Role of MADS-box Gene in the Development of Flower Organs in Pineapple of a Typical Collective Fruit

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Abstract

Background: MADS-box genes play crucial roles in plant vegetative and reproductive growth, especially in inflorescences, flower, and fruit. Pineapple is a typical collective fruit, and a comprehensive analysis of the MADS-box gene family in the development of floral organs of pineapple is still lacking.

Results: In this study, the whole-genome survey and expression profiling of the MADS-box family in pineapple were introduced. Forty-four AcMADS genes were identified in pineapple, 39 of them were located on 18 chromosomes and five genes were distributed in five scaffolds. Twenty-two AcMADS genes were defined as 15 pairs of segmental duplication events. Syntenic analysis showed that pineapple is closely related to monocotyledon plants. Most members of the type II subfamily of AcMADS genes had higher expression levels in floral organs compared with type I subfamily, thereby suggesting that AcMADS of type II may play more crucial roles in the development of floral organs of pineapple. Six AcMADS genes have significant tissue-specificity expression, thereby suggesting that they may participate in the formation of one or more floral organs.

Conclusions: Our findings not only benefit to reveal the functional characterization of MADS-box genes in the floral organ development of pineapple but also provide additional information for further understanding the formation and development collective fruit.

Background

The MADS-box transcription factors are one of the important families in higher plant [1] that play fundamental roles during plant development and floral organ differentiation [2]. The prominent feature of the MADS-box proteins is its MADS domain that consists of 56–58 amino acids [3]. The MADS domain can recognize the CArG-box with similar 10-bp A/T-rich DNA sequences [4]. In plants, MADS-box genes can be classified into two distinct groups, namely, types I and II, on the basis of the evolutionary relationships: type I members are SRF-like in plant, which only have a MADS (M) domain; type II MADS-box proteins are MEF2-like in plant, animal, and yeast, which contain a highly conservative DNA-binding domain (M), an intervening (I) domain, a semiconservative K domain, and a C-terminal region [5-6]. The type I proteins were further divided into Mo, Mi, and My subfamilies, and the type II group also were defined as MIKC-type proteins, which comprise MIKC⁵-type proteins, which comprise MIKC⁵⁵- and MIKC*-type proteins [7-8].

Flowering is a complex process that requires the cooperation and interaction of numerous genes. Previous reports have shown that the MADS-box genes can regulate the characteristics of floral meristems [9-10]. The ABCDE model completely explained the individual development of plant flowers and the determination of the identity of floral organs [11-12]. A genes determined the sepal development, petal development required A and B genes, stamen development needed B and C genes to work together, carpel development was ascertained by C genes, and ovule development was identified by C and D genes [12]. Class E genes need to assist other genes to participate in the determination of all flower organs and meristems [13]. In Arabidopsis, almost every gene from this model, such as A (APETALA1) [14], B
(PISTILLATA, AP3) [15], C (AGAMOUS) [16], D (AGAMOUS-LIKE 11) [17], and E (SEPALLATA 1, 2, 3,4) [18], belong to the type II subfamily, thereby suggesting that type II MADS-box genes play a vital role in the control of floral organ development.

Pineapple is one of important tropical fruits with great economic and research value [19-21]. This fruit is a typical collective fruit, and each pistil forms a separate little fruit, which is gathered on the enlarged torus. At present, few studies have been conducted on the morphological and physiological basis of collective fruits, especially the molecular mechanism. The physiological basis and molecular mechanism of the formation of collective fruits in pineapple should be understood. Previous studies had shown that the MADS-box genes are involved in various physiological processes, especially with the identification of floral organs. Thus, the exploration of the gene function of MADS-box and the molecular mechanism of pineapple flower organ development has received considerable interest.

In the present study, the comprehensive analysis, including the chromosomal localization, synteny analysis, and gene duplication, was investigated on the basis of the pineapple genome. Global expression analysis of MADS-box genes in different tissues and oral organs has been conducted by using RNA sequencing (RNA-Seq) and quantitative real-time polymerase chain reaction (qRT-PCR) to identify the specific MADS-box genes involved in the different biological processes. Six AcMADS genes demonstrated significant tissue specificity in different floral organs. These initiatives provided a reference for the functions of MADS-box genes in pineapple.

Results

Characteristics of Pineapple Flower

After the inflorescence of pineapple emerges, its inflorescence comes out from among leaf clumps and looks like a pine cone. The entire inflorescence development to bloom is approximately 1 month. The capitulum of pineapple is composed of 50 to 200 florets. The blossoming order of the capitulum is from the bottom to the top (Fig. 1A) [22]. The pineapple flowers are hermaphrodite and consist of three sepals, three petals, five stamens, and one pistil (Fig. 1B). The edible part of pineapple involves the fleshy axis of inflorescence and the ovary of florets.

Identification and Classification of MADS-box Genes in Pineapple

The MADS-box protein sequences were used to Hidden Markov Model (HMM) search, and 54 candidate genes were originally obtained. The sequence analysis indicated that ten candidate MADS-box genes containing incomplete MADS-box domains were removed. Forty-four MADS-box genes were selected and annotated in pineapple (Supplementary Table S1). Two maximum likelihood trees (ML) were further constructed on the basis of the full-length sequence alignment of all AcMADS genes together with grape and Arabidopsis to provide a reference for the evolutionary relationship of the MADS-box family in pineapple (Fig. 2). The 44 AcMADS genes of the pineapple can be divided into two categories, namely, type I (16) and type II (28). In the first ML tree, type I AcMADS genes were further classified into three
subclasses: Mα, Mβ, and Mγ (Fig. 2A). One MIKC*-type and 27 MIKC^C-type genes were showed in the second ML tree, and the MIKC^C-type genes were further classed into 11 major groups (Fig. 2B). These groups were named as follows: SVP (SHORT VEGETATIVE PHASE), AGL12, SEP, AGL6, AP1, FLC (FLOWERING LOCUS C), SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS), AG, PI/AP3, TT16 (TRANSPARENT TESTA16), and ANR.

Chromosomal Location and Duplication Analysis of Pineapple MADS-box Genes

Forty-four MADS-box genes were unevenly mapped to the 18 linkage groups (LGs) and five scaffolds and named from AcMADS1 to AcMADS44 according to their order on the LGs. The largest number of six genes (13.64%) was found in LG01, and the other LGs contained less than three AcMADS genes. Among the 44 AcMADS genes, 16 genes of type I were distributed on 10 chromosomes and three scaffolds, and 28 genes of type II were mapped to 16 chromosomes and two scaffolds. LG04 and LG13 only contained type I genes, while LG05, LG06, LG10, LG16, LG20, LG21, LG22, and LG24 only had type II genes (Fig. 3). Tandem duplication event refers to a region of chromosomes within 200 kb containing two or more genes [23]. The gene replication events of the MADS-box family found that one pair of genes (AcMADS17/AcMADS18) underwent tandem repeat events within the AcMADS gene family on LG7 (Fig. 3). These results showed randomness and nonuniform distribution of the AcMADS family in pineapple.

Syntenic Analysis of Pineapple AcMADS Genes

The segmental and tandem duplication events of the AcMADS genes family were identified to test the duplication effect in pineapple. In addition to the above-mentioned two tandem duplication events, twenty-two AcMADS genes were clustered into 15 segmental duplication events by BLASTP and MCScanX methods (Fig. 4, Supplementary Table S2). We found many copies of the segmental duplicated gene pairs from the same group, such as AcMADS08/15, AcMADS08/34 and AcMADS14/27 were from the ANR and SVP groups, respectively. AcMADS3/12 and AcMADS3/35 were from the AG group, AcMADS1/16 were from the AP1 group, and AcMADS38/39 were from SOC1. These AcMADS genes showed highly orthologous relationship, thereby indicating that they were obtained by gene duplication and may be from a common ancestor.

The comparative syntenic maps between pineapple with other five representative species (Arabidopsis, grape, banana, rice, and maize) were performed to further derive the origin and evolutionary mechanisms of pineapple MADS family (Fig. 5). Twenty-four AcMADS genes displayed syntenic relationship with those in banana, followed by maize (23, 52%), rice (22, 50%), grape (10, 32%), and Arabidopsis (6, 13%) through the whole genome-wide comparative analysis. The numbers of orthologous pairs between pineapple and other five species (banana, rice, maize, grape, and Arabidopsis) were 42, 42, 40, 14, and 12, respectively (Supplementary Table S3). Many collinear gene pairs were only found in monocots but not in dicots, thereby suggesting evolutionary difference between dicotyledonous and monocotyledonous plants. The mutual collinear pairs involving three AcMADS genes were identified between pineapple and all five other species. This finding indicates that these orthologous pairs may be derived from the same
ancestor, and duplication occurred before species divergence. These pairs may participate in the evolution of MADS family.

**Tissue-specific Expression Patterns of AcMADS Genes in Pineapple**

MADS-box genes were reported to participate in plant organ development, especially floral organ specification [2]. The expression patterns of 44 pineapple AcMADS genes in different tissues were obtained from the transcriptome data. The results showed that the transcriptional abundance of MADS genes in pineapple greatly varied in all detected samples (Fig. 6). The genes with high expression were mainly concentrated in type II subfamily. Twenty-six AcMADS genes (59%) were highly expressed in the flower of pineapple. Meanwhile, 14 AcMADS genes (32%) were highly expressed in the fruit of pineapple. Sixteen genes (36%) were expressed at low levels or not expressed in pineapple root, bud, leaf, flower, and fruit (Supplementary Table S4). Multiple of AcMADS genes were specifically expressed in flower and fruit. For example, five AcMADS genes (AcMADS5/10/16/21/24) showed high transcript abundance in flower and fruit, two genes (AcMADS06/13) only demonstrated high transcript abundance in flowers, and two genes (AcMADS36/4) only presented high expression in the roots (Fig. 6A). These implied MADS-box genes might participate in the flower development of pineapple, which was in line with previous reports [24].

The expressions of all 44 AcMADS genes in different floral organs were investigated to further verify the potential functions of the AcMADS genes in the formation of floral organs (Fig. 6B). Thirty-eight AcMADS genes exhibited relatively high expression in one or more flower organs of pineapple, and fifteen of them were highly expressed. Most AcMADS genes with high expression in floral organs originated from the type II subfamily, thereby suggesting that AcMADS genes of type II may play crucial roles in the development of floral organs of pineapple contrast with type I (Fig. 6B). Twenty-eight type II AcMADS genes were further performed by qRT-PCR to validate the RNA-seq results (Fig. 7). The qRT-PCR results showed that the expression profiles of most AcMADS genes were consistent with RNA-Seq. Many AcMADS genes showed obvious tissue specificity. For example, AcMADS12 only expressed in pistil, AcMADS03, AcMADS31, AcMADS33, and AcMADS35 had specifically high expression in the ovary, and AcMADS08 only expressed in the stamen (Fig. 8).

**Co-expression network of key AcMADS genes**

Plant flowering is a complex physiological process that requires multiple genes to work together. Studying gene co-expression networks contributes to the exploration of the potential functions of genes. The qRT-PCR and RNA-seq results indicated that six AcMADS genes with specific expressions in the floral organs of pineapple were selected to construct an interaction network through the String Protein Interaction Database (https://string-db.org/). A total of 129 protein pairs with interactions were detected. These interaction proteins were mainly involved in the floral organ developmental genes and floral induction, including AP1, CO, WUS, SEU, AP2, UFO, and LFY (Fig. 9). In the co-expression network diagram, AcMADS3 interacted with 10 known proteins with the largest number of interacting proteins. AcMADS35, AcMADS12, AcMADS31, and AcMADS33 interacted with 2, 4, 6, and 8 known proteins, respectively.
AcMADS8 only interacted with one known protein. These results will be beneficial to future research and verify its biological function on the basis of relevant experiments.

**Discussion**

Pineapple is a typical collective fruit, and research about its formation mechanism is still lacking. The *MADS-box* genes play crucial roles in the development of floral organs and had been investigated in multiple species [25-29]. However, previous studies only analyzed some functions of *AcMADS* genes in the CAM photosynthesis of pineapple [30]. In this study, 44 genes with typical MADS domains were defined as MADS-box family genes, which is inconsistent with previous research [30]. The number of MADS-box in pineapple (44) is much lower than that of *Arabidopsis* (106) [26], rice (75) [27], poplar (105) [28], apple (147) [29], and grape (54) [30]. The lack of pan-grass ρ whole-genome duplication (WGD) event and only having the σ-WGD event during pineapple evolution may affect the amount of *AcMADS* genes [31].

The *AcMADS* family of pineapple is classified into two categories, namely, types I and II; the type II genes are further divided into 11 subfamilies (Fig. 2B). However, the classification and number of type II *AcMADS* are slightly different among different species. The genome-wide duplication events are common in angiosperm evolution and generally result in the expansion of gene families [32]. Type II MADS genes originate from the whole genome replication, while type I genes are mainly duplicated by small-scale and recent duplications [33]. In our studies, 22 of the 44 *MADS-box* genes are associated with segmental duplication events, which is higher than that of the *Arabidopsis* [26]. *AcMADS* genes had more collinear gene pairs in monocotyledonous plants than dicotyledonous plants, thereby indicating that pineapple is closely related to monocotyledonous plants. The exploration of the genes related to flower development and flowering remains to be a major research topic. *MADS-box* genes play a major role in determining the identity of floral organs [34], and most type II genes are extensively expressed in the reproductive organs and lowly expressed in vegetative organs [35]. In this study, 38 *AcMADS* genes are expressed in one or more tissues, and 13 of them are specially expressed in flowers. The type II of *AcMADS* genes are highly expressed compared with type I in flower organs (Fig. 6B), thereby indicating that the type II MADS genes might play more important roles in the flower development of pineapple. This finding is consistent with previous reports [34]. The ABCDE model is a classical model of plant flower development [36,37]. In *Arabidopsis*, *AP1* act as a gene for flower meristem and organ morphology to promote the development of petals and sepals [13,38]. In pineapple, two homologous genes of class A genes (*AcMADS1* and *AcMADS16*) are identified. *AcMADS1* and *AcMADS16* are highly expressed in petals and sepals, respectively (Fig. 7), which is consistent with previous reports. The main function of class B genes (*AP3* and *PI*) is to determine the development of the second round of petals and the third round of stamens in *Arabidopsis* [36]. Two AP3-like (*AcMADS41* and *AcMADS22*) and one PI-like (*AcMADS13*) genes from the B class genes are identified in pineapple and had similar expression patterns (Fig. 7). The class B genes participate in the second and third rounds of floral organ formation of pineapple consistent with *Arabidopsis*. These genes might also participate in the formation of the fourth round of pistils in pineapple.
AG was a typical class C gene and essential for the identification of stamens and carpels [39]. Four AcMADS genes of class-C were detected in pineapple (Fig. 2B). AcMADS3, AcMADS12, and AcMADS35 were specifically highly expressed in the pistil, and AcMADS24 showed high expression in the stamen and pistil (Fig. 7). AcMADS3, AcMADS12, AcMADS3, and AcMADS35 were also clustered into the segment replication events (Fig. 4). These results showed that these paralogous genes of class C genes could derive from gene duplication and have similar functions in the development of stamen and pistil of pineapple. The homologous genes of AG were involved in the development of stamen and carpel in pineapple, which was consistent with previous reports [40]. The SEP genes, which are functionally important “adhesives” for A, B, C, and D genes, form higher order MADS-box protein complex multimers with other genes and play an effective role in protein–protein interactions [41,42]. Genetic and molecular studies had shown that class E genes (SEP1/2/3/4) had an obvious redundant function in flower development and were necessary to determine all four whorls of the flower organs [41,42]. In pineapple, two SEP-like genes (AcMADS21 and AcMADS5) were identified (Fig. 2B), and they were highly expressed throughout the floral meristem (Fig. 6B).

Six genes had significantly specific expression in one or two floral organs of pineapple (Fig. 8). AcMADS3, AcMADS12, and AcMADS35 belong to the AG subgroup, while AcMADS8, AcMADS31, and AcMADS33 belong to the ANR, TT16, and FLC subgroups (Fig. 2B). AcMADS8 was specifically expressed in the stamens; however, homologous gene AtAGL16, which negatively regulated flowering transition through FLOWERING LOCUS T (FT), was not found in stamens [43]. This finding indicated that AcMADS8 played a novel and key role in the development of pineapple stamens. TT16 (AT5G23260.2) was involved in the developmental regulation of the endothelium, which is essential for ovule development [44]. AcMADS31, a homologous gene of TT16, was specifically expressed in the ovary of pineapple, manifested that AcMADS31 was involved in the development of ovary, and was essential for the formation of female gametophyte of pineapple. AcMADS33, which was homologous with FLC that is flowering repressor in Arabidopsis [45], was also specifically expressed in the ovary of pineapple. However, the involvement of this gene in pistil development is yet to be reported. These AcMADS genes could directly or indirectly determine the formation of pineapple floral organs and provide a reference for further exploring the molecular mechanism of pineapple flower formation.

Materials And Methods

Plant Materials and Treatments

The pineapple plants (Ananas comosus L. cv. Comte de Paris) used in this study were grown in South Subtropical Crop Research Institute, Zhanjiang, China (21°10′2″N; 110°16′34″E). The different tissues of pineapple, including the bud, flower, fruit, leaf, and root, were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until further use. The floral organs of the pineapple flower, including petals, ovary, stamens, sepals, and stylet, were collected. All treated tissue samples were frozen in liquid nitrogen as quickly as possible and stored at −80 °C.
**Total RNA Isolation and qRT-PCR**

The total RNA was extracted from the pineapple tissues with the RNA extraction kit (Huayueyang, China) according to the manufacturer’s instructions. The concentration and quality of all purified RNA were checked on a 1% agarose gel and Bio Photometer Plus (Eppendorf, Germany). RNA (5 μg) was reverse transcribed to cDNA with the Revert Aid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

The quantitative RT-PCR assays were conducted in the Light Cycler 480 II (Roche, Switzerland) by using SYBR Green qPCR Master Mixes (Thermo Fisher Scientific, USA). Actin gene was used as the internal control of pineapple. The reaction mixture included 5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), a diluted cDNA template of 1 μL, and 1 μL of each primer in a final volume of 10 μL. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 15 s at 95 °C, 56°C for 15 s, and 72°C for 40 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of each gene. All primers were designed by the Primer Premier 5.0 [47] and listed in Additional file 4.

**Database Search and MADS-box Gene Family Identification in Pineapple**

The nucleotide and protein sequences of AtMADS genes were searched and obtained from TAIR (http://www.arabidopsis.org/) databases. We downloaded the pineapple genome and proteome sequences from the Pineapple Genomics Database (http://pineapple.angiosperms.org/pineapple/html/index.html) [21]. The genome data of grape, banana, rice, and maize were downloaded from Ensemble plants database (http://plants.ensembl.org/index.html). We downloaded the HMM file keeping with the MADS domain (PF00319) from the Pfam protein database (http://pfam.xfam.org, Pfam 31.0) and searched for the MADS-box genes in the pineapple genome database through HMMER 3.0. The e-value lower than 0.01 and the default parameters were selected. The MADS-box core sequences were confirmed by using the SMART database and the NCBI CDD web server (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Sequences without MADS-box domain will be deleted. The length of sequences, molecular weight, and isoelectric point (PI) of the MADS proteins were obtained by using the compute pi/Mw tool in the ExPASy server (http://web.expasy.org/protparam/). The subcellular localization of the identified MADS proteins was predicted by the cello web server (http://cello.life.nctu.edu.tw/).

**Phylogenetic Tree Construction and Classification in Pineapple MADS-box Genes**

MADS-box genes of Arabidopsis and grape were used as a reference to classify the MADS-box genes of pineapple. A single alignment of pineapple MADS domain by using the Clustal W program was built in MEGA6.0 software; a phylogenetic tree was then constructed by using maximum likelihood (ML) method [48] with the following parameters: 1000 bootstrap replications, partial deletion, and Jones–Taylor–Thornton (JTT) + gamma distributed (G) model.

**Chromosomal Location, Gene Duplication, and Syntenic Analysis**
The physical positions of the *AcMADS* genes on chromosomes were identified with TBtools [49] according to the gene location in the pineapple genome. The tandem duplication events were defined as the single chromosomal region contiguous homologous genes with the original repeat, while the duplicate of the whole blocks of genes between different chromosomes was defined as segmental duplication [50]. Gene duplication events were drafted with Multiple Collinearity Scan tool kit (MCScanX) [51]. In the syntenic analysis, the genome data of five representative species were downloaded from Ensemble plants database, and the diagrams were visualized using the TBtools with Dual Systeny Plotter.

**Conclusions**

In this study, the evolution and functional differentiation of the MADS-box genes of pineapple were comprehensively analyzed, and the expression profile of the *AcMADS* genes in the floral organ were proposed. The type II *AcMADS* genes played crucial roles in the development of floral organs of pineapple. *AcMADS3\12\35* of the AG subfamily and *AcMADS31* of the TTI6 subfamily were highly related to ovary development and pistil formation. *AcMADS8* of the ANR subfamily controlled the formation of stamens. Thus, these genes can be identified as candidate genes for vector construction and further functional analysis. These genes provided resources for exploring the regulation network of pineapple flowering and references for the genetic improvement of transgenic crops and traditional breeding.

**Declarations**

**Author’s Contributions:** X.P. wrote the manuscript. H.Z. designed the experiment and contributed to data analysis. D.Y. took charge the experimental materials treatment and collection. W.L. carried out RNA extraction and Q-PCR verification. X.Z. conceived the study. All authors have read and approved the final manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Figures
Figure 1

Different stages and organs of pineapple floral development. A: Different developmental stages of inflorescence. 1. Inflorescence emerging; 2. Inflorescence swelling out; 3. Early flowering stage; 4. Full-bloom stage; 5. Flower fading stage; 6. Panoramic view of the full-bloom stage. B: Floral organ structure of pineapple. 1. Sepal; 2. Petal; 3. Stamen; 4. Pistil.

Figure 2

Phylogenetic analysis of type I (A) and type II (B) MADS-box genes in Arabidopsis, grape, and pineapple. The phylogenetic trees were constructed using the ML method. The blue, dark green, and green triangles represent the MADS-box proteins from the grape, pineapple, and Arabidopsis, respectively. MADS-box proteins from the grape with the prefix “GSVIVT” indicate “VvMADS” and “At” means “AtMADS” in Arabidopsis.
Figure 3

Distribution of AcMADS genes on the pineapple chromosomes. The black vertical lines indicate the pineapple chromosome (LG), the number is above the LG, and the tandemly duplicated genes were connected by the green line. The different groups of AcMADS genes were color-coded.
Figure 4

Schematic representations for interchromosomal relationships and segmental duplication events of pineapple MADS genes. The gray lines represent all collinear blocks within the pineapple genome, and the red lines denote duplicated MADS gene pairs.
Figure 5

Syntenic analysis of MADS-box genes between pineapple and other five species. The gray lines represent the collinear blocks between pineapple and other plant genomes. The red lines indicate the collinear blocks of the pineapple MADS genes. Species names: A. comosus: Ananas comosus, A. thaliana: Arabidopsis thaliana, V. vinifera: Vitis vinifera, M. acuminate: Musa acuminate, O. sativa: Oryza sativa, Z. mays: Zea mays.
Figure 6

Expression patterns of the pineapple MADS genes. A. Hierarchical clustering of the expression patterns of AcMADS genes in the different tissues of pineapple. B. Expression profiles of AcMADS genes in pineapple floral organs.
Figure 7

Expression patterns of 28 type II AcMADS genes in pineapple floral organs by RT-qPCR. The error bars represent the standard deviations of three biological replicates. Br: Bract, Se: Sepal, Pe: Petals, St: Stamen, Ov: ovary, and Sty: Stylet.
Figure 8

Six tissue-specific AcMADS genes related to pineapple floral organ development. The blue module represents relatively low expression in floral organs. The red module represents relatively high expression in floral organs.
Figure 9

Co-expression relationship between AcMADS proteins and other related proteins according to the orthologs in Arabidopsis. Pink line: experimentally determined; green line: gene neighborhood; red line: gene fusions; blue line: gene co-occurrence; cyan line: text mining; black line: co-expression.

Supplementary Files

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