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Full Length Research Paper

Development of adjustable male sterile plant in broccoli by antisense *DAD*1 fragment transformation

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Male sterile line is very important in hybrid seed production in broccoli (*Brassica oleracea* L. var. *italica*). A fragment of *BoiDAD*1F was amplified from this specie using primers designed against the conserved *DAD*1F fragment of *DAD*1 genes in *Arabidopsis thaliana* and *Brassica rapa*. The *BoiDAD*1F sequence was 88 and 99% similar to the homologous genes in *Arabidopsis* and *B. rapa*, respectively. The antisense *BoiDAD*1F was then cloned onto pBI 121 vector to make the transformation construct, pBI-antiBoiDAD1F. Cotyledons with petiole were inoculated with *Agrobacterium tumefaciens* harboring the pBI-antiBoiDAD1F plasmid. Positive transformation was confirmed using polymerase chain reaction (PCR) and Southern blots. Twenty individual transformants were obtained, of which 15 showed suppressed DAD1 mRNA level. Pollens produced by the male sterile transgenic plants germinated poorly (≤ 10%). No seeds were produced on the flowering transgenic plants, and the siliquae were empty, or the few shrinked seeds, which failed to germinate. When pollinated with wild type plant pollen, the siliquas of the transgenic plants enlarged to produce normal seeds. No morphological differences were observed between the transgenic and wild type plants, except a few abnormal flowers on transgenic ones. To restore the fertility, exogenous JA were applied by dipping the floral buds into an aqueous solution containing 500 µM MeJA and 0.05% Tween 20 after removal of the opened flowers on the inflorescence. The treated flower produced normal pollens which germinated on the medium, and were fertile. T1 population consisted of fertile and sterile plants in the ratio of 3:1. The T2 population comprised of both complete fertile and sterile lines. These results confirmed the Mendel model of separation of the antisense RNA insert in the transgenic plants.

Key words: *Brassica oleracea* L. var. *italica,* DAD1, adjustable male sterility, jasmonic acid and antisense RNA.

NTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica*) is an important vegetable crop in the *Brassica* family. Due to lack of good male sterile line to be used for producing F_1 seeds, self-incompatibility is the only option presently in use. However, the self-incompatible line is very weak in vigour leading to an urgent need to develop male sterile plant materials. Previously, *Barnase* was used to create male sterile plants. For the leafy vegetable species, suppressing expression of this gene can restore fertility. However, the offsprings of the self- pollinated male sterile line can only maintain 50% male sterility. The other 50% fertile plants have to be mechanically removed. Konagaya et al. (2008) obtained efficient production of genetically engineered, male-sterile *Arabidopsis thaliana* using anther-specic promoters and genes derived from *Brassica oleracea* and *Brassica rapa*. There is also the problem of barnase gene. These systems cannot be used to produce F_1 seeds.

The *DAD*1 (defective in anther dehiscence1) gene in *A. thaliana* encodes for a phospholipase A1 (PLA1), which

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Abbreviations: *DAD*1, Defective in anther dehiscence1; **PLA1,** phospholipase A; **JA,** jasmonic acid; **CTAB,** cetylmethylammonium bromide; **PCR,** polymerase chain reaction; **MS,** Murashige and Skoog; **NAA,** naphthaleneacetic acid; **ABA,** abscisic acid; **NAA,** naphthaleneacetic acid; **6-BA,** 6-benzylaminopurine; **IAA,** indole-3-acetic acid; **AOC,** allene oxide cyclase; **NCBI,** national center for biotechnology information; **BLAST,** basic local alignment search tool.

#These authors contributed equally to this paper

catalyses the conversion from phospholipid into linolenic acid, the first step in the biosynthesis of jasmonic acid (JA). Ishiguro et al. (2001) inserted a T-DNA transposon into the gene and obtained a mutant *dad*1, which is male sterile. Further examination found that *dad*1flower buds contained 22% of JA content compared to the wild type plants. Exogenous application of JA or its precursor linolic acid completely restored the fertility (McConn and Browe 1996; Mandaokar et al., 2003). However, it has not been attempted to use the antisense *DAD1* expression vector for production of male sterile lines, followed by exogenous JA application to restore fertility.

In *A. thaliana*, mutation or defective in anther dehiscence can cause male sterility. This study initially cloned the conserved region of the homologues DAD1 gene, and the DNA sequence was used to make antisense expression construct, which was introduced into the maternal plants of elite F1 plants. The DAD1 gene regulates the biosynthesis of JA which control male organ development, and lowering JA content through introduction of antisense DAD1 gene fragment could lead to male sterility. During floral season of the self-pollinated male sterile plants, spraying JA could restore fertility enabling the production of seeds. Since fertility is restored by exogenous JA supplement, the genes causing male sterility will not be affected. The next generation was still not able to synthesize JA, and consequently remain 100% sterile. Such maternal line can be used for producing F1 seeds.

MATERIALS AND METHODS

Materials

Broccoli cv. Lvzhou with self-compatible character was used in this study. The *Escherichia coli* strain was DH5α, *Agrobacterium tumefaciens* strain was EHA105, and plant expression vector was pBI121. These materials were stored in the laboratory.

Primers design

The *DAD*1 sequences were retrieved for the genes cloned from *A. thaliana* (GenBank accession At2g44810) and *B. rapa* (GenBank accession AB073401) (Ishiguro et al. 2001). A conserved region of 700 bp in length from 402 to 1084 nucleotide was used to design the following primers: Forward primer P1 5′-GAATGGACACGTG GAGCTCAC-3′, and reverse primer P2 5′-GTCATCTCCTCCC GTGGAACC-3′.

Cloning *DAD1* fragment

DNA was extracted from leaf tissues using the cetylmethylammonium bromide (CTAB) method. Polymerase chain reaction (PCR) done was using the following condition: 35 cycles of 94°C 1min, followed by 50°C 1min, and then 72°C 2 min, with a final extension at 72°C for 10 min. After purification, the PCR products was ligated onto the pMD18-T vector (TaKaRa) and transformed into *E. coli* cells. Six positive clones were sequenced, and the sequences were compared to the nucleotide sequences that were deposited in National Center for Biotechnology Information (NCBI) GeneBank (BLAST, basic local alignment search tool) to determine the orientation of the cloned fragment. In addition, the cloned sequence was compared for their similarity with reported genes in the BLASTN program.

Construction of the antisense *DAD*1F expression vector and genetic transformation

Plasmid from the positive clones with antisense orientation was isolated, and double digested with *Xba* I and *Sma* I. The purified fragments were subcloned onto the pBI 121expression vector which was digested with the same enzymes to make the antisense expression construct.

For genetic transformation, the sterile cotyledons from 4-5 old seedlings were inoculated with *A. tumefaciens*. The pre-culture and co-culture medium was Murashige and Skoog (MS) + 0.2 mg·L^{-1} naphthaleneacetic acid (NAA) + 6 mg·L^{-1} 6-benzylaminopurine (6-BA) + 4 mg·L^{-1} $AgNO_3$ + 0.25 mg·L^{-1} abscisic acid (ABA). The culture medium was MS + 0.2 mg·L^{-1}NAA + 6 mg·L^{-1}6-BA + 4 mg·L^{-1} $AgNO_3$ + 0.25 mg·L^{-1} ABA + 200 mg·L^{-1} Cb. The selection medium was MS + 0.2 mg·L^{-1}NAA + 6mg·L^{-1}6-BA + 4mg·L^{-1} $AgNO_3$ + 0.25 mg·L^{-1} ABA + 200mg·L^{-1}Cb + 5.0 mg·L^{-1} Km ; and the rooting medium was MS + 0.2 mg·L^{-1} NAA + 0.1 mg·L^{-1} indole-3-acetic acid (IAA) + 5.0 mg·L^{-1} Km + 200 mg·L^{-1} Cef.

Molecular detection of the transgenic plants

The vector pBI 121 contains a *GUS* gene, which can be used to detect the transgenic plants. The positive plants should yield a 371 bp long PCR fragment. The primers for amplification of *GUS* gene were (Malek et al., 2002) P3: 5′-ACG TCC TGT AGA AAC CCC AAC C-3′, and P4: 5′-TCC CGG CAA TAA CAT TAC GGC GT-3′. Leaf tissues (3-5 g) were collected from single plant, and DNA was extracted using CTAB method, and used to amplify the GUS gene with P3 and P4 primer pair. The putative transgenic plants which produced the expected PCR fragment was used to extract DNA which was digested with *BamH*I at 37°C for 12 h. The digestion products were separated on agarose gel followed by transferring onto nylon membrane for Southern blot hybridization.

Determination of the fertility of the transgenic plants

At the flowering stage, the morphological characteristics of the floral organs of the transgenic plants were observed. Pollens were collected and cultured on 1% agarose medium containing 10% sucrose, 100 mg·kg^{-1} boric acid and 5 mg·kg^{-1} giberrelic acid. The ratio of germinated pollens under *in vivo* condition was used to determine pollen viability (Liu, 1985).

Restoration of fertility of the transgenic plants

After removal of the opened flowers on the transgenic plants, the floral buds were soaked for 1-2 min in 500 µM methyl jasmonate which was dissolved in 0.05% Tween 20 aqueous solutions. The pollen germination and fertilization on stamens and medium plate were observed after 3 days.

RESULTS

Cloning and sequence analysis of the *DAD*1 homologous fragment in broccoli

A DNA fragment of the expected 700 bp in length was

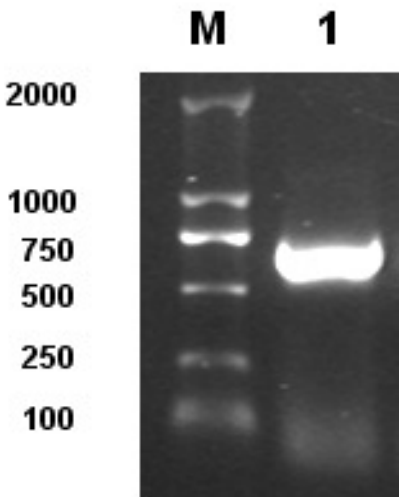

Figure 1. Detection of the PCR-amplified *DAD*1 fragments on agarose gel. M,marker DL2000; 1, *BoiDAD*1F from broccoli.

amplified from the broccoli plants (Figure 1). The band was gel purified, ligated onto vector and transferred into *E.coli* cell. The double enzyme digestion of the plasmid was used to confirm the recombinant. At the same time, 6 positive colonies were used for sequencing.

Sequence analysis result shows that the length of the insert was 678 bp. When searched in NCBI database, the cloned sequence was highly homologous to the *DAD*1F genes in *A. thaliana* and *B. rapa*, the similarity level was 88 and 99% respectively. These results confirmed that cloned frag-ment was a partial sequence of a homologous *DAD*1 (DAD1F) gene in broccoli. The clone was named *BoiDAD1F*. When the deduced amino acid sequence was compared to the *DAD*1 protein in *A. thaliana*, the cloned *BoiDAD1F* contained the signature contig GHSLG (83-87) as well as the amino acid residues of the catalytic triad, such as the serine (S 85), asparatic acid (D 142) and histine (H 208 or H 215). These results confirmed the existence of PLA1 in broccoli. The cloned sequence contained the signature fragment and catalytic centre of the lipase.

*DAD*1 protein catalyzes the first step in JA biosynthesis to convert phospholipid into linolic acid. Sequence alignment revealed that the gene sequences were very similar with very low degree of variation. This indicates that JA biosynthetic pathway is highly conserved in plants, especially among different *Brassica* species where expression of the key enzymes for JA metabolism is highly homogenous.

Construction of the antisense *DAD*1F expression vector and genetic transformation

The recombinant plasmid was enzyme digested to obtain the 700 bp fragment. Sequence analysis confirmed that the *BoiDAD1*F was inserted into pBI121 in both orient-tations. The antisense DAD1F expressions construct pBI-antiBoi *DAD*1F was selected to transform plants. For the transformation, a total of 20 plants were selected using kanamycin antibiotics. The putative transgenic and non-transgenic plants were all transferred into plastic greenhouse for field test.

Molecular detection of transgenic plants

A PCR reaction was conducted to amplify the GUS gene using P3 and P4 primer pair. The antisense pBI-antiBoi *DAD*1F plasmid DNA was used as the positive control, and the wild type leaf DNA was the negative control. When examined on agrose gel, no bands should be shown for the negative plants; those producing different sized bands compared to the positive control should be false positive. Only the putative transgenic plants that produced the same sized fragment as the positive control were positive transgenic plants (Figure 2). The PCR analysis provides the preliminary evidence for the putative antisense *BoiDAD*1F transgenic plants. A total of 15 such plants were obtained.

Next the *GUS* gene fragment was used as probe for Southern blot to validate the putative transformation. All the transgenic plants confirmed by PCR were also validated as positive by Southern blot analysis (Figure 3). These results indicate that the DNA fragment has been integrated into the genome of the transgenic plants. A total of 15 plants transformed with antisense *BoiDAD*1F were obtained. Most of them contained 2 copies of the insert such as the plant No. 4-10 in Figure 3. RT-PCR analysis showed that the expected band did not appear in 11-14 of the transgenic plants, suggesting that transcription of the foreign gene was not active in those plants (Figure 4). The other 12 plants had the bands same as the positive control, transcription of the antisense *BoiDAD*1F happened in these transgenic plants.

Detection of fertility in transgenic plants

The six plants, No 4, 6, 8, 9, 10 abd 12 that have been selected by RT-PCR bloomed normally, but the floral organs all appeared abnormal (Figure 5). The control plants produced uniform and well developed pollen, and the pollen germination rate was over 85%. However, the transgenic plants were seen to have the following symptoms: Degenerated anther, empty anther without pollen, or pollens of various sizes, shrunken pollen and underdeveloped, and pollen with low viability. The pollens could not germinate.

Among the transgenic plants bearing under developed flowers, 4 of them failed to bear any siliqua, or the siliquae were empty with no seeds. These plants were

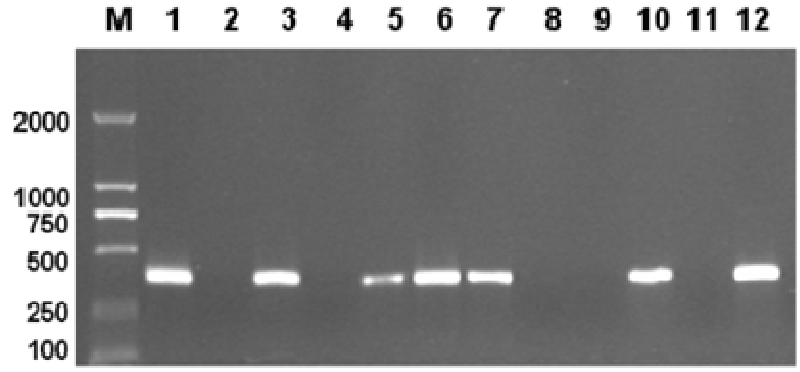

Figure 2. PCR analysis of the *GUS* gene in the transgenic *B. rapa* L. ssp *chinensis* var.*utilis* Tsen et Lee. M, DL2000; 1, positive control (plasmid PCR); 2, negative control (control plant); 3, 5, 6, 7, 10, 12, positive plants; 2, 4, 8, 9, 11, negative plants.

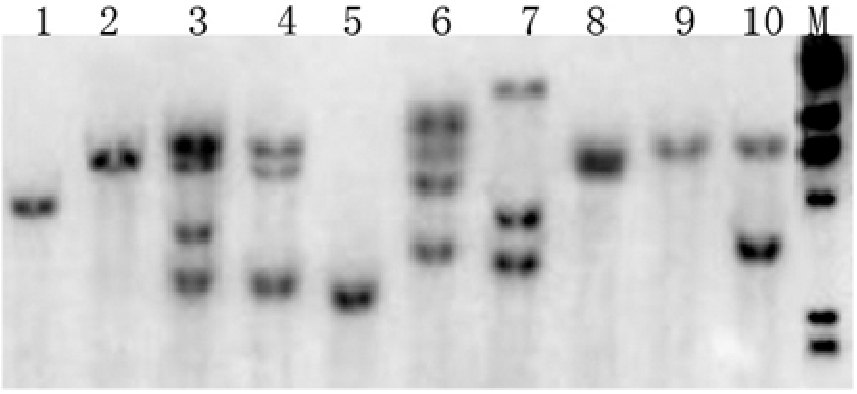

Figure 3. Southern blott of transgenic plant. M: Molecular marker; 1-10, transformed plant

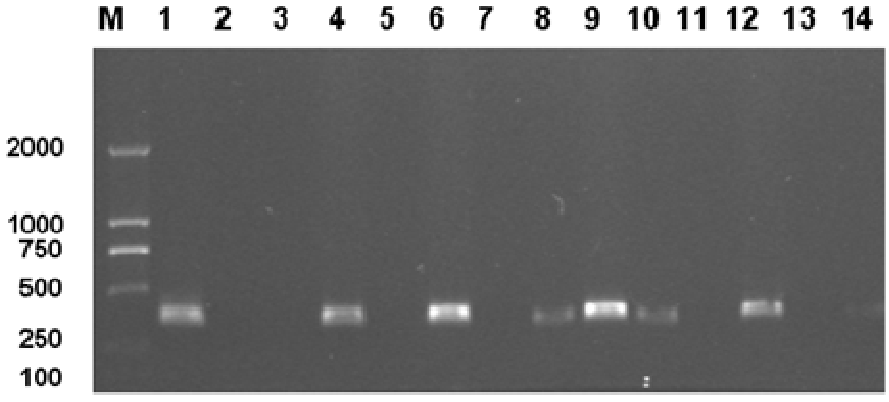

Figure 4. RT-PCR analysis of the transgenic plants. M: Marker; 1, positive control; 2, negative control; 3 - 14, transgenic plants.

sterile. Most of the ovaries did not enlarge. The few enlarged ovaries still could not bear seeds. The possible reasons why the seeds could not germinate include that interference of JA biosynthesis which disturbed the physiochemical metabolism of the seeds, and hence resulted in the loss of germination ability. Secondly, the

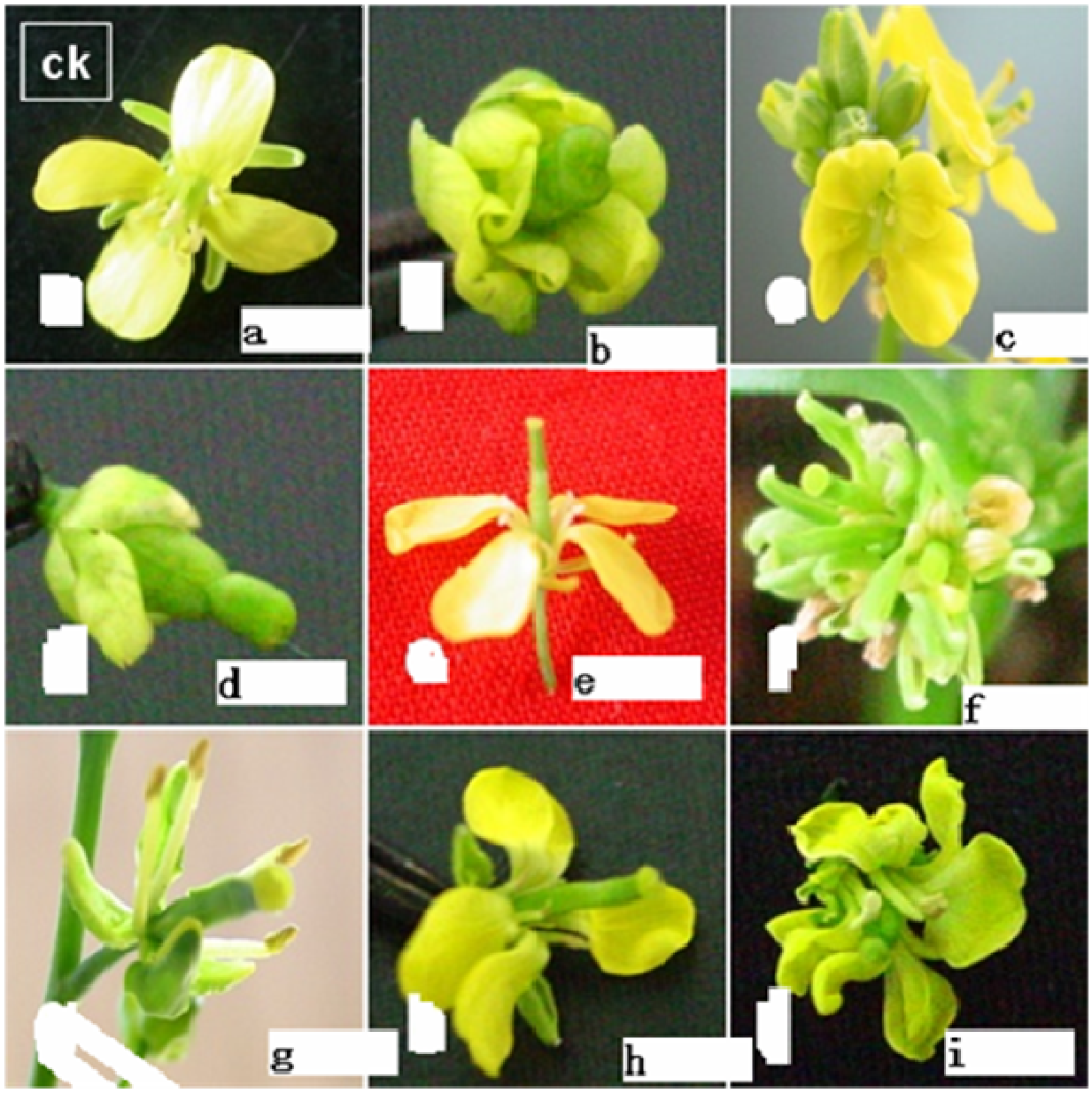

Figure 5. Flower phenotypic abnormity for a few transgenic plant. a, Normal flower; b, multiple petals and abnormal stigma; c, multiple petals; d, degenerated petals and abnormal stigma; e, degenerated stamen; f, multiple petals; g and h, wilted anther; I, multiple petals.

late maturation of the seeds was not enough. The seed viability will be investigated in future research.

Two of the transgenic plants produced flowers with enlarged ovaries and silique, but these siliques stopped growth when reaching 1/5th of the size of the wild type plants. Theses siliques were deformed and could not bear seeds. When the transgenic plants were pollinated by the wild type plants, their ovary started to enlarge within one week and produced normal siliques and seeds (Figure 6b). These results indicate that the stigma of the transgenic plants were well developed and was fertile. However, self-pollinated transgenic plants could not complete fertilization process due to degenerated anthers, and the plants became male sterile.

Restoration of the male sterility of the transgenic plants

The floral buds of the transgenic plants were treated with 500 µM methyl jasmonate for 1-2min. After 3 d, the pollen germinated normally on the stigma of the male sterile plants as well as on medium plates. Plants pollinated with these pollens were able to produce normal seeds (Figure 7).

Figure 6. The silique of transgenic plant. a, transgenic plant without pollination; b, trangenic plant with artificial pollination.

DISCUSSION

JA metabolism and its relationship with the fertility of plants

The biosynthesis of JA starts at linolenic acid, which is produced from phospholipid under the action of phospholipase. The next step is catalyzed by lipoxygenase (LOX) for the conversion of linolenic acid into (9Z, 11E, 15Z, 13S)-13-hydroperoxy-9, 11, 15-octadecatrienoic acid, which then forms 12-oxo-10, 15(Z)-octadecatrienoic acid catalized by allene oxide cyclase (AOC). Reduction of the 10,11-double bond by a NADPH-dependent OPDA-reductase then yields 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) which is believed to undergo three cycles of ß-oxidation to yield the end product of the pathway, i.e. JA. JA is converted into various jasmonates. Site mutation at multiple steps of this metabolic cycle have all being found to cause male sterile phenomenon, and application of exogenous JA restores the fertility of these plants.

In this study, the antisense *BoiDAD*1F transgenic plants exhibited male sterility. The pollen germination rate was very low, and no seed pods were produced. The seeds produced in very few pods did not germinate. The integration of the antisense *BoiDAD*1F into the plant genome effectively silenced the *DAD*1 gene, and interfered with JA metabolism, whereby inhibiting anther development and eventually causing male sterility.

Ishiguro (2001) reported that the floral organs of *dad*1 mutant had no obvious difference than wild type *A. thaliana* plants. However, the anther could not open and release the unviable pollens. He accordingly proposed the model that *DAD*1 gene control anther opening. At the late middle stage of the anther maturation, *DAD*1 gene expresses in the upper part of the faliment to produce JA. This region can induce cellular water absorption so that water can be transported from anther chamber through anther wall (the internal wall and connective tissues) to filament. The dryness in the chamber inhibits maturation of pollen through a yet defined mechanism. The *DAD*1 gene expression was detected in the cells of both the upper and basal parts of the filaments at late maturation stage to produce JA, which enhances water movement from the anther wall and flower stalk into the filament to be used for its elongation and anther opening. In addition, JA (or MeJA) in the filaments is also transported into petals to improve water absorption in order to induce petal growth and flower opening.

For the transgenic broccoli plants expressing the antisense *BoiDAD*1F, the anthers were open, and could release pollen, but the pollen had very low viability and majority of them did not germinate. The possible causative reasons for the difference between the previously reported and the current works include the following: Firstly, the transgenic *A. thaliana* was transformed *with* a *DAD*1 gene under its own original promoter that is expressed in filament. The construct used in this study was under the control of the constitutive CaMV 35S promoter, which could cause interference with the expression of other highly homologous genes. Secondly, gene expression varies among different plant species. JA biosynthesis is a complex process, and affected by many factors. Minor difference in the metabolic pathway can result in phenomenal changes. Results from this study indicate that the *DAD*1 is associated with anther development. Insertion of the antisense *DAD*1 fragment has led to pollen abortion which was restored by exogenous application of JA.

Antisense gene silencing technique and male sterility

Using the antisense RNA to interfere with gene expression

Figure 7. Selfing transgenic plants treated with 500 µM methyl jasmonate.

has been successfully applied to disturb normal function of target genes. This technology has been used in genetic engineering for long-shelf life tomato, and some other genetics studies. Transformation of petunia with antisense RNA constructs that specifically interferes with expression of genes encoding for the biosynthesis of flavonoids in anther has induced male sterile dwarf plants (Van der Meer and Stam, 1992). *CYP86MF* encodes for P450 which is associated with male sterility in *B. campestris* L. *ssp. chinensis* Makino. When a 460 bp antisense *CYP86MF* construct was transferred into regular broccoli and kale, some male sterile plants were produced (Huand, 2004). The expression of the antisense *BcMF* 9 gene also produced male sterile plants of broccoli (Ye, 2006), but none of those transgenic plants reached 100% sterile ratio.

In this study, antisense interference technique was used to interfere with the expression of *DAD*1 gene in order to stop the JA biosynthesis, whereby inducing male sterile plants. The resultant male sterile ratio was 99%. Meanwhile, the foreign gene had no side effect on plant growth and development of the transgenic plants. These plants can be used to produce F1 hybrid seeds. The antisense sequence was designed against the conserved region of *DAD*1 in *A. thaliana*. Sequence comparison revealed that the cloned *DAD*1 homologous gene fragment contained the amino acid residues of the catalytic triad of the phospholipase. The antisense construct effectively suppressed expression of the phospholipase gene, and resulted in good silencing effect. This result indicates that this conserved region contains the desirable sequence. Meanwhile, when the inserted foreign genes are cloned from the same species, it reduces the chances of other associated side effect caused by genetic variation. The resultant efficiency of antisense gene silencing can be very strong.

The copy number of the transgene and its expression

As validated in Southern blot, 1- 4 copies of the antisense gene construct were integrated into the genome of individual transgenic plants. More than one copy insertion happens on multiple sites. Generally speaking, single copy integeration will not cause co-inhibition phenomenon, which could happen in the plant carrying multiple

copy of the foreign gene. In this research, no difference was observed bwteen the plants carrying one and multiple copies of the antisence construct. It is possible that copy number of the foreign gene has disernalble effect only in the transgenic plants that are over expressing the foreign gene. The tranformation of the antisense gene does not have similar effect. Because the insert used in this study was an antisense construct, the copy number of the forign gene had no obvious correlation with the resultant inhibitory function.

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