Journal of Plant Growth Regulation (2019) 38:479–493
https://doi.org/10.1007/s00344-018-9863-2

Jasmonate Signal Receptor Gene Family *ZmCOIs* Restore Male Fertility and Defense Response of *Arabidopsis* mutant *coi1-1*

Likun An[1] · Ramala Masood Ahmad[1] · Hong Ren[3] · Jia Qin[1] · Yuanxin Yan[1,2]

Received: 17 March 2018 / Accepted: 3 September 2018 / Published online: 30 October 2018

Abstract

Jasmonates (JAs) play an important role in many developmental processes, such as root growth, leaf senescence, male fertility, and defense responses against insects and pathogens. The F-box protein COI1, which plays a central role in JA signal transduction, perceives the JA signal and is required for all the JA-mediated defense responses against biotic and abiotic stresses. JA signaling elements including COI1 have been extensively investigated in *Arabidopsis*e. However, the elements of the JA signaling pathway in maize are largely unknown. In this study, we identified four F-box protein genes from the maize genome, which share high homology with *AtCOI1*, designated as *ZmCOI1a, ZmCOI1b, ZmCOI1c*, and *ZmCOI2*, collectively *ZmCOIs*. To test whether or not the homologous genes of maize are functionally conservative in JA signaling, we over-expressed *ZmCOIs* in the *Arabidopsis coi1-1* mutant. The results showed that over-expression of *ZmCOI1a*, *ZmCOI1b* or *ZmCOI1c* in the *coi1-1* mutant resulted in the restoration of male fertility, indicating successful complementation of *coi1-1* sterility by *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c*. However, *ZmCOI2* was not able to restore male fertility of the mutant, indicating that *ZmCOI2* has a function diverged from JA signaling. Furthermore, over-expression of the *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* genes, except *ZmCOI2*, which, in the *coi1-1* mutant, caused restoration of resistance to the leaf pathogen *Botrytis cinerea* and the soil-borne pathogen *Pythium aristosporum*. In addition, a set of JA-dependent genes are highly induced by wounding in the transformants of *ZmCOIa, ZmCOI1b*, and *ZmCOI1c*, but not inducible in transformants of *ZmCOI2* or in the *coi1-1* mutant, indicating that *ZmCOIa*, *ZmCOI1b*, and *ZmCOI1c*, except *ZmCOI2*, which can compensate *coi1-1* mutation of *Arabidopsis* for the stress defense response. Putting all the data together, our results suggested that *ZmCOIa*, *ZmCOI1b*, and *ZmCOI1c*, but not *ZmCOI2*, act as *AtCOI1* orthologues in maize for JA signal transduction.

Keyword Maize *Coronatine Insensitive1* · Jasmonate · Male fertility · Defense response

Introduction

Jasmonic acid (JA) and derivatives, such as methyl jasmonate (MeJA) and jasmonoyl isoleucine (JA-Ile), are collectively referred to as jasmonates (JAs) (Schaller et al. 2004; Yan et al. 2013). JAs are lipid-derived hormone signals that regulate a wide range of biochemical and physiological processes in plants, ranging from vegetative growth to reproductive development, including seed germination, root growth, trichome development, leaf senescence, tendril coiling, anther dehiscence, pollen viability, fruit ripening, etc (Creelman and Mullet 1997; Yan et al. 2013; Wasternack and Hause 2013; Wasternack 2014; Huang et al. 2017; Zhai et al. 2017; Wasternack and Feussner 2018). In *Arabidopsis*, JA biosynthesis mutants, such as *fad3/7/8* (McConn and Browse 1996), *dad1* (Ishiguro et al. 2001), *dde2-2* (von Malek et al. 2002) or *aos* (Park et al. 2002), *dde1* (Sanders

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00344-018-9863-2) contains supplementary material, which is available to authorized users.

✉ Yuanxin Yan
yuanxin.yan@njau.edu.cn

[1] State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China

[2] Jiangsu Collaborative Innovation Center for Modern Crop Production, Nanjing 210095, China

[3] Dryland Crop Institute of Guizhou Province, Guiyang 550006, China

et al. 2000) or *opr3* (Stintzi and Browse 2000) or *opr3-3* (Chini et al. 2018), and *acx1/5* (Schilmiller et al. 2007), exhibited defects in filament elongation, anther dehiscence, and pollen maturation leading to male sterility. Exogenous application of JA/MeJA on these JA-biosynthetic mutants can restore the stamen development of *fad3/7/8* (McConn and Browse 1996), *dad1* (Ishiguro et al. 2001), *aos* (Park et al. 2002), *opr3* (Stintzi and Browse 2000) and *opr3-3* (Chini et al. 2018), demonstrating that JA is an essential signal for stamen development and pollen maturation in *Arabidopsis*. Mutants impairing JA signal transduction, such as *coi1* (Feys et al. 1994), *myb21* (Mandaokar et al. 2006), and *myb21myb24* (Mandaokar et al. 2006), are also male-sterile because of reduced filament elongation and lack of anther dehiscence. Over-expression of Jas domain-mutated JAZ protein genes, such as *JAZ1Δ3A* (Thines et al. 2007), *jai3-1* (Chini et al. 2007), *JAZ10/JAS1* (Yan et al. 2007), and *JAZ10.4* (Chung and Howe 2009), impaired JA signal transduction in the transformants, which exhibited a male-sterile phenotype similar to *coi1*. In monocotyledonous plants, JA is also an important hormone signal for reproductive growth of flowers and seeds (Yan et al. 2012; Cai et al. 2014). In maize, the JA-deficient mutant *opr7opr8* displays a feminized tassel, which consists of pistillate spikelets (female florets) instead of staminate spikelets (male florets) (Yan et al. 2012), indicating that JA is a key signal for sex determination of maize tassels. In rice, the JA biosynthesis mutants *hebita* and *cpm2* (both are the mutants of the single copy *AOC* gene in rice genome), and the JA signaling mutant *Osjar1* showed male sterility due to no anther dehiscence and abnormality of floret architecture (Riemann et al. 2013; Xiao et al. 2014), indicating that JA is required for floret development in rice.

In particular, JAs are a critical hormone signal for plant defense against herbivore insects and pathogens. JA biosynthesis mutants, such as *Arabidopsis fad3fad7fad8, aos, opr3* or JA perception mutants *jar1*, and *coi1*, as well as those from other plant species, such as tomato *jar1* and maize *opr7opr8*, are highly susceptible to insect attack (McConn et al. 1997; von Malek et al. 2002; Stintzi et al. 2001; Staswick et al. 1998; Xie et al. 1998; Li et al. 2004; Yan et al. 2012). On the other hand, JA-pathway over-expressing mutants, such as *cev1*, *cex1*, and *fou2*, are highly resistant to insect and pathogen attacks (Ellis and Turner 2001; Xu et al. 2001; Bonaventure et al. 2007). Exogenous application of JA or MeJA decreased the suitability of foliage for herbivorous insects in tomato (Thaler et al. 1996; Pauwels et al. 2009). For disease resistance, JA has been demonstrated to be an indispensible signal for resistance/susceptibility to several diseases caused by fungal, bacterial, and viral pathogens (Staswick et al. 1998; Vijayan et al. 1998; Yan and Xie 2015; Wasternack and Strnad 2016; Zhang et al. 2017). The JA perception mutant *coi1* displays enhanced susceptibility to the necrotrophic fungi *(A) brassicicola*, *(B) cinerea*, *P. cucumerina* and *F. oxysporum* (Thomma et al. 1998; Rowe et al. 2010; Thatcher et al. 2009), while the JA biosynthesis mutant *aos* as well as the signaling mutant *coi1* are also highly susceptible to *B. cenerea* (Rowe et al. 2010). The JA biosynthesis mutants *fad3fad7fad8* and *jar1* exhibit enhanced susceptibility to the soil-borne pathogen *Pythium* spp. (Vijayan et al. 1998; Staswick et al. 1998). In maize, the double mutant *opr7opr8*, deficient in JA biosynthesis, showed extreme susceptibility to *Pythium aristosporium* (Yan et al. 2012). In addition, JAs have also been reported for their important roles in plant responses to abiotic stresses (Kazan 2015; Per et al. 2018), such as heavy metals (Maksymiec et al. 2005), drought (Brossa et al. 2011), heat stress (Clarke et al. 2009), salt (Zhao et al. 2014) and ozone stresses (Sasaki-Sekimoto et al. 2005).

All the actions of JA are completed in plants by the JA signal transduction machinery, called the JA signaling pathway. The core signaling module of this pathway consists of four major components: a bioactive JA signal, such as JA-Ile, the SCF-type E3 ubiquitin ligase SCF^{COI1} complex, jasmonate ZIM-domain (JAZ) repressor proteins, and transcription factors that promote the expression of JA-responsive genes. When JAZ proteins were discovered as the true targets of the SCF^{COI1} complex simultaneously by three research groups (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007), the JA signaling model was established: (1) at low intracellular levels of the JA signal, the SCF^{COI1} complex (JA receptor) has no E3 ubiquitin ligase activity, resulting in accumulation of JAZ proteins which repress the activity of transcription factors, such as MYC2 that positively regulates JA-responsive genes; and (2) at high levels of the JA signal, such as when a plant is attacked by insects or pathogens, the rapidly accumulated JAs promote SCF(COI1)-mediated ubiquitination of JAZ proteins and subsequently cause them to be degraded via the 26S proteasome. Removal of JAZ proteins causes the release of JAZ-repressed transcription factors, such as MYC2, MYB21, ERF1, etc., and subsequent activation of a number of early JA-responsive genes (Chini et al. 2007, 2016; Thines et al. 2007; Browse and Howe 2008; Katsir et al. 2008; Kazan and Manners 2008; Fonseca et al. 2009; Sheard et al. 2010; Wasternack and Hause 2013; Howe et al. 2018).

Coronatine Insensitive 1 (COI1) is an F-box protein component of the Skp1-Cul-F-box protein (SCF) complex (Devoto et al. 2002) which recruits JAZ proteins and other co-repressor proteins, such as TOPLESS for JA perception and signal transduction (Pauwels et al. 2010). The *COI1* gene was identified and cloned from *coi1-1*, *coi1-15*, and *coi1-18* mutants (Xie et al. 1998), which are insensitive to the bacteria phytotoxin coronatine (a JA analog) and JAs (Feys et al. 1994). Loss-of-function mutants of *COI1* in *Arabidopsis*, such as *coi1-1*, are completely deficient in all

the JA responses (Feys et al. 1994; Xie et al. 1998) due to lack of JA perception. Similar to JA biosynthesis mutants, such as *aos* (Park et al. 2002), and *opr3* (Stintzi and Browse 2000), the JA signaling mutant *coi1-1* showed phenotypes of inhibited filament elongation, reduced pollen development and lack of anther dehiscence leading to complete male sterility (Feys et al. 1994; Xie et al. 1998). A number of *coi1* alleles have been isolated, and all the knock-out mutants of them, such as *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-9*, *coi1-10*, etc., share similar phenotypes, such as male-sterile, susceptible to insect damage (He et al. 2012; Huang et al. 2014). To date, the *COI1* genes have been identified and characterized from several plant species (Li et al. 2004; Wang et al. 2005, 2014; Peng et al. 2009; Lee et al. 2013) and some of them have been tested experimentally for their function in the JA signaling pathway (Wang et al. 2005; Lee et al. 2013). *Arabidopsis* has only one copy of the *COI1* gene, whereas three *AtCOI1* orthologues (*OsCOI1a*, Os01g0853400; *OsCOI1b*, Os05g0449500; and *OsCOI2*, Os03g0265500) have been reported in rice (Yang et al. 2012; Lee et al. 2013). Although maize (*Zea mays* L.) is an economically important crop in the world, little is known about JA biosynthesis and signaling in this species compared to greater advances in dicot plants, such as *Arabidopsis*. A study of the *opr7opr8* mutant showed that JAs have tremendous roles in a number of developmental and defense processes in maize (Yan et al. 2012). Recently, the lipoxygenase pathway and JA function were reviewed by Borrego and Kolomiets (2016); however, knowledge of JAs and other oxylipins in maize is still limited. In this study, we identified the *COI1* orthologues (*ZmCOIs*) of maize, and their function was analyzed by complementation of the *Arabidopsis coi1-1* mutant. Our results indicated that three of four *ZmCOIs* genes play a crucial role in JA signal transduction in maize.

Materials and Methods

Identification and Phylogenetic Analysis of COI1 Orthologues in Maize and Other Plant Species

To identify the *COI1* orthologous genes in maize, we performed a number of blasts against the genome database of maize (https://www.maizegdb.org/) and "Gramene" (http://www.gramene.org/) using the amino acid sequences of *Arabidopsis COI1* gene (*AtCOI1*) and rice *COI* genes (*OsCOI1a*, *OsCOI1b*, and *OsCOI2*) as the blast queries. To find the *COI1* orthologous genes in other plant species, we searched the databases of NCBI (https://www.ncbi.nlm.nih.gov/), PlantGDB (http://plantgdb.org/cgi-bin/blast/PlantGDBblast) using the sequences of reported *COI1* genes, such as *Arabidopsis COI1* (Xie et al. 1998), tomato *COI1* (Li et al. 2004), *OsCOIs* (Lee et al. 2013), *GmCOI1* (Wang et al. 2005), *HbCOI1* (Peng et al. 2009) and *AsCOI1* (Liao et al. 2015) as search queries. Multiple sequence alignment and phylogenetic analysis was performed by MEGA5.0 software.

Plant Material and Growth Conditions

The 3rd leaf of maize inbred line B73 was used to extract genomic DNA or total RNA, which were used to amplify genomic and cDNA sequences of *ZmCOIs*. The cDNA sequences were used for construction of over-expression vectors. *Arabidopsis coi1-1* heterozygous and wild-type (columbia-0) seeds were kindly provided by Dr. Daoxin Xie (Tsinghua University, Beijing, China).

Maize seeds were planted in 2-L pots filled with mixed soil (vermiculite: organic substrate: loam = 1:1:1). Maize seedlings were grown in the greenhouse, with controlled conditions (temperature was controlled at 25–35 °C). *Arabidopsis* seeds were surface-sterilized, and planted in 0.3-L pots with artificial mixed soil (vermiculite: organic substrate: peat: perlite = 3:3:3:1). The pots were placed under 16 h day and 8 h night cycles at 22 °C in a growth chamber.

Construction of Over-Expression Vectors of *ZmCOIs*

The full-length coding region of the *ZmCOIs* was PCR-amplified from the maize B73 cDNA with gene-specific primers. The PCR products were cloned into the pEASY-Blunt Zero vector (Transgen Biotech, pEASY-Blunt Zero cloning kit) for sequencing. The sequencing-verified sequences of *ZmCOIs* were used for over-expression vector construction. The verified clones of *ZmCOIs* were subsequently cloned into the pANIC6E vector (Mann et al. 2012) by gateway cloning techniques using BP Clonase™ Enzyme Mix (Invitrogen, Catalog no. 11789-013) and LR Clonase™ Enzyme Mix (Invitrogen, Catalog no. 11789-019). The construction procedure was performed according to the manufacturer's instructions for the cloning kits. In the final over-expression construction, the gene of interest was controlled under a ZmUbi1 promoter. The final constructions (pANIC6E-*ZmCOI1a*, *-ZmCOI1b*, *-ZmCOIc*, *-ZmCOI2*) were transformed into *Agrobacteria* strain GV3101 that was used to transform *Arabidopsis*.

Selection of *coi1-1* Homozygote Transformants

coi1-1 heterozygous plants were used to transform with the constructions of *ZmCOIs*. The *coi1-1* homozygous segregants were selected by PCR genotyping using primers P1&P2 and restriction enzyme Xcm I (Xie et al. 1998).

Gene Expression by qPCR and RT-PCR

To test the *ZmCOIs* expression level in the tissues of maize plant, total RNA was extracted from tissues including ear leaf, young cob, young tassel, brace roots, internode, silk, etc. To test the effect of hormones on the gene expression of *ZmCOIs*, the V3-stage-seedlings of B73 were sprayed with 100 µM of JA, ABA, ACC, NAA, and GA3 and 2.5 mM of SA solution containing 0.1% Tween-20. The 3rd leaves of the treated plants were harvested at the time points of 0, 6, 12, 24, 48, and 72 h after treatment and frozen in liquid nitrogen for further use to isolate RNA. To test the gene expression of JA-dependent genes in over-expression transformants, the leaves of WT, *coi1-1* and transformants were mechanically wounded and samples harvested at 0, 0.5, 1.5, and 6 h after wounding for further RNA extraction.

The total RNA of all the samples was extracted using TRIzol™ Reagent (Thermo Fisher Scientific, Catalog No. 15596018). The first strand of cDNA was synthesized using EasyScript first-strand cDNA synthesis super mix (Transgen Biotech, Catalog No. AE301-02). The gene expression of *ZmCOIs* was detected by real-time quantitative PCR (qPCR) using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Catalog No. A25741). The maize *EIF4α* gene was used as the reference gene of qPCR. In the transgenic *Arabidopsis* plants, JA-dependent gene expression in response to mechanical wounding was detected by semi-quantitative RT-PCR according to Marone et al. (2001) using the *β-actin2* gene as the reference gene.

Pathogen Inoculation of *ZmCOIs*-Over-Expressed Plants

To test the defense response of transgenic plants, two pathogen species (the necrotrophic pathogen *Botrytis cinerea* and the soilborne root pathogen *Pythium aristosporum*) were applied to *Arabidopsis* WT, *coi1-1* and over-expressed transformants of *ZmCOIa*, *ZmCOI1b*, and *ZmCOI1c* in the *coi1-1* mutant.

Forthe *Botrytis cinerea* culture and inoculation, purified *B. cinerea* was obtained from Chunhao Jiang (Nanjing Agricultural University, Nanjing, China). A piece of *B. cinerea* culture was transferred to a PDA (potato dextrose agar) plate grown at 25 °C for 10–15 days under a fluorescence light of < 1000 lx illumination. The conidia were collected and suspended in sterile water containing 0.025% Tween-20. The whole plants were inoculated by spraying a spore suspension (5×10^5 spores /ml) until the leaves were fully covered by the suspension. Inoculated plants were kept at 25 °C in a box covered with a piece of plastic membrane for 1 day and then they were put back in the growth chamber for 3–5 days until the typical symptom appeared.

For the *Pythium aristosporum* culture and inoculation, *P. aristosporum* was isolated from the rotted roots of the maize JA biosynthesis mutant *opr7opr8* (Yan et al. 2012). The isolate was inoculated to a carrot–agar medium plate and grown at room temperature with 200–1000 lx illumination until oospores formed in the medium. The medium culture was blended with sterile water and passed through two layers of gauze. The denseness of suspension was adjusted with sterile water to a concentration of 10^5 oospores/ml, which was counted under a microscope using a hemocytometer. The plants were inoculated by adding the suspension to the soil to infect the roots. Each plant received 10 ml of the *Pythium* suspension. The inoculated plants were kept at 25 °C with illumination at about 1000 lx. At 3–5 days, photographs for the symptoms of disease were taken.

Wound Treatment

Mechanical wound treatment was conducted as described by Reymond et al. (2000). The samples were harvested at 0, 0.5, 1.5, and 6 h after wounding.

Results

Identification of the *COI1* Orthologues in the Maize Genome

The *COI1* gene has been identified from the *Arabidopsis* mutant *coi1-1*, *coi1-15* and *coi1-18* (Xie et al. 1998) and the genome has only a single *COI1* gene copy (Lee et al. 2013). Three *COI1* orthologues in rice, *OsCOI1a* (Os01g0853400), *OsCOI1b* (Os05g0449500), and *OsCOI2* (Os03g0265500), have been reported (Lee et al. 2013). To identify the *COI1* orthologous genes in maize, we searched the maize genome by performing blasts against the genome database of maize (https://www.maizegdb.org/) and "Gramene" (http://www.gramene.org/) using the amino acid sequences of the *Arabidopsis COI1* gene (*AtCOI1*) and rice *COI* genes (*OsCOI1a*, *OsCOI1b*, and *OsCOI2*) as the blast queries and four *COI1* orthologous genes in maize genome have been identified: GRMZM2G125411, GRMZM2G151536, GRMZM2G353209, and GRMZM2G079112, designated as *ZmCOI1a, ZmCOI1b, ZmCOI1c, ZmCOI2* respectively, collectively called *ZmCOIs*.

The amino acid (AA) sequence alignment of ZmCOIs with AtCOI1 showed that ZmCOIs share 54.6–57.2% AA identity with AtCOI1 (Fig. 1; Fig. S1), indicating that ZmCOIs share a low homology with AtCOI1. However, the protein domains ZmCOIs and AtCOI1 share conservative F-box and leucine-rich repeats (LRRs) (Fig. 1), indicating that they may share a similar molecular function. The COI1 protein is a critical component of the SCF(COI1)-JAZ

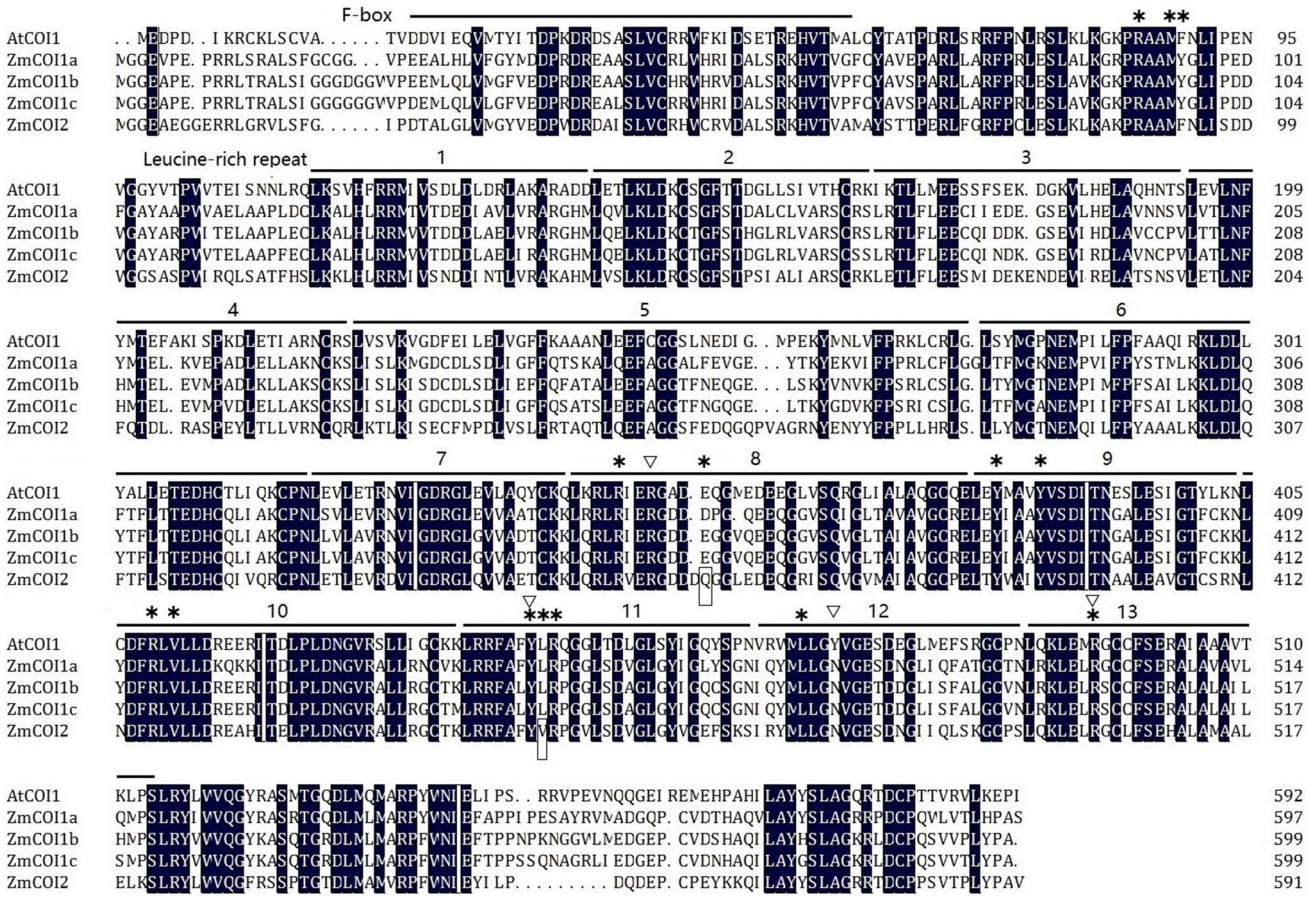

Fig. 1 Amino acid sequence alignment of AtCOI1 and ZmCOIs. Deduced amino acid sequences of AtCOI1 and ZmCOIs were aligned using the DNAMAN7.0 program. *Black shaded letters* indicate identical residues. ZmCOIs and AtCOI1 share conservative F-box and Leucine-rich repeats (LRRs). *Asterisks* indicate the binding sites of coronatine/JA-Ile in the COI1-JAZcomplex (Yan et al. 2009; Sheard et al. 2010). *Triangles* indicate JAZ-binding sites involved in the COI-JAZ interaction (Sheard et al. 2010). *Solid boxes* indicate the amino acid residues in ZmCOI2 which are divergent from AtCOI1 and ZmCOI1a/b/c

complex, the core machinery of JA perception in plants (Katsir et al. 2008; Sheard et al. 2010). The COI1 protein possesses 16 key amino acid residues (asterisks or triangles in Fig. 1), which are supposed to be the binding sites of JA-Ile or JAZ proteins with COI1 (Yan et al. 2009; Sheard et al. 2010). We found that ZmCOI1a, ZmCOI1b, and ZmCOI1c share 13–14 conservative key amino acid residues with AtCOI1 (Fig. 1), but ZmCOI2 has two additional divergent points of these key amino acids (solid boxes in Fig. 1), suggesting ZmCOI1a, ZmCOI1b, and ZmCOI1c may have a conservative molecular function as AtCOI1, but ZmCOI2 might have evolved into a new divergent function category.

The AA sequence alignment of ZmCOIs showed that the members of the ZmCOIs family share higher AA identity with each other. The AA identity is 78.4% between ZmCOI1a and ZmCOI1b, 78.9% between ZmCOI1a and ZmCOI1c or 93.5% between ZmCOI1b and ZmCOI1c (Fig. S2). The AA identities of ZmCOI2 with ZmCOI1a, ZmCOI1b, and ZmCOI1c are 60.9%, 61.6% and 60.1%, respectively (Fig. S2), indicating that *ZmCOI2* is a highly different gene from *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* in the maize genome. We have also blasted "Gramene" using the amino acid sequences of ZmCOIs as the blast queries and identified the *COI1* orthologous genes in purple false brome *Brachpodium distachyon*, millet (*Setaria italica*), and sorghum (*Sorghum bicolor*). Rice, *Brachpodium*, millet, and sorghum all have three *COI1* orthologous genes in their genomes (Fig. S3). AA sequence analysis showed that *ZmCOIs* are highly similar to the *COI1* orthologous genes in rice (*OsCOIs*), *Brachpodium* (*BdCOIs*), millet (*SiCOIs*), and sorghum (*SbCOIs*) (Fig. S3), indicating that *COI1* genes are highly conserved in cereals. ZmCOIs and SbCOIs have AA identity of more than 93% (Fig. S3), indicating that sorghum is a highly close species to maize for the genome evolution.

Phylogenetic Analysis of *COI1* Orthologous Genes in Plants

The COI1 protein, a critical component of the JA-perception complex (Katsir et al. 2008; Sheard et al. 2010), must be an essential protein for all higher plants, in which it acts as an indispensable growth regulator for varied developmental processes and defense responses (Yan et al. 2013). To determine the evolutionary relationship among COI1 proteins in plants, the AA sequences of COI1 orthologues from 36 plant species were obtained by searching the NCBI database (https://www.ncbi.nlm.nih.gov/) and "Gramene", using AtCOI1, OsCOIs and ZmCOIs as the searching queries and an unrooted phylogenetic tree of these COI1s was constructed with maximum likelihood (ML) algorithms by the software MEGA5.0 (Fig. 2). This phylogenetic tree displayed that all the COI1 proteins we applied here clustered into two clades: dicots and monocots (Fig. 2), indicating that dicotyledonous and monocotyledonous *COI1* genes have their own ancestral lineage. The dicotyledonous clade can be divided into five groups (I–V) and AtCOI1 belongs to group II. The monocotyledonous COI1 proteins clustered into two groups (VI–VII) (Fig. 2). Group VI contains COI1a and COI1b and maize COI1c. Group VII includes COI2 of the monocotyledonous species. On this tree, we also noticed that maize COIs are closer to sorghum COIs than other cereals, revealing a close phylogenetic relationship between these two species.

Tissue-Specific Expression of *ZmCOIs* Genes and Their Responses to Hormone Treatments

The maize genome contains four *COI1* orthologous genes (Fig. 1), but some of them may not be expressed. To know which of the *ZmCOIs* are expressed, we analyzed the expression levels of *ZmCOIs* in the leaf, internode, brace roots, ear cob, young tassel and silk using the quantitative RT-PCR technique. The results showed that all the members of *ZmCOIs* are constitutively expressed in all the tissues tested (Fig. S4), among them *ZmCOI1a* and *ZmCOI1b* are highly-expressed genes whereas *ZmCOI1c* and *ZmCOI2* are less expressed, indicating that maize may largely depend on the function of *ZmCOI1a* and *ZmCOI1b* for JA signal transduction. To further verify our results, we downloaded the data of RNA-Seq expression from MaizeGDB (https://www.maizegdb.org/), and the results confirmed that *ZmCOI1a* and *ZmCOI1b* are highly-expressed and *ZmCOI1c* and *ZmCOI2* less-expressed genes in 79 tissues of maize plants (Fig. S5). Interestingly, these data showed that *ZmCOI2* is highly expressed in anther and in seed endosperm (Fig. S6), indicating that *ZmCOI2* may be involved in anther or seed development.

The *COI1* gene is a critical gene for JA signal transduction (Feys et al. 1994; Xie et al. 1998). To understand if the expression of *ZmCOIs* responds to plant hormones, the transcript level of *ZmCOIs* was analyzed by quantitative RT-PCR in the leaves sprayed with JA, ABA, ACC, GA_3, NAA, and SA (see "Materials and Methods"). The results showed that *ZmCOI1a* and *ZmCOI1b* genes were strongly induced by JA and ABA (Fig. 3), but there was no significant induction by ACC, GA_3, NAA, and SA (Fig. 3). For JA treatment, the expression of *ZmCOIa* and *ZmCOI1b* was increased more than 15 times in 12 h after treatment (Fig. 3). For ABA treatment, *ZmCOIa* and *ZmCOI1b* increased more than 8 and 5 times, respectively, in 12 h after treatment (Fig. 3). This result indicates that the expression level of *ZmCOI1a* and *ZmCOI1b* may be regulated by endogenous JA and ABA. *ZmCOI1c* and *ZmCOI2* genes are less-expressed genes in maize tissues (Fig. 3) and they are just slightly induced by JA and ABA, but not induced by ACC, GA_3, NAA, and SA (Fig. 3), suggesting that *ZmCOI1c* and *ZmCOI2* are low-expressed but constitutively expressed genes in maize.

Over-Expression of *ZmCOI1a/b/c* in *Arabidopsis coi1-1* Mutant Restores Male Fertility of the Mutant

To efficiently test whether the *ZmCOIs* are functionally similar to *Arabidopsis COI1*, we transformed each of the *ZmCOIs* into the *Arabidopsis coi1-1* mutant to test whether *ZmCOI1a*, *ZmCOI1b*, *ZmCOI1c*, and *ZmCOI2* can complement the JA-insensitivity-related phenotypes of *coi1-1*. Because *coi1-1* homozygous is male-sterile and is not suitable to be transformed by floral dip (Clough and Bent 1998), we transformed each *ZmCOIs* gene into *coi1-1* heterozygous plants. The transgenes existence in the genome of transformant plants (T1) was confirmed by PCR amplification using *ZmCOIs*-specific primers (Fig. S10), and the mRNA of the transgenes was detected by RT-PCR amplification using *ZmCOIs*-specific primers (Fig. S10). In the segregation generation (T_1) of transformants, we selected only *coi1-1* homozygous transformants containing a transgene gene (*ZmCOI1a*/*ZmCOI1b*/*ZmCOI1c*/*ZmCOI2*) by PCR genotyping (see "Materials and Methods"). A large portion (15.6–36.3%) of transgenic *coi1-1* mutant plants transformed with *ZmCOI1a* or *ZmCOI1b* or *ZmCOI1c* showed fertile flowers (Table 1; Fig. 4) and good seed-bearing (Table 1), whereas transgenic plants with *ZmCOI2* were male-sterile and non-seed-bearing (Table 1; Fig. 4). These results indicated that *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* are capable of complementing the *Arabidopsis coi1-1* mutant but *ZmCOI2* is not, suggesting that *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* have similar functions as the *Arabidopsis COI1* gene whereas *ZmCOI2* has a divergent function from *COI1*. All the T_2 generation plants of the *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* T_1 generation (*ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* transformants) showed fertility and seed-bearing (seeds/silique) similar to T_1 plants (Table 1).

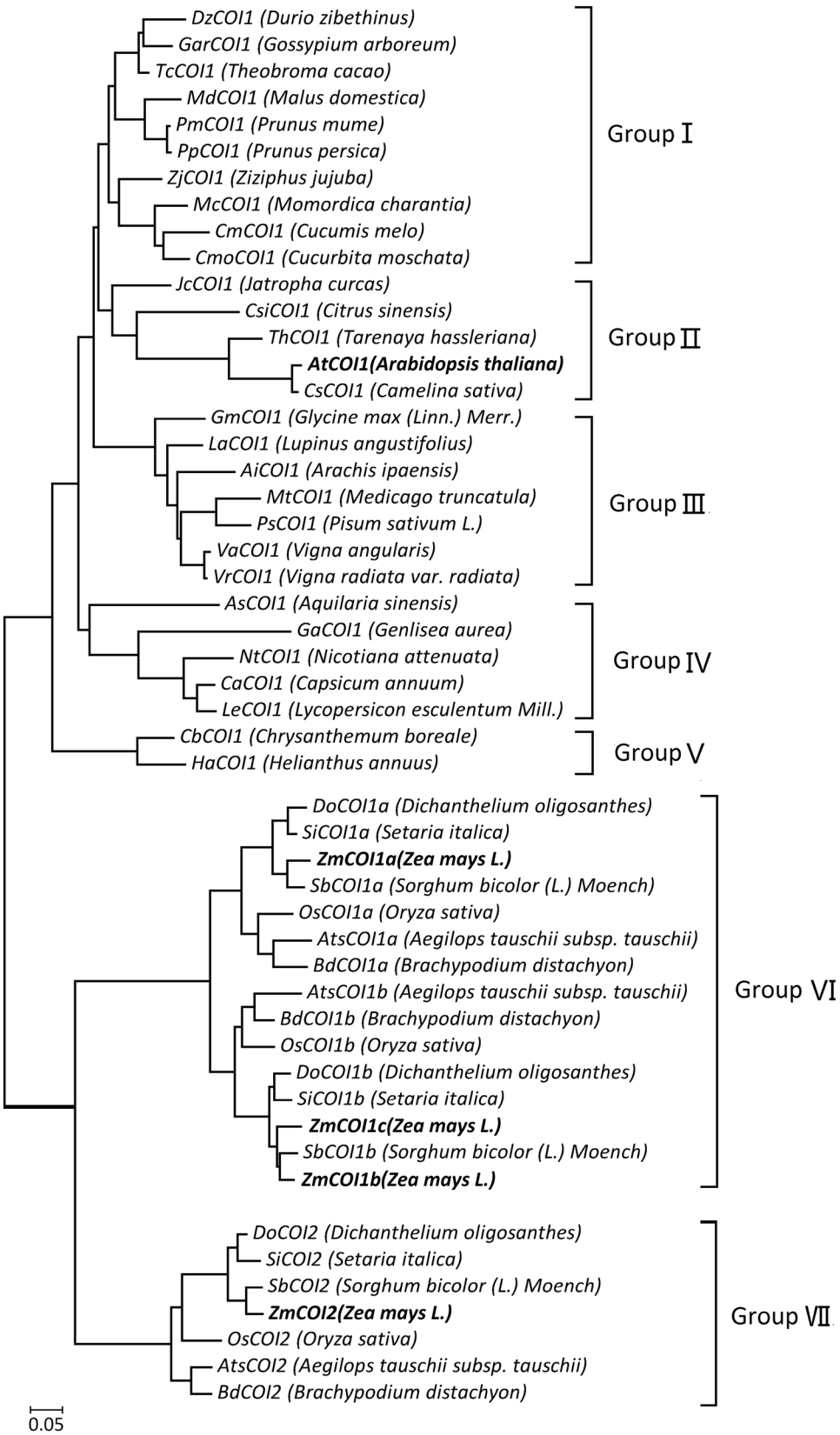

Fig. 2 A phylogenetic tree of amino acid sequences of COI1 proteins in 36 plant species was drawn with maximum likelihood (ML) algorithms by the software MEGA5.0 with 1000 bootstrap replicates. The accession numbers in GenBank of COI1 genes used in this analysis are listed in Table S1

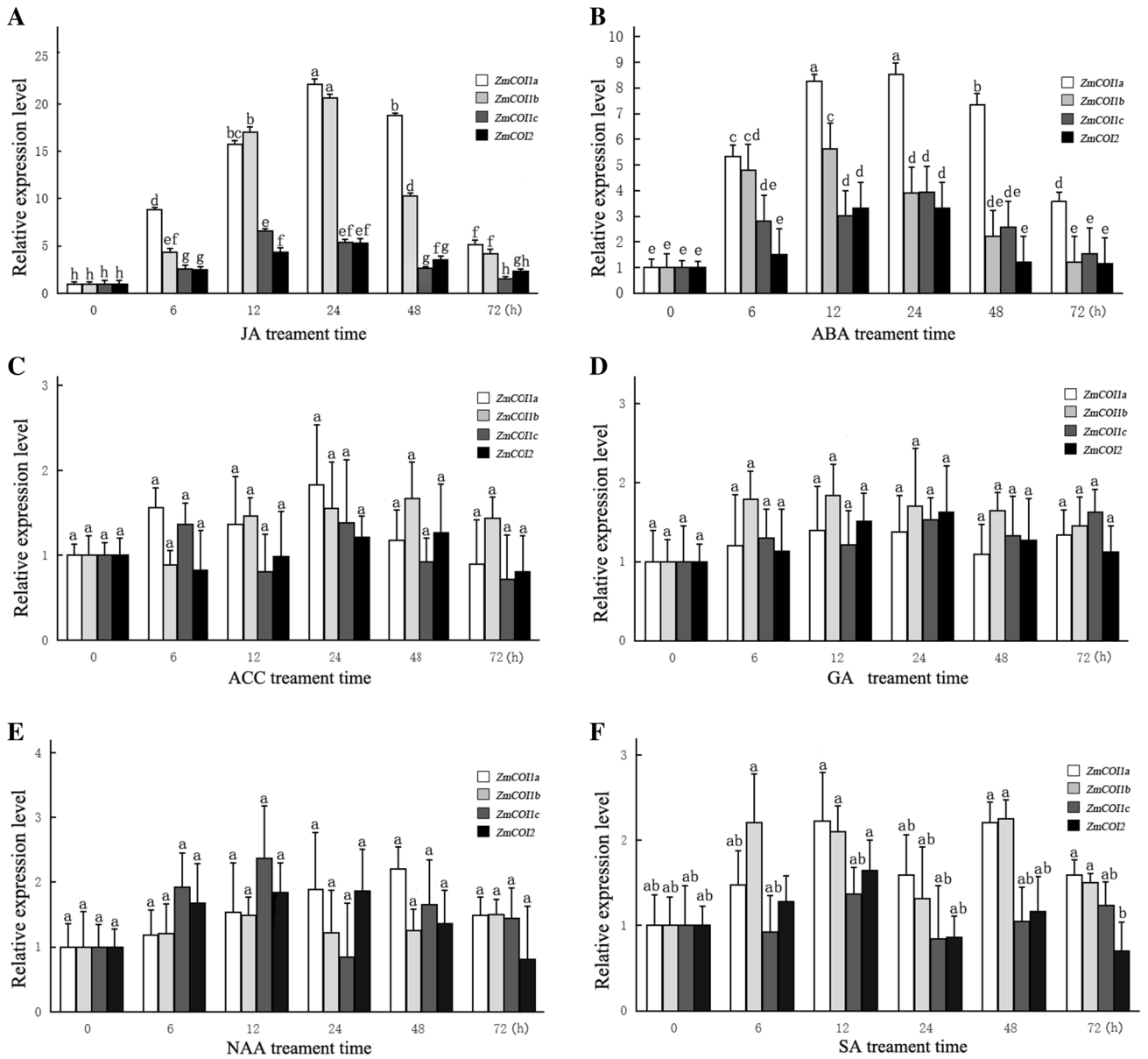

Fig. 3 The expression analysis of ZmCOIs in the leaf in response to hormone treatments. The hormone solutions of **a** jasmonic acid (JA, 100 μM), **b** abscisic acid (ABA, 100 μM), **c** 1-aminocyclopropane-1-carboxylic acid (ACC, 100 μM), **d** gibberellic acid3 (GA3, 100 μM), **e** 1-naphthylacetic acid (NAA, 100 μM). **f** Salicylic acid (SA, 2.5 mM) was sprayed to B73 seedlings at the V3 stage. The expression levels were detected by quantitative RT-PCR. Three biological replicates were performed. *EIF4α* was used as a reference gene. *Error bars* indicate the standard deviations (SD) of the mean value of three biological replicates. *Different letters* represent means statistically different ($p < 0.05$)

Over-Expression of *ZmCOI1a/b/c* in *coi1-1* Mutant Causes Immunity Recovery Against Necrotrophic Pathogen *Botrytis cinerea* and Oomycete Pathogen *Pythium aristosporum*

JA is one of the major defense hormones in plants (Browse 2009). The *Arabidopsis* JA biosynthesis mutant *aos* and JA signaling mutant *coi1-1* are highly susceptible to the necrotrophic pathogen *Botrytis cinerea* (Rowe et al. 2010). In this study, we tested whether *ZmCOIs* are able to complement the *coi1-1* mutant for its defense ability against *B. cinerea*. The transgenic plants (homozygous *coi1-1* transformed with one of *ZmCOIs*) as well as WT were inoculated with *B. cinerea* spore suspension. The results showed that the transgenic plants of *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* are highly resistant to *B. cinerea* as WT (Fig. 5), but the transgenic plants of *ZmCOI2* remained susceptible to *B. cinerea* just like the *coi1-1* mutant (Fig. 5), indicating that the transgene

Table 1 Fertile plant percentage of *coi1-1* homozygous plants transformed with *ZmCOIs*

Transgene	*Transgenic coi1-1* homo. plants[a]	Fertile plants[b]	Fertile plant %	Seeds/silique of fertile plants[c]
ZmCOI1a	22	8	36.3% (100)	15.6 ± 7.46 (16.3 ± 5.73)
ZmCOI1b	29	7	24.1% (100)	17.7 ± 5.23 (17.8 ± 3.64)
ZmCOI1c	32	5	15.6% (100)	17.4 ± 3.93 (16.9 ± 6.47)
ZmCOI2	27	0	0	0
Empty vector	18	0	0	0
WT				42.6 ± 9.34

The number in parentheses indicates the fertile plant percentage or the seed-bearing (seeds/silique) of the transgenic plants of the T_2 generation. *coi1-1/ZmCOI2* T_1 generation plants remain male-sterile and no T_2 plants were obtained

[a]The *coi1-1* homozygous segregants (T_1 generation) containing a *ZmCOI* gene were identified by PCR genotyping using primers p1&p2 and restriction enzyme Xcm I (Xie et al. 1998). *coi1-1* heterozygous plants were transformed by floral dip (Clough and Bent 1998) and one-quarter of T_1 plants (phosphinothricin-resistant) will be the *coi1-1* homozygous plants plus a *ZmCOI* transgene

[b]Fertile lines: if a transgenic *coi1-1* homozygous plant has 10% or more fertile flowers, it was seen as a fertile line. The fertility of flowers was observed under a microscope

[c]50 siliques of fertile lines were used to account for the seed-bearing rate (seeds/silique)

Fig. 4 The flower morphological phenotypes of *coi1-1* homozygous mutants transformed with *ZmCOI1a*, *Zm-COI1b*, *ZmCOI1c*, and *ZmCOI2*, respectively, at the T1 generation. **a** Inflorescences of 8-week-old plants of wild-type (*WT*) and transformants. **b** The flowers of 6-week-old plants of WT and transformants. **c** Fully developed siliques of WT and transformants. **d** The seeds in a siliques of WT and transformants

ZmCOI1a, or *ZmCOI1b*, or *ZmCOI1c* in transformants, but not *ZmCOI2*, imitates *AtCOI1* in the COI1-mediated defense response against necropathogens.

Pythium spp. are soilborne pathogens and are able to infect a wide variety of plants. The *Arabidopsis* triple mutant *fad3 fad7 fad8* cannot accumulate jasmonate and is extremely susceptible to root rot caused by the fungal root pathogen *Pythium mastophorum* (Vijayan et al. 1998). The *Arabidopsis* JA signaling mutant *jar1-1* is highly susceptible to a soil oomycete *Pythium irregular* (Staswick et al. 1998). To further demonstrate the function of *ZmCOIs* for defense response, we inoculated the transgenic plants with the suspension of the root pathogen *Pythium aristosporum* by dropping the suspension to the soil of each plant. Our results showed that the *Arabidopsis coi1-1* mutant is highly susceptible to *Pythium aristosporum* (Fig. 5) and WT is resistant to this pathogen. Over-expression of *ZmCOI1a, ZmCOI1b*, and *ZmCOI1c* in the *coi1-1* mutant provided resistance to the *coi1-1* mutant and *ZmCOI2* is not able to compensate the susceptibility to *P. aristosporum*, indicating again that *ZmCOI1a, ZmCOI1b*, and *ZmCOI1c* but not *ZmCOI2* are functional orthologues of

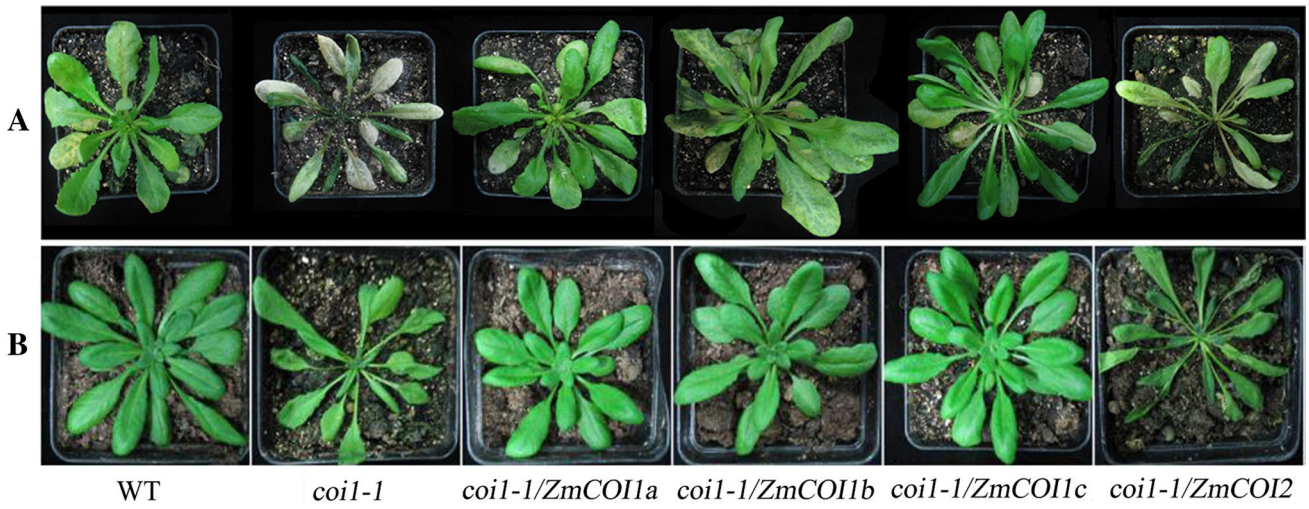

Fig. 5 The susceptibility of *coi1-1* transformants with *ZmCOI1a*, *ZmCOI1b*, *ZmCOI1c*, and *ZmCOI2*, respectively, to *Botrytis cenerea* and *Pythium aristospor*. **a** The 4-week-old plants of genotypes indicated were sprayed with spore suspension of *Botrytis cenerea*. The picture was taken at 72 h after inoculation. **b** Four-week-old plants of genotypes indicated were inoculated at the root system with a suspension of *Pythium aristosporum*. The pictures were taken at 72 h after inoculation

AtCOI1 in COI1-mediated defense against the soilborne pathogen *Pythium* spp.

Over-Expression of *ZmCOI1a/b/c* in *Arabidopsis coi1-1* Mutant Restores the Wound Response of JA-Dependent Genes

The *COI1* gene is required for expression of all the JA- or wound-inducible genes in *Arabidopsis* (Devoto et al. 2005). In this study, we tested the expression of a number of JA-dependent genes in *ZmCOIs* transformants. The results showed that the eight typical *COI1*-dependent genes chosen here are not inducible by wounding in the *coi1-1* mutant, but induced in WT (Fig. 6). All the eight genes are highly induced by wounding in the transformants of *ZmCOIa, ZmCOI1b*, and *ZmCOI1c* in the *coi1-1* background, but not in the *coi1-1/ZmCOI2* (Fig. 6), indicating that *ZmCOIa, ZmCOI1b*, and *ZmCOI1c* but not *ZmCOI2* can compensate the *coi1-1* mutation in *Arabidopsis* for the wound-induced defense response. These gene expression results supported that *ZmCOIa, ZmCOI1b*, and *ZmCOI1c* are the functional orthologous genes in maize to *AtCOI1* in *Arabidopsis* and *ZmCOI2* may not have the typical function of the SCF^{COI1} complex genes in defense and development processes.

Discussion

Identification of ZmCOIs and Orthologues in Other Monocots and Comparison Analysis with AtCOI1

In this study, we isolated and characterized the *COI1* orthologous genes in maize. Using the nucleotide sequence and amino acid (AA) sequence of the *Arabidopsis COI1* gene (*AtCOI1*) and rice *COI1* orthologues (*OsCOI1a*, *OsCOI1b*, and *OsCOI2*) we blasted the maize genome (http://www.MaizeGDB.org) and found four *COI1* orthologous genes in the maize genome. According to the nomenclature used in rice *COI1* orthologues (Lee et al. 2013), we designated the *COI1* orthologues of maize as *ZmCOI1a*, *ZmCOI1b*, *ZmCOI1c*, and *ZmCOI2*. This designation means that the first three of the four genes are the closest orthologues of *AtCOI1*, and the last one (i.e. *ZmCOI2*) is a less similar orthologue with *AtCOI1* than the first three but is still highly homologous to *AtCOI1*. To understand better why *Arabidopsis* just has one *COI1* gene but monocots, such as rice and maize, have many copies of *COI1*, we have carried out two analyses in this study: (1) how many copies of *COI1* orthologues are in other monocots? And (2) what is the homology among all the *COI1* genes in monocotyledonous and dicotyledonous plants? Using the AA sequences of ZmCOI1s and OsCOI1s, we blasted the genomes in the database Gramene (http://www.gramene.org/), and three *COI1* genes were found in *Brachypodium distachyon*, *Setaria italic*, and *Sorghum bicolor*, respectively. Using similar nomenclature to *OsCOIs* or *ZmCOIs*, we designated them as *BdCOI1a*, *BdCOI1b* and *BdCOI2* for the *Brachypodium*, *SiCOI1a*, *SiCOI1b* and *SiCOI2* for foxtail

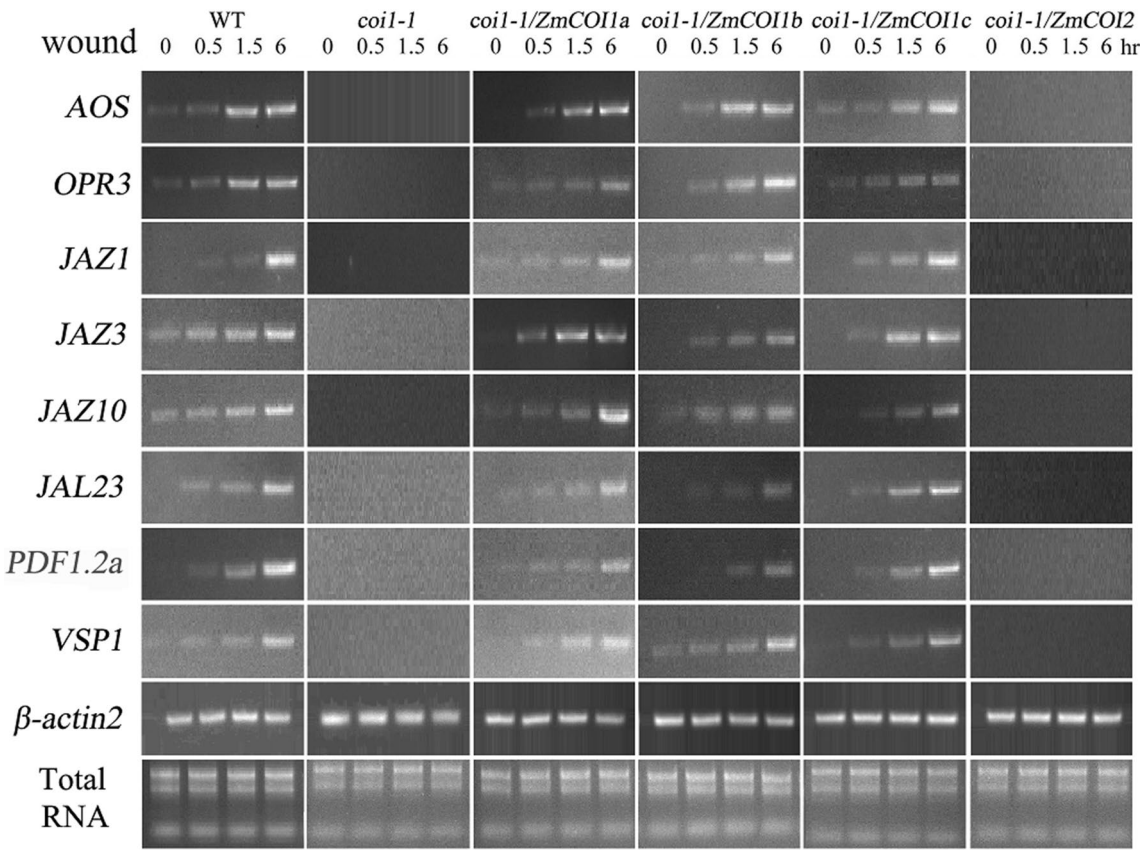

Fig. 6 The expression of a set of JA-dependent genes in *coi1-1* transformants with *ZmCOI1a*, *Zm-COI1b*, *ZmCOI1c*, and *ZmCOI2*, respectivel,y were detected by RT-PCR upon wounding. The leaves of the plants of the genotypes indicated were wounded with forceps and the mRNA levels of eight JA-dependent genes (AOS, OPR3, JAZ1, JAZ3, JAZ10, JAL23, PDF1.2a, and VSP1) were detected after wounding at the time points indicated. The accession numbers of the eight genes are listed in Table S1

millet and *SbCOI1a*, *SbCOI1b*, and *SbCOI2* for sorghum. Amino acid sequence alignment showed that AtCOI1 shares 53.9–57.6% AA identity with the COI1 orthologues in the five monocotyledonous species (Fig. S1), suggesting that, in the cereals, COI1 orthologues are substantially different from AtCOI1. Furthermore, we analyzed COI1 orthologues in the five monocots. The alignment showed that ZmCOI1s share more than 78% AA identity with OsCOI1s, BdCOI1s, SiCOI1s and SbCOI1s (Fig. S3). These results indicated that *COI1* genes in monocots are highly conservative. Especially, the *COI1* genes in maize and sorghum are so close to each other that they share more than 93% AA identity (Fig. S3).

Phylogenetic Analysis and Evolution of *COI1* Genes in Plants

To understand the evolutionary relationship of *COI1* genes in plants, the sequences of the COI1 orthologues in 36 plant species were searched and identified from a number of genomic databases (Gramene, PlantGDB, NCBI, Phytozome, Rice Genome Annotation Project, MaizeGDB). Phylogenetic analysis of these COI1 orthologues showed that all the AA sequences fall into two separate clades (Fig. 2). The first clade contains all the *COI1* genes from dicotyledonous species and the second clade from monocotyledonous plants (Fig. 2). Placing the AA identity of COI1s between maize and other species into plant evolutionary lineages (Fig. S9), we found that COI1 is a good marker for speciation of the evolutionary lineage. If the divergence time is earlier, the AA identity of COI1s between maize and a species is higher, and vice versa. For example, the divergence between maize and sorghum happened 13 Mya and the AA identity between them is about 94%, whereas between maize and *Brachpodium* is 48 Mya and the AA identity about 80% (Fig. S9). Monocotyledonous plants diverged from dicotyledonous at 140–150 Mya (Chaw et al. 2004) and the AA identity between maize and *Arabidopsis* is about 55% (Fig. S9).

In the phylogenetic analysis, we noticed that dicots have only one *COI1* gene but that monocots have 3 or more. This phenomenon may not be a divergence feature between dicots and monocots, and is just because of the limited sequences

of *COI1* genes in dicots. *Arabidopsis* (2n = 10, diploid) has a single copy of *COI1* (Lee et al. 2013). Tomato (*Solanum lycopersicon*, 2n = 24, diploid) also has a *JAI1* gene (*COI1* orthologue in tomato) (Li et al. 2004). Using *JAI1* and *AtCOI1* to blast the tomato genome in Gramene, we get only one gene, Solyc05g052620.2, which is a 100% match to *JAI1*, suggesting the tomato genome just has a single copy of the *COI1* gene. Wild tobacco (*Nicotiana attenuate*, 2n = 24, diploid) was found to have one *COI1* gene by Southern blot analysis (Paschold et al. 2007). A *COI1* gene has been isolated from cultivated tobacco (*Nicotiana tabacum*, 2n = 4x = 48, allotetraploid) (Shoji et al. 2008). Using *AtCOI* and *JAI1* as the query to blast the tobacco genome, we can obtain four loci orthologous to *AtCOI1* or *JAI1*, indicating that there are four *COI1* genes existing in the tobacco genome. For soybean (*Glycine max*, 2n = 40, paleotetraploid), there is a *COI1* gene (Wang et al. 2005). Searching the soybean genome in Gramene, we found four *COI1*-like genes (GLYMA11G34940, GLYMA18G03420, GLYMA14G06740, GLYMA02G42150) existing in the soybean genome. In *Brassica napus* (2n = 4x = 38, allotetraploid), there are 8 COI1-like genes (Wang et al. 2015). Putting all the information together, we can conclude that the diploid dicots have a single copy of the *COI1* gene, whereas the polyploid or paleopolyploid dicots may possess a number of *COI1* orthologues in their genomes. We also noticed that the monocotyledonous species applied here have three or more *COI1* orthologues. This must be because of the species evolution of the monocots. A number of studies for species evolution have concluded that all grass genomes are derived from a shared paleopolyploid ancestor ($n = 12$) (Eckardt 2008; Devos 2010; Zhang et al. 2012), which underwent further whole genome duplication events and nested chromosome fusion events to form the genomes of the cereals such *Brachypodium*, rice, maize, sorghum, wheat, etc. (Zhang et al. 2012). This means that the modern cereals must have the features of paleopolyploids, indicating that most genes in these genomes are duplicated or multiple-copied. Our data suggest that the paleopolypoidy of *Brachypodium*, rice, maize, sorghum and millet is the reason that they comprise several copies of *COI1* orthologues in their genomes.

The Conservative Function of ZmCOI Orthologues

Our major concern in this study is the function of the *ZmCOI1s*. Maize possesses four orthologues of *AtCOI1*. In this study, we applied the "mutant complementation" approach to characterize the function of the *ZmCOI1* genes. This approach has been successfully used for functional analysis of *GmCOI1* (Wang et al. 2005) and *OsCOI1s* (Lee et al. 2013). Here, we used this approach to carry out the functional analysis of *ZmCOI1s* by overexpression of a single *ZmCOI1* orthologue into the *Arabidopsis* mutant *coi1-1*, in which the *AtCOI1* function was completely lost (Xie et al. 1998). The *Arabidopsis* mutant *coi1-1* is impaired in JA responses, resulting in male sterility of flowers and insensitivity of roots and shoots to JA/MeJA treatment (Xie et al. 1998). Our results showed that *coi1-1* transformants with overexpressed *ZmCOI1a*, *ZmCOI1b* or *ZmCOI1c* are male-fertile but transformants with *ZmCOI2* remain male-sterile (Fig. 4), indicating that *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* have a similar function as *AtCOI1*, while *ZmCOI2* may not be a functional *COI1* orthologue in maize. Furthermore, necrotrophic pathogen assays and caterpillar feeding assays showed that *coi1-1* transformants with overexpressed *ZmCOI1a*, *ZmCOI1b* or *ZmCOI1c* have higher resistance to the pathogen fungi and chewing insect *Spodoptera exigua* than *coi1-1* mutants, but *coi1-1* transformants with *ZmCOI2* are just like *coi1-1* mutants, which are highly susceptible to the pathogen *B. cinerea* or the insect *S. exigua* (Fig. S7). These results further indicated that *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c* function similar to *AtCOI1* for JA signaling, but *ZmCOI2* does not show the function of *AtCOI1* in *Arabidopsis*. *ZmCOI1a*, *ZmCOI1b*, *ZmCOI1c*, and *ZmCOI2* shared about 55% AA identity with *AtCOI1*. Interestingly, *ZmCOI2* has just 1.8% less AA identity than *ZmCOI1b* or *ZmCOI1c* and it cannot complement the *Arabidopsis coi1-1* mutant. Our results are in accordance with results from a *OsCOI1s* study (Lee et al. 2013), which showed overexpression of either *OsCOI1a* or *OsCOI1b* in the *Arabidopsis coi1-1* mutant resulting in the restoration of JA signal transduction and successful complementation of the *coi1-1* mutation phenotypes, but *OsCOI*2 was not able to successfully complement the *coi1-1* mutant, although the OsCOI2 protein interacted with a few OsJAZs (Lee et al. 2013). Lee et al. (2013) showed that the H391 of the OsCOI2 protein is a crucial site of OsCOI2 function divergence. They further showed that a single mutation that replaced the conserved His (H)-391 residue of the OsCOI2 protein with a Tyr (Y)-391 enabled *OsCOI2* to complement the *coi1-1* mutant (Lee et al. 2013). Similarly, our results showed that *ZmCOI2* cannot complement the phenotypes of the *coi1-1* mutant. However, "H391"does not exist in ZmCOI2 (the corresponding site of H391 in ZmCOI2 is Y, not H) (Fig. S8). And the corresponding site of "H391" in ZmCOI1a, ZmCOI1b, and ZmCOI1c are Y, not H (Fig. S8). Therefore, the hypothesis that H391 of OsCOI2 significantly changed the protein function does not applied to ZmCOI2. For ZmCOI2 function, we have a similar hypothesis that a single amino acid substitution can significantly change the protein physiological function. With this hypothesis, we analyzed the ZmCOI2 AA sequence with all the *coi1* alleles in *Arabidopsis* which have been reported (He et al. 2012; Huang et al. 2014). This analysis showed that none of the AA changes in the *coi1* alleles is equal to the amino acid substitution in ZmCOI2 in comparison to *ZmCOI1a*, *ZmCOI1b*, and *ZmCOI1c*.

However, the *coi1-7* site may be important for ZmCOI2. The *Arabidopsis coi1-7* allele is a loss-of-function mutant caused by an amino acid substitution: G155E. The corresponding site of *coi1-7* in the ZmCOI2 or OsCOI2 protein is S instead G (Fig. S8), indicating that, if ZmCOI*2* evolved a divergent function rather than mediating JA signaling, this S may be an important amino acid residue which might significantly change the function of *ZmCOI2*. Noticeably, further studies are needed to elucidate the biological function divergence of *ZmCOI2* as well as other *COI2* genes in cereals.

In conclusion, four *ZmCOIs* were identified from the maize genome and their functional features have been characterized in this study. JA is an essential phytohormone that controls many aspects of plant development and defense in response to endogenous developmental cues and environmental stimuli. COI1s are one of the critical components for JA signal perception in response to diverse stimuli. Our results provided evidence that *COI1* genes may be functionally conserved in monocotyledonous and dicotyledonous plants, suggesting that *COI1* genes from different plant species share similar basic functions for JA signal transduction. In addition, it is possible that some orthologues of plant *COI1* genes, such as *ZmCOI2* and *OsCOI2*, may have evolutionarily diverged and gained new functions for other signal transductions rather than JA signaling.

Accession Numbers of Genes

The *ZmCOI1a*, *ZmCOI1b*, *ZmCOI1c*, and *ZmCOI2* genes can be found in the dababase of http://www.maizeGDB.org or http://www.gramene.org by their B73_RefGen_v3 (or v4) version ID numbers: *ZmCOI1a*, GRMZM2G125411 (or Zm00001d042833); *ZmCOI1b*, GRMZM2G151536 (or Zm00001d010082), *ZmCOI1c*, GRMZM2G353209 (or Zm00001d038273), and *ZmCOI2*, GRMZM2G079112 (or Zm00001d028543). The accession numbers of other genes in Genbank used in this study were listed in Table S1.

Acknowledgements This work was supported by the National Natural Science Foundation of China (31571580), the Fundamental Research Funds for the Central Universities (KYTZ201402 and KYRC201404), the Outstanding Scientific Innovation Team Program for Jiangsu Universities (2015), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We are grateful to Dr. Chunhao Jiang (Nanjing Agricultural University, Nanjing, China) who provided the culture of *Bortytis cinerea* and to Dr. Daoxin Xie (Tsinghua University, Beijing, China) who provided the *coi1-1* heterozygous *Arabidopsis* for the experiments of this study. We greatly thank Dr. Muhammad Aslam, University of Agriculture Faisalabad, Pakistan, for proof-reading the manuscript.

Author Contributions The work presented here was carried out in collaboration among all the authors. LA performed the experiments, analyzed data and contributed to write the manuscript. YY designed the project, analyzed data, wrote the manuscript and obtained funds to support the project. RMA contributed to the experiment performance and the manuscript writing. HR and JQ helped to perform the experiments. All authors have read and approved the final manuscript.

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