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Arabidopsis *lox3 lox4* double mutants are male sterile and defective in global proliferative arrest

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Abstract Fertility and flower development are both controlled in part by jasmonates, fatty acid-derived mediators produced via the activity of 13-lipoxygenases (13-LOXs). The *Arabidopsis thaliana Columbia-0* reference genome is predicted to encode four of these enzymes and it is already known that one of these, LOX2, is dispensable for fertility. In this study, the roles of the other three 13-LOXs (LOX3, LOX4 and LOX6) were investigated in single and double mutants. Four independent *lox3 lox4* double mutants assembled with different mutated *lox3* and *lox4* alleles had fully penetrant floral phenotypes, displaying abnormal anther maturation and defective dehiscence. The plants were no longer self-fertile and pollen was not viable. Fertility in the double mutant was restored genetically by complementation with either the *LOX3* or the *LOX4* cDNAs and biochemically with exogenous jasmonic acid. Furthermore, deficiency in *LOX3* and *LOX4* causes developmental dysfunctions, compared to wild type; *lox3 lox4* double mutants are taller and develop more inflorescence shoots and flowers. Further analysis revealed that developmental arrest in the *lox3 lox4* inflorescence occurs with the production of an abnormal carpelloid flower. This distinguishes *lox3 lox4* mutants from the wild type where developmentally typical flower buds are the terminal inflorescence structures observed in both the laboratory and in nature. Our studies of *lox3 lox4* as well as other jasmonic acid biosynthesis and perception mutants show that this plant hormone is not only required for male fertility but also involved in global proliferative arrest.

Keywords Lipoxygenase · Jasmonate · Male sterility · Carpelloid terminal flower

Abbreviations

LOX	Lipoxygenase
AOS	Allene oxide synthase
DAD1	Defective in anther dehiscence1
JA	Jasmonic acid
LNA	α-linolenic acid
CaMV 35S promoter	Cauliflower mosaic virus promoter
WT	Wild type
FA	Fatty acid
GPA	Global proliferative arrest

Introduction

Lipoxygenases (LOXs) are found in most eukaryote lineages where they typically catalyze the oxygenation of fatty acids (FAs) containing 1(*Z*), 4(*Z*) pentadienyl motifs (Schneider et al. 2007). In plants oxygenation can occur on

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carbon number 13 of the FA (reactions catalyzed by the 13-LOX family), or in 9-LOX on carbon number 9 (Andreou and Feussner 2009). Through these first canonical LOX reactions hydroperoxy-fatty acids are produced to serve as the precursors of diverse oxygenated fatty acids (oxylipins) including jasmonates in plants. Biologically active jasmonates such as jasmonoyl-L-isoleucine (JA-Ile) are derived through the activity of 13-LOXs and have been shown to be involved in many aspects of defense and development in many plants (Avanci et al. 2010; Browse 2009; Fonseca et al. 2009; Wasternack 2007). Concerning reproduction, the biosynthesis of jasmonates in Arabidopsis during anther development is required to ensure male fertility through controlling stamen filament elongation, pollen development and anther dehiscence. In fact, jasmonate and coronatine (a JA-Ile mimic) perception mutants such as *coronatine insensitive1-1* (*coi1-1*; Feys et al. 1994) and jasmonate biosynthetic mutants such as the enzymes *allene oxide synthase* (*aos (dde2)*; Park et al. 2002; von Malek et al. 2002), *12-oxophytodienoic acid reductase3* (*opr3 (dde1)*; Stintzi and Browse 2000; Sanders et al. 2000) and the lipase mutant *defective in anther dehiscence1* (*dad1*; Ishiguro et al. 2001) are male sterile. Interestingly, the *DAD1* gene is itself a target of AGAMOUS (AG), a homeotic protein involved in stamen and carpel development that also plays a secondary post-homeotic role at later stages of flower development involving jasmonate signaling (Ito et al. 2007).

Regarding tissue growth control, it is notable that, in contrast to promoting anther elongation, jasmonates repress petal growth in Arabidopsis (Brioudes et al. 2009). Jasmonates also appear to impact post-fertilization growth events in Arabidopsis since seeds of the *aos* mutant are larger than those of wild type (WT; Farmer and Dubugnon 2009). The JA-Ile synthesis mutant *Osjar1* in rice has a strong floral phenotype: the plant is male sterile and, if fertilized with WT pollen, the glumes can no longer cover the developing seed, thus exposing it to the environment (Riemann et al. 2008). All these reported effects concern the impacts of jasmonates on pre- and post-fertilization flower development and seed weight in plants with bisexual flowers. However, in maize, a monoecious plant, jasmonate synthesis was found to be essential for male inflorescence development. Mutations in a *LOX* gene involved in jasmonate biosynthesis prevented male inflorescence development and resulted in a 'default pathway' to female inflorescence development in the place of male tassels (Acosta et al. 2009). Finally, it is notable that female fertility in Arabidopsis is not affected as strongly as male fertility whereas the opposite is true for tomato (Li et al. 2001). Together, these findings all underscore the fact that the effects of jasmonate in reproductive development in plants are complex and likely to be somewhat species-dependent. In particular, the recent work on rice and maize raises the possibility that other jasmonate-dependent phenotypes exist in dicotyledons like Arabidopsis, particularly at the level of inflorescence formation and flower development.

The Arabidopsis *Columbia-0* (*Col-0*) accession encodes four predicted 13-LOXs: LOX2, LOX3, LOX4 and LOX6 (Bannenberg et al. 2009) and little is known about their specific contributions to jasmonate synthesis. However, recent work has shown that one of these, LOX2, is not required for fertility. Instead, a major role of LOX2 in leaves appears to be the synthesis of jasmonate precursors for incorporation into secondary metabolites known as arabidopsides (Glauser et al. 2009; Seltmann et al. 2010). The roles of the other 13-LOXs in Arabidopsis remain unknown. In this report, we attribute new biological roles to LOX3 and LOX4 demonstrating that these LOX isoforms are necessary for Arabidopsis male fertility. Moreover, we find that these LOXs are required for WT inflorescence development, and indirectly for inflorescence proliferative arrest.

Materials and methods

Plant materials

Arabidopsis plants were grown at 22°C, under a 12 h light/day photoperiod. T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center (ABRC), and genotyped by allele-specific PCR. At least two different alleles were isolated for each T-DNA insertion mutant, for *LOX3* (At1g17420), allele *lox3A* corresponds to line SALK_119404, allele *lox3B* corresponds to line SALK_147830, allele *lox3D* is line SALK_062064, for *LOX4* At1g72520, *lox4A* is SALK_071732, *lox4B* is SALK_017873, for *LOX6* At1g67560, *lox6A* is SALK_138907 and *lox6B* is SALK_083650. The silenced *LOX2* At3g45140 line and the cognate control line are available as line CS3748 and CS3749, respectively. The *dad1* mutant was from Ishiguro et al. (2001), and the *aos* mutant was from Park et al. (2002). The terminal inflorescences of wild Arabidopsis plants (96 individuals) growing on the University of Lausanne campus were observed on May 1, 2010. Only plants that had completed flowering were scored.

Cloning *LOX3* and *LOX4* cDNAs and plant transformation

LOX3 and *LOX4* cDNA clones in the Gateway vector pENTR223 (G21585 and G11274, respectively), were ordered from ABRC. The clones were sequence-verified

prior to use. Then via an LR Clonase™ reaction (Invitrogen), each cDNA was introduced into the pMDC32 destination vector (Curtis and Grossniklaus 2003), downstream of the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al. 1985). *LOX3*/pMDC32 or *LOX4*/pMDC32 were introduced into competent *Agrobacterium tumefaciens* (strain GV3101::pMP90) using a freeze thaw method (Weigel and Glazebrook 2002). Positive colonies were re-streaked onto fresh YEB medium containing Rifampicin (25 mg/ml), Kanamycin (50 mg/ml), and Gentamycin (25 mg/ml) and checked by PCR for the presence of the *LOX3* or *LOX4* cDNAs. Double mutants *lox3 lox4* plants were transformed according to the standard floral dipping protocol. Transformed seedlings were identified approximately 10 days later, by their growth on ½ Murahige and Skoog (MS) medium containing 15 mg/ml of hygromycin.

Recovery of fertility experiments: JA and LNA treatment and pollen viability tests

Flowers of sterile *dad1*, *aos* and double *lox3 lox4* mutants were dipped in an Eppendorf tube containing either water (control) or 1 mM JA (jasmonic acid; Sigma–Aldrich) or 0.1% (v/v) LNA (α-linolenic acid; Sigma–Aldrich), both dissolved in 0.05% aqueous Tween 20, for 5 consecutives days.

The pollen viability of WT *Col-0* plants, single mutants *lox3*, *lox4*, double mutants *lox3 lox4* and *aos* mutants was evaluated using a double staining procedure with fluorescein diacetate (FDA) and propidium iodide (PI) as described by Mandaokar and Browse (2009). Pollen grains were examined under a fluorescence microscope (Leica MZ16FA, GFP3 filter for FDA: excitation 470/40 nm, emission 525/50 nm, and DsRED filter for PI: excitation 545/12 nm, emission at 620/60 nm). Viable pollen grains fluoresce bright green, while the non-viable pollen grains fluoresce red. In total, 675 WT (*Col-0*), 872 *lox3*, 1075 *lox4*, more than 700 for either *lox3 lox4*, or *aos* pollen grains were scored and the percentage of viability calculated.

Substrate binding pocket virtual analysis

The three-dimensional structures of the soybean LOX1 and LOX3 (pdb entries 1YGE, Minor et al. 1996; and 1IK3, Skrzypczak-Jankun et al. 2001, respectively) were superposed using Swiss-PdbViewer (Guex and Peitsch 1997). The primary sequences of the six distinct Arabidopsis LOXs were directly imported into the workspace from Uniprot (Boutet et al. 2007) (accession ids: Q06327, P38418, Q9SMW1, Q9FNX8, Q9LUW0, Q9CAG3; LOX1-6, respectively) and aligned onto the structures using the Align with MUSCLE option of the Fit menu. The residues within 5.0 Angstroms of the 13(S)-hydroperoxy-9(*Z*),11(*E*)-octadecadienic acid binding site present in the soybean LOX3 were selected and defined as substrate binding pocket (Suppl. Fig. 1), and the corresponding residues of the Arabidopsis LOXs identified from the multiple sequence alignment. Percent identity between the aligned LOXs for the whole sequence and the active site (Fig. 1) have been obtained directly from the Swiss-PDB Viewer alignment window.

Results

Male sterility phenotypes in *lox3 lox4* double mutants

LOX3, LOX4 and LOX6 are components of the same phylogenetic clade in Arabidopsis, while LOX2 is disparate (Bannenberg et al. 2009). LOX3 and LOX4 are highly similar, sharing 85% overall amino acid identity (Fig. 1a and Suppl. Fig. 1). The amino acid sequences of the substrate binding pocket of LOX3 and LOX4 are even more conserved (97%, Fig. 1b) with only one synonymous amino acid change of a leucine in LOX3 to a valine in LOX4. The strong similarities of LOX3 and LOX4 prompted us to make double mutants for these proteins. We first identified T-DNA insertion mutants for *LOX3 (lox3A, lox3B and lox3D)*, for *LOX4* (*lox4A, lox4B*), for *LOX6* (*lox6A, lox6B*) and the co-suppression line of *LOX2* (*lox2.S12*; Bell et al. 1995). All 13-*LOX* single mutants were fertile (Fig. 2a and Suppl. Fig. 3). We then produced four independent *lox3 lox4* double mutants using different insertion mutant lines: *lox3B lox4A,*

(A) Full length amino acids sequence identity

	LOX3	LOX4	LOX6	LOX2	LOX5	LOX1
LOX3	100%					
LOX4	85%	100%				
LOX6	53%	53%	100%			
LOX2	45%	45%	49%	100%		
LOX5	45%	45%	42%	42%	100%	
LOX1	45%	45%	45%	45%	64%	100%

(B) Sequence identity in the substrate binding pocket

	LOX3	LOX4	LOX6	LOX2	LOX5	LOX1
LOX3	100%					
LOX4	97%	100%				
LOX6	76%	76%	100%			
LOX2	69%	69%	79%	100%		
LOX5	66%	62%	62%	62%	100%	
LOX1	66%	62%	62%	62%	97%	100%

Fig. 1 Amino acid sequence identity of Arabidopsis LOX in percentage. **a** Full length sequence identity. **b** Sequence identity of the 29 residues composing the substrate binding pocket

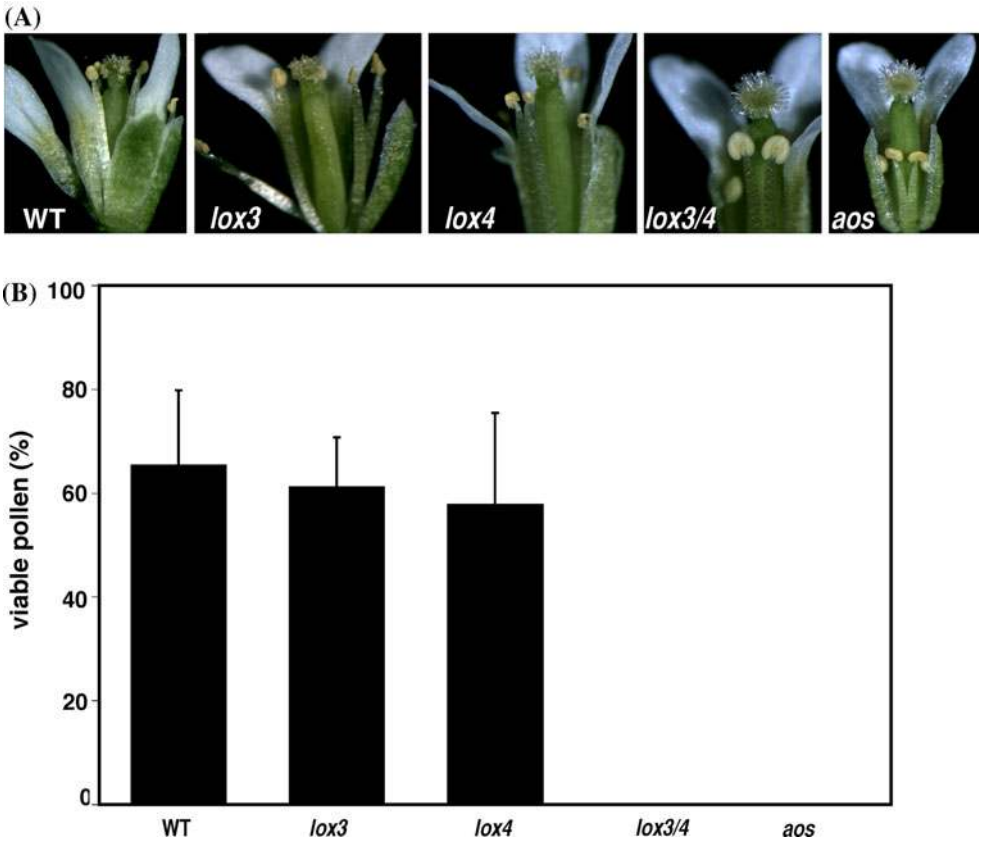

Fig. 2 **a** Anther phenotype of WT and *LOX* mutants. **b** % of pollen viability. WT (*Col-0*), *lox3*, *lox4*, *lox3 lox4*, and *aos*

lox3D lox4B, lox4A lox3D, and lox4B lox3B. All these mutants were male sterile (Fig. 2a). We also produced *lox3 lox6* and *lox4 lox6* double mutants, but these plants were found to be fully fertile (Suppl. Fig. 4). Importantly, all *lox3 lox4* double mutants showed the same flower phenotype: shorter anther filaments, indehiscent anthers, and longer stigma papillae than those in WT flowers. We then compared the floral morphology of *lox3 lox4* double mutants to that of *aos*. As shown in Fig. 2a we observed that both mutants show a similar flower phenotype. Moreover, the pollen contained in the indehiscent anthers of *lox3 lox4* double mutants and of *aos* mutants was not viable, while pollen viability in the WT (65%) was similar to that in *lox3* (61%) and *lox4* (58%) single mutants (Fig. 2b). Efforts to self-fertilize all *lox3 lox4* double mutants with pollen extracted from their own anthers failed. These results indicate clearly that *LOX3* and *LOX4* are both required for anther and pollen development.

Restoration of fertility to the *lox3 lox4* double mutant

In order to confirm the hypothesis that *lox3 lox4* double mutants were sterile because they are unable to produce JA indispensable for anther maturation, we applied exogenous JA to the sterile flowers to restore fertility. The flowers were treated either with water, or with 0.1% (v/v) α-linolenic acid (LNA) or with 1 mM JA. LNA was reported to rescue fertility in the lipase mutant *dad1*, but it did not do so in *aos* nor in *lox3 lox4*. In contrast, JA restored fertility in all mutants tested (Fig. 3). Therefore, JA production in the flowers of *lox3 lox4* double mutants is impaired, resulting in male sterility. Furthermore, the presence of either *LOX3* or *LOX4* is necessary and sufficient to confer fertility to Arabidopsis, as all the single *lox3* and *lox4* mutants are fertile. This is supported by the fact that T1 plants of *lox3 lox4* double mutants expressing the cDNAs of either *LOX3* or *LOX4* (driven by the strong CaMV 35S promoter) are male fertile (Fig. 4).

Inflorescence morphology

The JA synthesis mutant *aos* and all *lox3 lox4* double mutants were compared and found to share many morphological characteristics. All of these mutants were significantly taller than the WT or the single *LOX* mutant plants. Under our growth conditions nine-week old WT or single 13-*LOX* mutant plants measured approximately 35 cm in height while *lox3 lox4* double mutants or *aos* plants measured more than 50 cm (Fig. 5a). Moreover, *aos* or *lox3 lox4* double mutant plants developed more floral shoots (on average about 30), compared to WT and single mutants (average around 20). Male-sterile mutants also produced more than 65 flowers compared to 45 flowers for the single mutants or WT

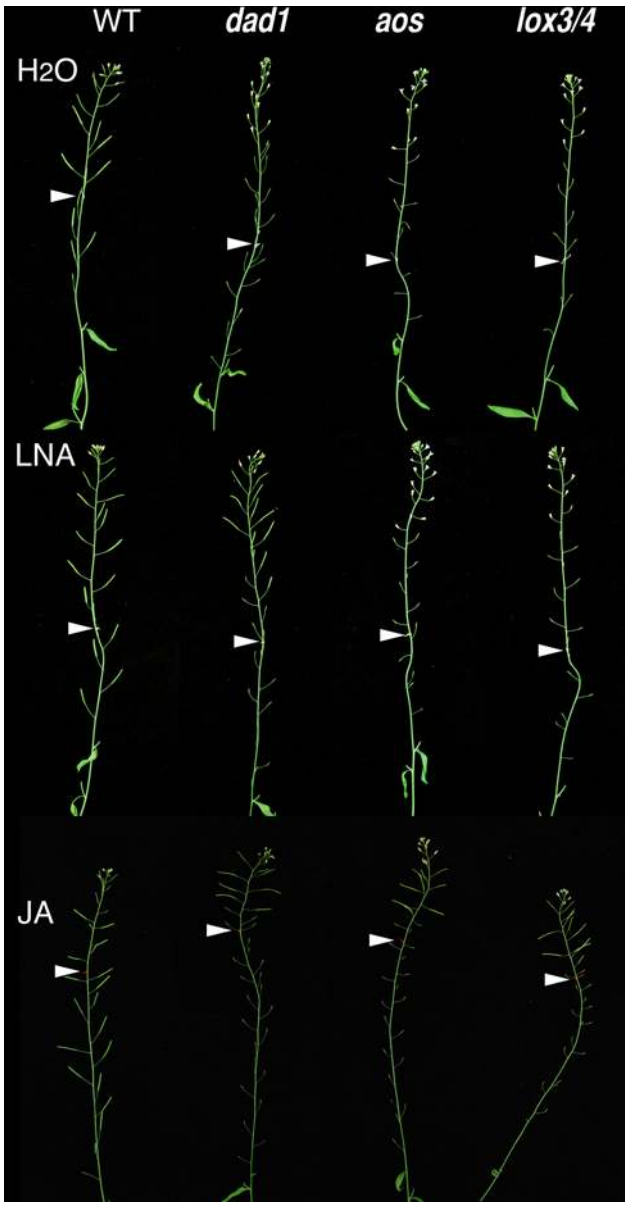

Fig. 3 Restoration of fertility experiments. *Arrow heads* point to the location where the different treatments were initiated. H_2O: water, LNA: 0.1% (v/v) α-linolenic acid, JA: 1 mM jasmonic acid. The plants used in the experiments were WT (*Col-0*), *dad1*, *aos*, and *lox3 lox4*

(Fig. 5c). This was due to the fact that *aos* and *lox3 lox4* double mutant continued flowering 1–2 weeks longer than the WT and the single mutants. Consequently, *lox3 lox4* and *aos* plants have a longer life span than the WT under our growth conditions (data not shown).

Floral morphology: the terminal flower of the *lox3 lox4* inflorescence has carpelloid and staminode structures

In WT plants that have completed flowering and have filled their siliques with seeds, we observed that inflorescence meristematic activity terminated in the production of floral buds (Fig. 6a). In contrast, in the *lox3 lox4* double mutant inflorescences, proliferation arrest produced a fully developed abnormal structured flower (Fig. 6e–f). This flower completely lacked petals and sepals which were transformed into open carpelloid structures with exposed ovules and bearing stigmatic papillae. Moreover, the number of stamens was irregular and reduced compared to the WT. Staminode (stamen-like) structures that were larger than WT anthers and of an irregular shape were often present (Fig. 6d–g). We then found the same to be true of *dad1*, *aos*, *opr3*, *coi1-1* (data not shown), and in WT (*Col-0*) plants rendered sterile by the surgical removal of fruits (Fig. 6b–d).

Discussion

LOX3 and *LOX4* contribute to male fertility in Arabidopsis

The role of JA in anther maturation was firstly established by showing that the triple *fatty acid desaturase fad3-2 fad7-2 fad8* mutant was male sterile and that fertility could be recovered by treatment with exogenous JA (McConn and Browse 1996). Similar effects were also observed for JA biosynthetic mutants: *12-oxophytodienoic acid reductase3* mutants (*opr3,* Stintzi and Browse 2000; *dde1,* Sanders et al. 2000), *dad1* lipase mutants (Ishiguro et al. 2001) and, finally, *aos* mutants (Park et al. 2002; *dde2-2*, von Malek et al. 2002). The role of LOX isoforms in male fertility has not been examined previously, and here we demonstrated that among all of the Arabidopsis 13-LOXs, LOX3 and LOX4 are the isoenzymes necessary for anther maturation, whereas LOX2 and LOX6 are not necessary for this process and do not contribute to male fertility. LOX3 and LOX4 share a nearly identical substrate binding pocket (Fig. 1b), and act redundantly in JA synthesis to ensure male fertility. In fact, single *lox3* and *lox4* mutants are fertile and so are double mutants *lox3 lox4* complemented either with *LOX3* or *LOX4* (Fig. 4). The next closest LOX from a sequence point of view is LOX6 (Bannenberg et al. 2009, and Fig. 1), but *lox6* single mutants (Suppl. Fig. 3) as well as *lox3 lox6* and *lox4 lox6* double mutants are all fertile (Suppl. Fig. 4) indicating that LOX6 plays no major role in male fertility. LOX2 is phylogenetically the most diverse among the Arabidopsis 13-LOXs. Plants homozygous for a *lox2* null allele are fertile and a major role of this LOX in the vegetative tissues of Arabidopsis was found instead to be in the synthesis of arabidopsides (Glauser et al. 2009; Seltmann et al. 2010).

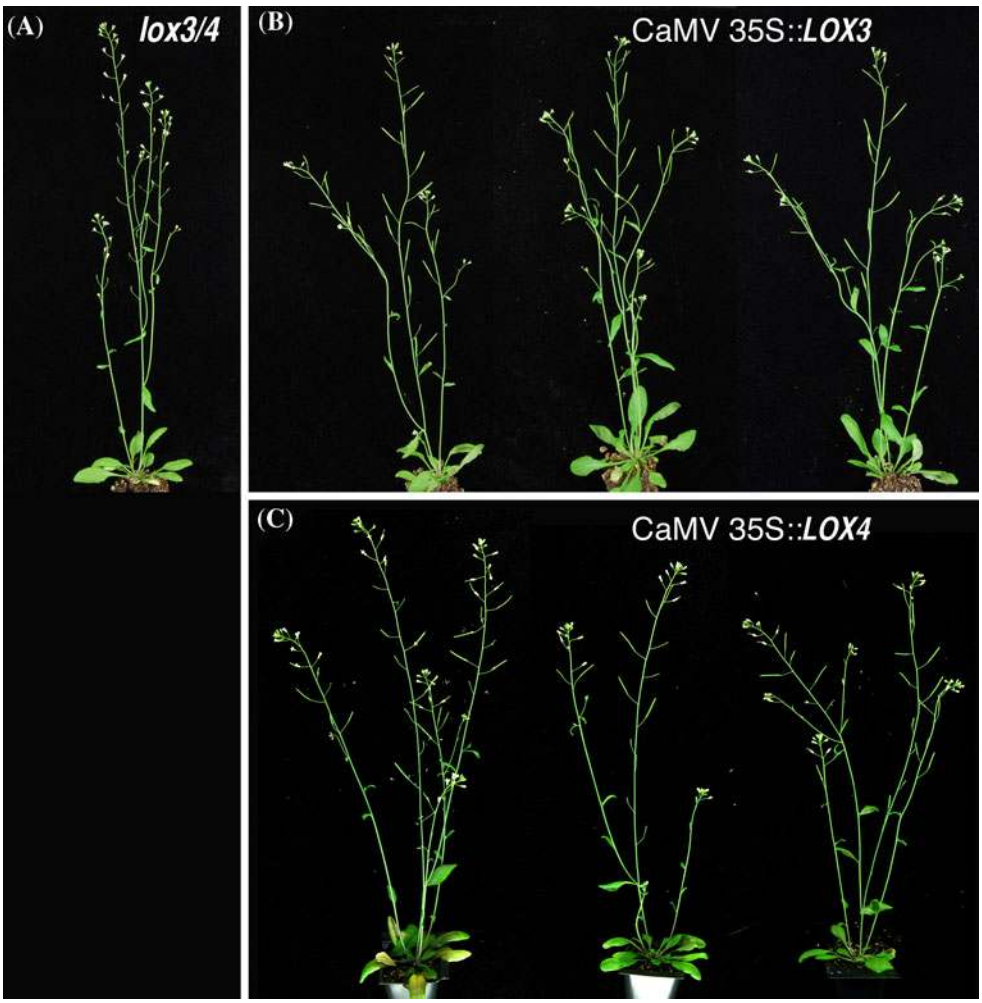

Fig. 4 Phenotype of the double mutant *lox3 lox4* and complemented lines. **a** *lox3 lox4*; note the absence of siliques. **b** T1 generation of *lox3 lox4* lines transformed with CaMV35S::*AtLOX3*. **c** T1 generation of *lox3 lox4* lines transformed with CaMV35S::*AtLOX4*

The effects on male fertility that we observed in *lox3 lox4* mutants were found to be the same as those reported for other Arabidopsis jasmonate synthesis and perception mutants (Feys et al. 1994; Ishiguro et al. 2001; Park et al. 2002; Sanders et al. 2000; Stintzi and Browse 2000; von Malek et al. 2002), that is an impairment in anther filament elongation, anther dehiscence, and pollen viability. Through further examination of *lox3 lox4* double mutants as well as *coi1-1*, *aos*, *dad1* and *opr3* mutants, we found that these effects were systematically observable in all flowers except the terminal one (and rarely, in the penultimate flower). We also studied inflorescence growth in *lox3 lox4* double mutants compared to *aos*, WT, and 13-*LOX* single mutants. After flowering, *lox3 lox4* and *aos* plants were taller than WT, and developed more floral shoots and more flowers. Instead 13-*LOX* single mutants developed in a similar way as the WT (Fig. 5). Thus, *lox3 lox4* mutations affect many aspects of plant growth.

lox3 lox4 double mutant inflorescences terminate in abnormal flowers

In WT plants, inflorescence meristematic activity invariably terminates with the production of unopened buds (Fig. 6a). This has been observed in the laboratory and in each of the 96 inflorescences of wild *Arabidopsis thaliana* collected in the field. In contrast, the terminal flower of *lox3 lox4* double mutants develops abnormally (Fig. 6e–g), this flower does not develop petals, nor sepals, has a reduced number of stamens, and shows carpelloid and staminode structures. This same structure was then observed in a variety of other JA synthesis and perception mutants (*dad1*, *aos*, *opr3* and *coi1-1*). It seems that in all these male sterile mutants the signal for correctly terminating meristematic activity is missing, and the inflorescence meristem undergoes irreversible differentiation, terminating in an abnormal flower. We noticed that the

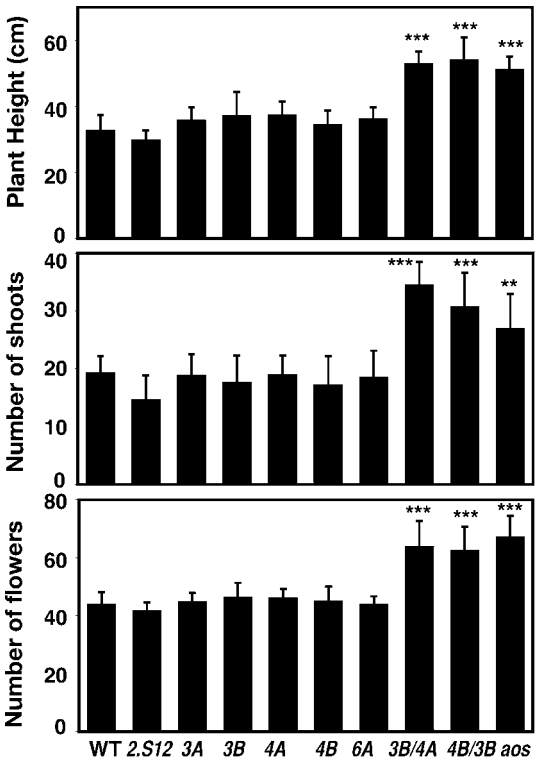

Fig. 5 Morphology of *LOX* mutants, 9 weeks old. **a** Plants height. **b** Number of floral shoots. **c** Number of flowers. 2.S12 is the silenced *LOX2* line. 3A, 3B, 4A, 4B, 6A, 3B/4A, 4B/3B correspond to the respective lines: *lox3A*, *lox3B*, *lox4A*, *lox4B*, *lox6A*, double mutant *lox3B lox4A*, *lox4B lox3B*. Each *bar* represents an average of 12 measurements. Significance level of the *t*-test, ** = $P<0.01$; *** = $P<0.001$

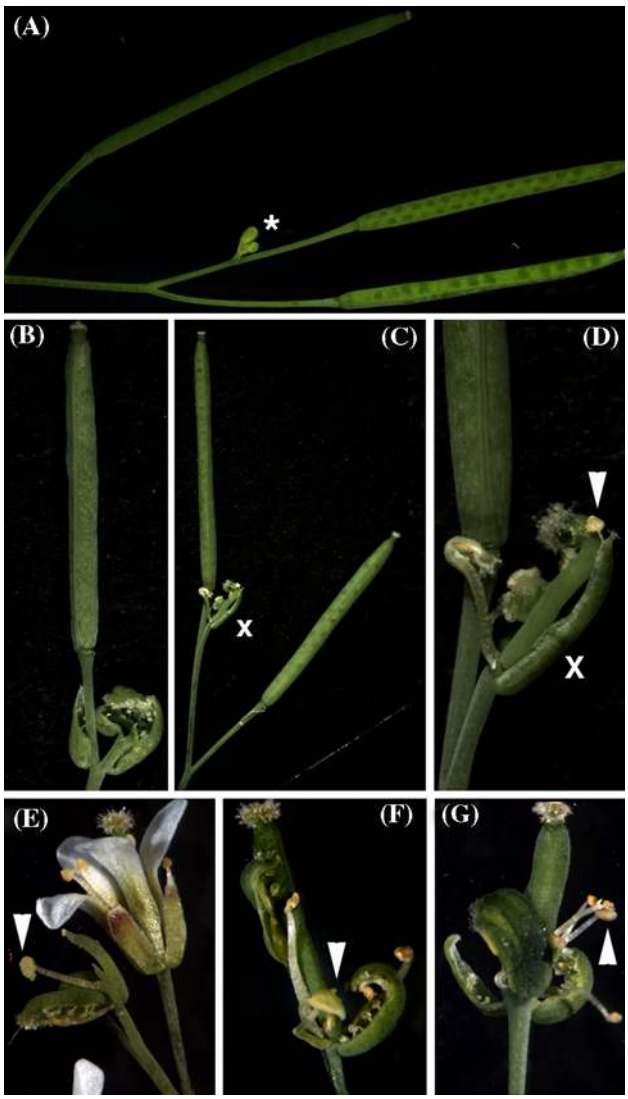

Fig. 6 Apical flower arrest. **a** WT (*Col-0*), the *asterisk* shows the terminal flower buds; **b**, **c** ultimate flower of WT (*Col-0*) plant in which fruits where surgically removed; **d** enlargement of the terminal flower is indicated by *X* in (**c**); **e**, **f**, **g** three distinct ultimate flowers of *lox3 lox4* double mutant. *Arrowheads* indicate staminode structures

phenotype of this aberrant flower resembles that of the *apetala2* mutant (*ap2*; Bowman et al. 1989; Jofuku et al. 1994). In *ap2* mutants the phenotype is linked to the de-repression of the homeotic gene *AGAMOUS* (*AG*; Drews et al. 1991; Bomblies et al. 1999). Similarly to the double mutant *lox3 lox4*, the inflorescence of *AG* over-expressing plants also terminated with the production of fully developed abnormal flower consisting of a few carpelloid organs (Mizukami and Ma 1997).

Jasmonates indirectly control global proliferative arrest

Interestingly, Arabidopsis plants with a similar terminal flower phenotype to that of the *lox3 lox4* double mutants have been observed in a different context. The *male sterile1-1* (*ms1-1*) mutant shows an aberrant terminal flower phenotype (Hensel et al. 1994). Moreover, a similar phenotype can be produced in WT plants through the systematic removal of developing fruits (Hensel et al. 1994, and Fig. 6b–d). Developing fruits control the capacity of inflorescence meristems to produce additional flowers and, in Arabidopsis, a threshold of greater than 30% of WT seed per silique is necessary to induce an unknown signal that causes what is termed as 'global proliferative arrest' (GPA) in the inflorescence (Hensel et al. 1994). In order for GPA to operate correctly, a type of systemic signal must move over the comparatively long distances from the developing siliques to the apex. Mutants in the production or perception of other plant hormones, auxin, ethylene, abscissic acid and gibberellin were tested by Hensel et al. and found to have no effect on GPA (Hensel et al. 1994). Our findings show that, by assuring seed production, jasmonates indirectly determine the fate of the inflorescence meristem. In the absence of JA, no seed is set and the apex grows to a greater height than in the WT, terminating in a fully differentiated structure. In conclusion, this study shows that

LOX3 and *LOX4* control male fertility and inflorescence structure in Arabidopsis.

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