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Isolation, characterization and expression analysis of the genes—*GhAOS*, *GhAOC* and *GhOPR3*: Encoding the key enzymes involved in jasmonic acid biosynthesis in *Gladiolus hybridus*

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ABSTRACT

Jasmonic acid (JA) plays a regulatory role in plant development such as tuber and corm formation and their responses to environmental stresses. The action of JA in regulating plant growth and stress responses often requires the elevation of endogenous levels by *de novo* synthesis. Allene oxide synthase (AOS), allene oxide cyclase (AOC) and 12-oxo-phytodienoic acid reductase 3 (OPR3) are the key enzymes involved in jasmonic acid biosynthesis in plants. Here, we isolated the AOS, AOC and OPR3 genes (*GhAOS*, *GhAOC* and *GhOPR3*) in full length from *in vitro* developing corms of *Gladiolus hybridus*. The subcellular localization analysis indicated that both GhAOS and GhAOC were chloroplast localization proteins whereas GhOPR3 was localized within the peroxisome. Real-time quantitative PCR showed that *GhAOS*, *GhAOC* and *GhOPR3* genes were expressed constitutively in all organs with different levels having a relatively higher level in corms and cormels. Additionally, we observed that MJ treatments contributed to increasing the amount of three genes, mRNA level and endogenous MJ content thus promoting corm formation and enlargement. These results revealed that *GhAOS*, *GhAOC* and *GhOPR3* were genes of significant importance and their expression contributed to JA biosynthesis thus playing an important role in corm formation and enlargement.

1. Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as JAs, constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues (Balbi and Devoto, 2008; Turner et al., 2002; Wasternack, 2007). These signaling molecules affect various plant processes including fruit ripening (Creelman and Mullet, 1997), production of viable pollen (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000), root elongation (Staswick et al., 1992), tendril coiling (Devoto and Turner, 2003), tuber enlargement (Kolomiets et al., 2001), corm formation (He et al., 2008; Lian et al., 2011), response to wounding (Zhang and Turner, 2008), abiotic stresses, defense against insects (McConn et al., 1997) and necrotrophic pathogens (Thomma et al., 1999).

In plants, oxylipin biosynthesis starts with the release of α-linolenic acid (α-LeA) from chloroplast membranes (Wasternack, 2007). This fatty acid can be metabolized by the action of 13-lipoxygenase (13-LOX) producing (13*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT). The first step in JA biosynthesis is carried out by an allene oxide synthase (AOS) leading to an unstable allene oxide. This intermediate is converted into (9*S*, 13*S*)-12-oxo phytodienoic acid (OPDA) by an allene oxide cyclase (AOC). The subsequent step, reduction of the cyclopentenone ring, is catalysed by an OPDA reductase 3 (OPR3). Three rounds of β-oxidative side-chain shortening starting with 3-oxo-2-(pent-2′-enyl)-cyclopentane-1-octanoic acid (OPC-8) *via* 3-oxo-2-(pent-2′-enyl)-cyclopentane-1-hexanoic acid (OPC-6) and 3-oxo-2-(pent-2′-enyl)- cyclopentane-1-butanoic acid (OPC-4) are achieved for the synthesis of JA (Meinicke et al., 2008).

Conversion of 13-HPOT, the first step in JA biosynthesis, is carried out by 13-AOS, a cytochrome P450 enzyme, leading to an unstable allene oxide (Vick and Zimmerman, 1987). AOSs belong to the family of CYP74A enzymes, which are independent from molecular oxygen and NADPH, exhibit low affinity to CO and use the hydroperoxide group as a source for reducing equivalents and oxygen (Song et al., 1993; Feussner and Wasternack, 2002; Howe and Schilmiller, 2002). Both transcript and protein levels of the *Arabidopsis* AOS increased after wounding or treatment with

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jasmonates (Laudert et al., 1996; Laudert and Weiler, 1998). A knock-out mutant defective in CYP74A was isolated (Park et al., 2002), which contained reduced amounts of JA and was male sterile. Male sterility could be rescued by exogenous application of methyl jasmonate or by complementation with constitutive expression of the wild-type *AOS* gene—a gene that regulates JA synthesis.

In addition, a crucial step in the biosynthesis of JA is the formation of its stereoisomeric precursor, cis-(+)-12-oxophytodienoic acid (OPDA), which is catalyzed by AOC (EC 5.3.99.6). *In vivo*, it occurs specifically forming the 9 (*S*), 13 (*S*)-12-oxo-PDA isomer. Since the halflife of the intermediate allene oxide is very short, association of AOS and AOC is likely to occur (Léon and Sánchez-Serrano, 1999). Among the JA biosynthetic enzymes, AOC is important, since this enzyme establishes the naturally occurring enantiomeric form of jasmonate (Ziegler et al., 2000).

OPR3 is a flavin mononucleotide (FMN)-dependent oxidoreductase that catalyzes the reduction of the cyclopentenone (9*S*, 13*S*)-12-oxophytodienoate [(9*S*, 13*S*)-OPDA] to the corresponding cyclopentanone in the biosynthesis of the plant hormone jasmonic acid (Breithaupt et al., 2009). The JA-deficient phenotype of OPR3 loss-of-function mutants in *Arabidopsis* (Sanders et al., 2000; Stintzi and Browse, 2000) and tomato (Stintzi and Bosch, unpublished) indicates that other OPR isoforms cannot substitute for OPR3 in JA biosynthesis.

All genes encoding enzymes of JA biosynthesis are JA-inducible (Wasternack, 2006), and promoters analysed so far increase their activity upon JA treatment (Sanders et al., 2000). which leads to the suggestion that JA biosynthesis is regulated by a positive feedback.

Gladiolus hybridus cultivar 'Rose Supreme' is the most widely planted flower bulbs in China. The cultivar is propagated through corms and cormels. However, repeated cycles of vegetative propagation in the field resulted in decline in the performance of cultivars. Therefore, lifespan of such commercially important cultivars becomes limited, which can be extended by maintaining disease-free propagules in culture. A large number of plants/propagules can be produced in a relatively shorter time from disease-free cultures. In view of this, a large number of studies have concentrated on *in vitro* propagation of *Gladiolus* using micropropagation techniques for cormel formation.

At present, the growth and development characteristics of corms, stolons and cormels of *Gladiolus* in the field have been made clear (Chen and Yi, 2004), while the mechanism of the formation and enlargement in *Gladiolus* corm is still unclear. In our previous research, we found that JAs had important effects on the corm formation and enlargement of *Gladiolus*. It suggested that *GhLOX1* might regulate the growth and development of *Gladiolus* corm through affecting JAs biosynthesis cycles and thus resulting in carbohydrate accumulation (He et al., 2008). However, relatively little is known about the induction processes of JA biosynthesis and its role in corm formation of *Gladiolus*. Furthermore, AOS, AOC and OPR3 are the key enzymes involved in JA biosynthesis, and *AOS*, *AOC* and *OPR3* genes might regulate the growth and development of *Gladiolus* corm through affecting JAs biosynthesis cycles. In this study, we isolated three full-length cDNA of *AOS*, *AOC* and *OPR3* from *G. hybridus* cultivar 'Rose Supreme'. Furthermore, the *GhAOS*, *GhAOC* and *GhOPR3* expressions both in different organs and under MJ treatments were investigated using real time quantitative RT-PCR, which correlated positively to endogenous MJ content. At the same time, the promotion treatment of different MJ concentrations confirmed that the expression of *GhAOS*, *GhAOC* and *GhOPR3* might promote corm formation by increasing endogenous MJ content. This work might provide a basis for promoting the trait of corm formation and enlargement of *G. hybridus* by gene engineering.

2. Materials and methods

2.1. G. hybridus growth condition and stress treatments

G. hybridus cultivar 'Roses Supreme' was planted in the scientific garden of China Agricultural University, Beijing. Different samples [leaf (tender leaf), flower (petal), root, stolon, corm and cormel] were taken 25 weeks after planting to detect *AOS*, *AOC* and *OPR3* genes expression and endogenous MJ content. The cormels of 'Rose Supreme' (diameter: 0.6–1.0 cm) were used as explants, which were harvested and stored in refrigerators at 4 °C to break dormancy. After regular disinfection, explants were cultivated on primary culture medium of MS + 0.5 mg/L NAA + 2.0 mg/L 6-BA + 60 g/L sucrose for 4 weeks, followed by 4 weeks of plantlet culture on secondary MS culture medium. A group of plantlets from secondary culture with similar height were selected for corm induction. The basic culture medium was MS + 60 g/L sucrose. MJ: 0.1, 0.2 and 0.5 mmol/L. The MS culture medium was used as a control.

2.2. Cloning of AOS, AOC and OPR3 from Gladiolus

The total RNA was extracted from the developing corms of *Gladiolus in vitro* with MyLab RNA extraction reagent box (Mylab, China), and cDNA was synthesized by Superscript II (Invitrogen, USA) with oligo nucleotide AP1.

The *AOS* cDNA fragment was amplified by the degenerative primers of AOSPF1 and AOSPR1 (all primers sequence shown in Table 1 in supplementary material). The single PCR product was subcloned into pMD18-T (TaKaRa, Japan) for sequencing. The cDNA fragment sequence was used to design the forward gene-specific primers (AOSGSP1 and AOSGSP2) and the reverse gene-specific primers (AOSGSP3 and AOSGSP4) for 3′-RACE and 5′-RACE PCR (TaKaRa, Japan), respectively. For the 3′ sequence of this gene, two adaptor primers AP2 and AP3 were designed to apply nested PCR with gene-specific primers (AOSGSP1 and AOSGSP2), respectively. AOSGSP3 and AOSGSP4 were used for 5′-RACE primers. The RACE reactions were performed according to the manufacturer's protocol (TaKaRa 5'-Full RACE kit, Japan). A single full-length, cDNA sequence was formed by combining the 5′-RACE fragment and 3′-RACE fragment. Finally, forward primer AOSGSP5 and reverse primer AOSGSP6 were used to amplify the entire *AOS* coding sequence. The thermal cycling conditions were shown in Table 2 in supplementary material. After testing by agarose gel electrophoresis, the PCR products were ligated into pMD-18T vector for sequencing. Then sequences of nucleotides and deduced protein were used as query for blast researches in NCBI for further analysis.

AOC and *OPR3* genes were isolated from the developing corms of *Gladiolus* as described previously. All primers and PCR thermal cycling conditions in this study were shown in Tables 1 and 2 in supplementary material, respectively.

2.3. Multiple alignment and phylogenetic analysis

Alignment and phylogenetic analyses were carried out using DNAStar software (Madison, USA). The phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal method using DNAStar software. Chloroplast targeting of the GhAOS and GhAOC protein were assessed by the computer analysis of the first 100 amino acids using the ChloroP version 1.1 (http://www.cbs.dtu.dk/services/ChloroP) (Emanuelsson et al., 2003) and the TargetP program version 1.1 (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al., 2000).