate immune system of Arabidopsis plants,
loss-of-function mutants of *AtCPK1* exhibit higher susceptibility to pathogen infection compared to wild-type plants [32]. In *Arabidopsis*, *AtCPK3* plays extensive roles in various biological and environmental responses [51]. *OsCPK12*-overexpressing plants exhibited increased tolerance to salt stress and increased susceptibility to blast fungus [18]. Overexpression of *OsCPK7* enhanced induction of some stress-responsive genes in response to salt/drought, but not to cold [21]. A total of 5 pineapple genes and 11 *Arabidopsis* genes were identified as syntenic orthologs between pineapple and *Arabidopsis*. Including pineapple, *Arabidopsis* and rice, the diversity of gene combinations reveals complex regulatory relationships. Some *CPK* genes present in these species, including pineapple, *Arabidopsis* and rice, could not be mapped into any syntenic gene pairs, it might due to multiple rounds of important chromosomal rearrangement and fusions happened in their genomes since the speciation of pineapple and *Arabidopsis* or rice, followed by selective gene loss, which can obscure the identification of chromosomal syntenies [49, 52].

In non-stressed pineapple plants, about half of *CPK* family members were expressed wildly in almost all the tissues and developmental stages according to pineapple expression pattern (Additional file 2: Figure S2). The duplicate genes may have different transcription levels in different tissues, development stages and even response to various stresses. The gene pair *AcoCPK3* and *AcoCPK6*, for example, had different transcriptional levels in different tissues: *AcoCPK6* displayed high expression level in leaf and flower, while *AcoCPK3* showed opposite expression profile. Specifically, *AcoCPK6* was down-regulated, whereas *AcoCPK3* was up-regulated and reached peak at 12 h dpi under hot stress, but these two genes were sensitive to the salt and drought stress (Figs.5, 6 and 8). These results indicated that the plants CPKs have evolved more specified functions through gene divergence to meet a broader array of lineage-specific requirements.
To understand the specific functions of pineapple CPKs, we randomly selected eight CPK genes for function analysis and the genes AcoCPK2, AcoCPK4, AcoCPK7, AcoCPK9 and AcoCPK12 could not be cloned by PCR. We found that the successfully cloned three CPK genes (AcoCPK1, AcoCPK3 and AcoCPK6) belonged to Group I subfamily and we further focused on these three CPK genes. Subcellular locations of three CPK genes (AcoCPK1, AcoCPK3 and AcoCPK6) were identified, and we found their protein are mainly expressed at plasma membrane and small partial localized at nucleus (Fig. 7), indicating that they are primarily soluble enzymes with the potential to target to the nucleus. Similar results had been found in Arabidopsis CPK family, with cytosolic and nuclear location for AtCPK3 and 4, a plasma membrane location for AtCPK7, 8, 9, 16, 21, and 28 and a peroxisome membrane location for AtCPK1 [53]. An increasing number of evidences have confirmed that CPKs play important roles in plant stress response and in related signaling pathways [6]. As shown in Figs. 5 and 6, some CPK genes shared similar expression patterns in response to a

![Fig. 8](image)

**Fig. 8** Effect of salt and drought stress on transgenic Arabidopsis plants overexpression AcoCPK1, AcoCPK3 and AcoCPK6. **a** Representative image of 5-day-old seedlings of wild-type and AcoCPK1, AcoCPK3 and AcoCPK6 overexpression transgenic lines under salt and drought stress treatments. **b** Root length and fresh weight of 5-day-old seedlings of wild-type and AcoCPK1, AcoCPK3 and AcoCPK6 overexpression transgenic lines under salt and drought stress treatments.
specific stress in the pineapple genome, for example, similar expression levels were observed among AcoCPK1, AcoCPK3, AcoCPK11, AcoCPK14 and AcoCPK17 in response to cold stress and AcoCPK6 and AcoCPK17 in response to mealybugs. Some genes can be induced by both biotic and abiotic stresses, such as AcoCPK17 response to cold and mealybugs, AcoCPK2 response to salt and mealybugs. Similar cross-talk between plant responses to biotic and abiotic stresses have been found to be mediated by CPK genes; for example, PaCPK1 was activated by cold, wounding, and pathogen challenge [54], and OsCPK12 functions in multiple signaling pathways, and it promotes tolerance to salt stress by reducing the accumulation of ROS and negatively modulates blast fungus resistance [18]. Thus, the cross-talk may help regulate the signaling network of AcoCPK in response to various types of stresses. Three genes, AcoCPK1, AcoCPK3 and AcoCPK6 were repressed by the abiotic stresses, and they also function in pathogen resistance (Figs. 8 and 9). Based on the phylogenetic tree, AcoCPK1 was clustered with Arabidopsis AtCPK2, and AcoCPK3 and AcoCPK6 was clustered with Arabidopsis AtCPK1. They have found that AtCPK1 and AtCPK2 were induced by drought, high-salt but not by low-temperature stress or heat stress [32, 55], while AcoCPK3 and AcoCPK6 were sensitive to drought and high-salt stress and they were also susceptible to S. sclerotiorum. When response to S. sclerotiorum, several marker genes related to ET, JA and ABA were suppressed indicating that phytohormone signaling pathway might be involved in the resistance response process. Genes with the same function are often closely related [49], indicating that AcoCPK1, AcoCPK3 and AcoCPK6 play important roles in different signal transduction
pathways and in the adaption of pineapple to changeable environments and stresses. The distinct roles of pineapple and Arabidopsis CPK genes might be caused by the divergence of monocotyledones and dicotyledones during evolutionary.

Conclusions
In summary, we identified 17 CPK genes in the pineapple genome that were distributed in eight chromosomes unevenly. According to the analysis of phylogenetic tree and gene structure, AcoCPKs are divided into four groups. Between pineapple and Arabidopsis, we identified 7 segmental duplication events and 5 syntenic blocks from CPKs, 8 between pineapple and rice. Expression profiles of AcoCPKs in response to various stresses implied their pivotal roles in participate in multiple signaling pathways. Our analyses provide an important foundation for understanding the potential roles of AcoCPKs in regulating pineapple response to biotic and abiotic stresses.

Methods
Identification of CPKs in pineapple and other species
Sequences from the pineapple genome database were downloaded from http://pineapple.angiosperms.org/pineapple/html/index.html. The 34 Arabidopsis CPKs protein sequences were obtained from http://www.Arabidopsis.org/. The rice and grape CPK sequences were downloaded from https://phytozome.jgi.doe.gov/pz/portal.html.

Phylogenetic analysis of AcoCPK proteins
We performed sequence alignments of pineapple, rice, grape and Arabidopsis CPKs by using muscle (http://www.ebi.ac.uk/Tools/msa/muscle/). As previous study described, phylogenetic and molecular evolutionary analyses were generated using MEGA 6.0 software (http://www.megasoftware.net) and the RAxML program (http://www.phylo.org/) [49].

Protein properties and sequence analyses
The ExPASy proteomics server (http://expasy.org/) [56] was used to identify the molecular weight and isoelectric points of predicted AcoCPK proteins. The MEME program (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) was used to predict the conserved motifs in full-length pineapple CPK proteins with the following parameters: maximum number of motifs was 10 and the optimum width of motifs was set between 10 and 50 [57]. Gene structure analysis of AcoCPK was displayed with Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/).

Synteny analysis and chromosome localization
The syntenic blocks used for constructing a synteny analysis map within the pineapple genome and between the pineapple and Arabidopsis or rice genomes, were obtained by using BLASTP with the reference E < 1e-5 and top 3 matches [49]. All data used to analyze the expansion patterns of the AcoCPK family were shown in Additional file 6: Tables S3, Additional file 7: Table S4 and Table Additional file 8: S5. Diagrams were generated using the Circos program (version 0.69) (http://circos.ca/). Based on the comparative synteny map between the pineapple and Arabidopsis or rice genomes, the synonymous (Ks) and non-synonymous (Ka) nucleotide substitutions between orthologous gene pairs were calculated using ClustalW [58], PAL2NAL [59] and yn00 program of the bio-pipeline (https://github.com/tanghaibao/bio-pipeli ne).

Expression profiles based on the estimation of expression levels from RNA-Seq data and qRT-PCR
We got the estimated expression levels, RPKM (reads per kilobase per million reads) values, for each AcoCPKs from 9 different tissues and developmental stages from https://de.iplantcollaborative.org/de/?type=data&folder=/iplant/home/cmwai/coge_data/Pineapple_tissue_RNAseq. The expression level under abiotic stress treatments was obtained by quantitative real-time PCR (qRT-PCR) analysis.

Plant materials and treatments
Pineapple (Ananas comosus) cultivar MD2 seedlings were grown in a greenhouse at 28 °C, 60–70 mmol photons m−2 s−1, a relative humidity of 70%, and a 16-h light/8-h dark photoperiod and the materials were provided by the pineapple breeding group in Fujian Agriculture and Forestry University.

Two-month old pineapple plants were treated with 400 mM NaCl and 400 mM mannitol to perform salt and drought stress, respectively, for 0, 2, 6, 12, 24 and 48 h. Heat treatment was carried out in a growth chamber at 45 °C, and cold treatment was performed in a growth chamber at 4 °C. For the biotic-stress treatment, two-month-old pineapple plants were infected with mealybugs (Dysmicoccus brevipes) [60]. The leaves were harvested at the indicated time points for the preparation of total RNA.

RNA extraction and quantitative real-time PCR
We used Trizol method (Invitrogen, Carlsbad, CA, USA) to extract total RNA, and the PrimeScript RT-PCR kit (TaKaRa) was used to do the reverse-transcribed experiment. Real-time PCR was performed to analysis the relative transcript levels of selected genes according to the Bio-Rad Real-time PCR system (Foster City, CA, USA) and the SYBR Premix Ex Taq II system (TaKaRa Perfect Real Time), and the primers used has been listed in Additional file 15: Table S12. The qRT-PCR program
was: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s; 95 °C for 15 s [61, 62]. The relative transcript levels of the analyzed pineapple genes were normalized to the transcript levels of AcoActin.

Vector constructs and pathogenicity assay
35S: AcoCPK1/3/6: GFP were generated by amplifying CDS sequence from wild-type pineapple cDNA using the primers listed in Additional file 14: Table S11. The PCR fragments were then cloned into the pENTR/D-TOPO vector (Invitrogen). pENTR clones were then recombined into the destination vector pGWBS06 using LR Clonase II (Invitrogen). The three constructs were transformed in Arabidopsis Col-0 plants using the vacuum infiltration method [63]. Wild-type S. sclerotiorum isolate was cultured exclusively on minimal medium for 3 days before inoculation. An agar plug (about 2.5 mm in diameter), which contain the advancing edge of S. sclerotiorum mycelia, was used to inoculate Arabidopsis leaves. A single rosette leaf of 4- to 6-week-old Arabidopsis plants was inoculated for 12 h and then was harvested for measurement of lesion areas and RNA extraction.

Subcellular localization
To examine the localization of AcoCPK1/3/6 in planta, the plasmid containing 35S: AcoCPK1/3/6 construction transformed Agrobacterium tumefaciens GV3101 culture was resuspended in infiltration media before infiltration into 32d old leaves of N. benthamiana. 2 days later, leaf discs were observed of GFP under a confocal microscope (Leica TCS SP8X DLS) with laser 488 nm.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-020-6501-8.

Additional file 1: Figure S1. 10 conserved motifs
Additional file 2: Figure S2. Expression profile of the pineapple CPK genes in different tissues and development stages
Additional file 3 Figure S3. The relative expression level of CPK genes in transgenic Arabidopsis plants. a The expression level of AcoCPK1 and the homologous AtCPK11 in transgenic Arabidopsis plants of OX-AcoCPK1. b The expression level of AcoCPK3 and the homologous AtCPK1 in transgenic Arabidopsis plants of OX-AcoCPK3. c The expression level of AcoCPK6 and the homologous AtCPK1 in transgenic Arabidopsis plants of OX-AcoCPK6
Additional file 4: Table S1. Characteristics of CPKs in pineapple
Additional file 5: Table S2. Synteny blocks of CPK genes within pineapple genome
Additional file 6: Table S3. Synteny blocks of CPK genes between pineapple and Arabidopsis genomes
Additional file 7: Table S4. Synteny blocks of CPK genes between pineapple and rice genomes

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Author contributions
MZ performed phylogenetic analysis and vector construction. YL annotated the genes on chromosomes and conducted the evolution analysis. QH and MC calculated all the data. YH and FC performed qRT-PCR analysis. X.W. and Y.L. provided the materials and analyzed the data. HC and YQ designed the research. MZ and YQ wrote the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interest.

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