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ORIGINAL ARTICLE

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Pleiotropic effects of the *male sterile33* (*ms33*) mutation in *Arabidopsis* are associated with modifications in endogenous gibberellins, indole-3-acetic acid and abscisic acid

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Abstract Earlier, we reported that mutation in the *Male Sterile33* (*MS33*) locus in *Arabidopsis thaliana* causes inhibition of stamen filament growth and a defect in the maturation of pollen grains [Fei and Sawhney (1999) Physiol Plant 105:165–170; Fei and Sawhney (2001) Can J Bot 79:118–129]. Here we report that the *ms33* mutant has other pleiotropic effects, including aberrant growth of all floral organs and a delay in seed germination and in flowering time. These defects could be partially or completely restored by low temperature or by exogenous gibberellin A_4 (GA_4), which in all cases was more effective than GA_3 Analysis of endogenous GAs showed that in wild type (WT) mature flowers GA_4 was the major GA, and that relative to WT the *ms33* flowers had low levels of the growth active GAs, GA_1 and GA_4, and very reduced levels of GA_9, GA_{24} and GA_{15}, precursors of GA_4. This suggests that mutation in the *MS33* gene may suppress the GA biosynthetic pathway that leads to GA_4via GA_9 and the early 13-H C_{20}GAs. WT flowers also possessed a much higher level of indole-3-acetic acid (IAA), and a lower level of abscisic acid (ABA), relative to *ms33* flowers. Low temperature induced partial restoration of male fertility in the *ms33* flowers and this was associated with partial increase in GA_4. In contrast, in WT flowers GA_1 and GA_4 were very much reduced by low temperature. Low temperature also had little effect on IAA or ABA levels of *ms33* flowers, but did reduce (> 2-fold) IAA levels in WT flowers. The double mutants, *ms33 aba1-1* (an ABA-deficient mutant), and *ms33 spy-3* (a GA signal transduction mutant) had flower phenotypes similar to *ms33*. Together, the data suggest that the developmental defects in the *ms33* mutant are unrelated to ABA levels, but may be causally associated with reduced levels of IAA, GA_1 and GA_4, compared to WT flowers.

Keywords *Arabidopsis* · Gibberellin · Indole-3-acetic acid · Male sterility · *ms33* mutant · Pleiotropy

Abbreviations ABA: Abscisic acid · GA: Gibberellin · GC-MS-SIM: Gas chromatography-mass spectrometry-selected ion monitoring · IAA: Indole-3-acetic acid · *ms33*: *Male sterile33* mutant · PP333: Paclobutrazol · WT: Wild type

Introduction

Analyses of various nuclear-coded genic (GMS) and cytoplasmic (CMS) male-sterile mutants in plants have shown that pollen development in angiosperms is controlled by a large number of nuclear and mitochondrial genes (reviewed in Kaul 1988; Shivanna and Sawhney 1997). In *Arabidopsis*, nearly 900 male-sterile mutants, induced by T-DNA insertion or ethyl methane sulfonate (EMS) mutagenesis, had defects in anther and pollen development, including aberrations in anther dehiscence (Sanders et al. 1999). Although many genes expressed during anther development are housekeeping genes, some are designated as pollen-specific and are expressed at early and late stages of microsporogenesis (Mascarenhas 1990). Additionally, in a few male-sterile mutants, other aspects of plant development are also affected, suggesting that some of the genes controlling pollen

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development may not be specific, i.e., they have pleiotropic effects. For example, the single gene *7B-1* mutant in tomato is male-sterile, but it also exhibits reduced de-etiolation of hypocotyl growth and increased resistance to abiotic stresses i.e., high osmoticum, various salts, and low temperature (Fellner and Sawhney 2000; Fellner et al. 2001). Conversely, some of the dwarf mutants in maize (Phinney and Spray 1982; Dellaporta and Calderon-Urrea 1994), tomato (Koornneef et al. 1990) and *Arabidopsis* (Koornneef and van der Veen 1980) are also male-sterile and have pleiotropic effects, e.g., delayed seed germination and flowering. This suggests that certain genes controlling pollen and stamen development may also be expressed at other stages of plant development, and that their effects may be mediated via common factors required for several developmental processes.

Plant hormones are implicated in various developmental processes, including seed germination, vegetative growth, flowering induction and reproductive organ development (Davies 1995). The various roles of plant hormones have been determined by research involving application, both in situ and in vitro, and analyses of endogenous hormones (see, e.g., Pharis and King 1985; Sawhney and Shukla 1994). In addition, mutants exhibiting defects in hormone biosynthesis or hormone signaling have been used to support the role of hormones in developmental processes, e.g., dwarfing, delayed seed germination and male sterility (Koornneef and van der Veen 1980; Koornneef et al. 1990; Jacobsen and Olszewski 1991, 1993; Wilson and Somerville 1995). For example, the gibberellin (GA) deficient, *ga1-1*and *ga1-3* mutants, and the GA-insensitive, *gai* mutant of *Arabidopsis* exhibit multiple defects, including delayed seed germination and flowering, loss of apical dominance, effects on plant stature and defects in male and female reproductive organ development (Koornneef et al. 1982; Wilson et al. 1992; Sun and Kamiya 1994; Goto and Pharis 1999). Similarly, male-sterile mutants in tomato, *Brassica* spp. and rice possess altered levels and/or altered metabolism of hormones e.g., GAs, cytokinins, and abscisic acid (ABA), suggesting that stamen and pollen development requires a critical balance of several key hormones (reviewed in Sawhney and Shukla 1994). A recent study with transgenics containing constructs of *CKX1*, a cytokinin oxidase gene, and *GAI*, a gene that regulates sensitivity to GA, has provided molecular evidence in support of the role of hormones in stamen and pollen development (Huang et al. 2003).

Pollen and stamen development is also influenced by environmental factors. In a number of plant species temperature, photoperiod and water stress can affect the expression of male sterility (e.g., Sawhney 1983; Murai and Tsunewaki 1993; Saini 1997) and it has been suggested that these effects may be mediated through changes in endogenous hormones (Singh et al. 1992; Singh and Sawhney 1998). That said, conflicting results have been reported with respect to the role played by environmental factors in different male sterile systems. For example, low temperature restores partial to complete fertility in the *sl-2* mutant of tomato (Sawhney 1983) and in the *msp* mutant of soybean (Carlson and Williams 1985). In contrast, in the *nap* and *pol* male-sterile mutants of *Brassica*, it is high temperature that stimulates normal pollen development (Fan and Stefansson 1986). Thus, the interrelationships of environmental factors and hormones with pollen development are not entirely clear.

A single gene recessive *male sterile33* (*ms33*) mutant in *Arabidopsis*, isolated by EMS mutagenesis, has defects in both stamen filament elongation and pollen development (Dawson et al. 1993). We showed earlier that the inhibition of filament growth in *ms33* was related to inhibition of rapid cell elongation immediately before anthesis, and that exogenous GA or low temperature was able to restore normal filament growth (Fei and Sawhney 1999). Pollen development in *ms33* is impaired near the maturation stage and this was, in part, related to increased vacuolation of pollen grains, likely as a result of inadequate pollen desiccation (Fei and Sawhney 2001).

The objectives of the present study were to determine (1) whether the *ms33* mutant in *Arabidopsis* has pleiotropic effects, i.e., other than those on pollen and stamen development, (2) whether hormones and temperature influence the expression of various phenotypic traits in the mutant, and if so, (3) whether the pleiotropic effects in the *ms33* mutant are related to changes in endogenous hormone content and/or hormone signaling.

Materials and methods

Plant material and growth conditions

Seeds of wild type (WT) *Arabidopsis thaliana* Landsberg *erecta* and the *ms33* mutant, produced by EMS mutagenesis (Dawson et al. 1993), were provided by B. Mulligan of the University of Nottingham, United Kingdom. Seeds of the *spy-3* mutant (Columbia ecotype) were obtained from the Arabidopsis Biological Research Center, Ohio State University, Columbus, Ohio, and seeds of the *aba1-1* mutant (Landsberg *erecta*=ler, ecotype) were obtained from the Nottingham Arabidopsis Stock Centre. Seeds were sown in 15 cm diameter plastic pots containing Tera-lite Redi-earth mix (Grace, Ajax, Ontario, Canada). Pots were then exposed to 4°C in the dark for 3 days prior to transfer to a growth chamber under 22°C/18°C (day/night) temperatures and a 16h/8 h light/dark photoperiod. Fluorescent tubes provided the light source (Osram Sylvania, Versailles, Ky.) at 120–150 μmol m^{-2} s^{-1}.

Seed germination

Fifty seeds of WT and pure line of the *ms33* mutant (obtained from low temperature treatment as described below) were germinated in light or in the dark at a con-

stant 24°C for 7 days in 6 cm diameter Petri dishes lined with two layers of filter paper. Each dish contained 2 ml distilled water (control) or 2 ml of one of the following solutions (all solutions had 0.02% (v/v) Tween-20); 10^{-4} M GA_3, 10^{-3} M GA_3, 10^{-4} M GA_4, 10^{-3} M GA_4, 10^{-4} M paclobutrazol (PP333) and a mixture of 10^{-4} M GA_4 and 10^{-4} M PP333 solution. For low temperature treatments, Petri dishes were exposed to either 4°C or 15°C in the dark for 3 days, and then transferred to 24°C in the dark for germination. Germination for dark experiments was examined under a dim green light. Each experiment was repeated three times.

Analyses of endogenous plant hormones

Sample collection

The mature flowers of *ms33* mutant and WT plants grown at normal temperature (22°C/18°C) and low temperature (15°C/11°C) were collected separately prior to anthesis. The samples were ground in liquid N_2 and freeze-dried and the lyophilized flowers were stored at −80°C until subsequent analysis.

GA analysis

Extraction Dry sample (1 g) was extracted with 20 ml 80% (v/v) aqueous methanol, with the following deuterated internal standards of GAs: $[^2H_2]$-$GA_1$10 ng, $[^2H_2]$-$GA_4$20 ng, $[^2H_2]$-$GA_8$20 ng, $[^2H_2]$-$GA_9$20 ng, $[^2H_2]$-GA_{15}20 ng, $[^2H_2]$-GA_{20}20 ng, $[^2H_2]$ GA_{24} 20 ng, $[^2H_2]$-GA_{44}20 ng and $[^2H_2]$-GA_{53}20 ng, added to the methanolic extract for subsequent quantification of endogenous GAs by the isotope dilution method using gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) for appropriate diagnostic *m*/*z* ions as in Fujioka et al. 1988 (modified by D. Pearce, cited in Zhang 1990). After an initial filtration, the tissue residue was re-extracted twice with 20 ml 80% aqueous methanol. All filtrates were then combined and taken to dryness in vacuo on a rotary flash evaporator at 35°C. The dried residue was subsequently dissolved in 5 ml 80% aqueous methanol for further purification.

Purification The 5 ml sample solution was loaded onto a column filled with 3 g Prep C_{18} (125 Å, 55–105 μm). The column was eluted with 20 ml 80% aqueous methanol. The eluate was collected and dried as above. The residue was then dissolved in methanol and transferred into a 50 ml beaker containing 1 g Celite. After drying with a warm air blower, the Celite + sample was loaded onto a column made of 5 g ICN-deactivated (20% water by weight) Silica (32–100 mesh). This column was eluted with 80 ml ethyl acetate:hexane (95:5, v/v) saturated with 0.5 M formic acid and the eluate was dried (Koshioka et al. 1983). The dried residue was then dissolved in 0.4 ml methanol and 0.6 ml 1% (v/v) aqueous acetic acid, then the $[^3H]$-GAs ($[^3H]$-GA_1, $[^3H]$-GA_4 and $[^3H]$-GA_9, about 3 million Bq each) were added as radiotracers. The sample solution was then subjected to reversed phase high performance liquid chromatography (HPLC) with a Radial-Pak μBondapak C_{18} cartridge (RCM, 8×100 mm, 5 μm, Waters, Milford, Mass.). After the sample was injected, the column was eluted isocratically with a solvent mixture of 10% methanol and 100% methanol (40:60, v/v) for 40 min at a flow rate of 1 ml/min. The eluate was collected in 1 ml fractions and a 10 μl aliquot of each fraction was mixed with 5 ml scintillation cocktail to detect radioactivity. The fractions were then grouped based on peaks of radioactivity. All fraction groupings were dried separately and each residue was subsequently dissolved in 1 ml methanol:acetic acid (99.9:0.1 v/v). The samples were further purified using a Nucleosil $N(CH_3)_2$ HPLC column (4.6×150 mm, 5 μm; Alltech, Deerfield Ill.), eluted with methanol:acetic acid (99.9:0.1 v/v) for 40 min at a flow rate of 1 ml/min. The eluate was collected as 1 ml fractions and the radioactivity in each fraction was measured and combined as above, then dried.

Methylation and silylation of GAs

GA_9 and GA_{15} were methylated with diazomethane. GA_1, GA_3, GA_4 and GA_{20} were first methylated with diazomethane, and then derivatized with bis-trimethylsilyltrifluoroacetamide (BSTFA). The methylation procedure was accomplished by first dissolving the sample in 10 ml methanol, then adding 90 ml ethereal CH_2N_2. The mixture was left at room temperature for 15 min and the solvent removed under a flow of N_2. For silylation, the samples were dissolved in 50 μl GC-MS grade pyridine followed by 50 μl BSTFA with 1% trimethylchlorosilane (TMCS). The reaction vial was flushed with N_2 then left at 80°C for 30 min. The samples were dried under a flow of N_2.

Quantitative analysis

Each GA was quantified by GC-MS-SIM by dissolving each sample in two drops of hexane and a 2 μl aliquot was then introduced into a GC-MS [Hewlett Packard (HP) 5890 II; MS, HP 5970 A] by on-column injection into a retention gap of a 0.5 m ×0.32 mm deactivated fused silica capillary DB1-15 N column (15 m ×0.25 mm, 0.25 μm methyl silicone film). The oven was heated from 60°C to 200°C at 20°C/min and from 200°C to 280°C at 5°C/min. Data acquisition was controlled by a HP 300 Series computer.

Analyses of indole-3-acetic acid and abscisic acid

Extraction and purification

The procedures for extraction and purification by a reversed-phase C_{18} open column for indole-3-acetic acid

(IAA) and abscisic acid (ABA) were the same as those used for GAs, except that 200 ng [$^{13}C_6$]-IAA and 100 ng [$^{2}H_6$]-ABA were added as quantitative stable isotope labeled internal standards.

The dry residue from the last step was dissolved in 10 ml 1% acetic acid. The solution was partitioned with 10 ml ethyl acetate (saturated with 2% acetic acid) three times. The ethyl acetate solutions were combined and dried. The residue was dissolved in 1 ml methanol and 1 ml 1% acetic acid. About 3 million Bq each of [^{3}H]-IAA and [^{3}H]-ABA of high specific radioactivity was added as a radiotracer for use in locating fractions after HPLC. The combined fraction expected to contain ABA and IAA was loaded onto the Sep-Pak C_{18} Cartridges with a syringe. The column was eluted with 15 ml 50% aqueous methanol, and the eluate collected and dried. Subsequently it was dissolved in 0.1 ml methanol, and then mixed with 0.9 ml 1% acetic acid. The sample solution was injected into HPLC with a C_{18} RCM (see above) column (8×110 mm, 5 μ). The solvent system for elution was 10% methanol in Pump A and 100% methanol in pump B at a flow rate of 2 ml/min. The following elution program was set: 0–10 min, 100% of pump A; 10–40 min, 30% of pump A and 70% of pump B with a linear gradient; 40–50 min, 0% pump A and 100% pump B. The first four fractions were 10 ml, followed by three fractions of 5 ml, five fractions of 2 ml and seven fractions of 5 ml. The radioactivity in each fraction was measured as above and radioactive fractions combined and dried as detailed above.

Methylation and quantification

IAA and ABA were methylated with diazomethane and quantified by GC-MS-SIM using the isotope dilution method as detailed above for GAs. All equipment used was the same as that for GAs. The temperature in GC was programmed from 15°C to 195°C at 15°C/min, and from 195°C to 275°C at 5°C/min.

Construction of double mutants

Homozygous recessive *ms33* mutant plants were crossed with homozygous recessive *aba1-1* and *spy-3*mutants, separately, to generate *ms33*/*ms33 aba1-1*/*aba1-1* and *ms33*/*ms33 spy-3*/*spy-3* double mutants. The F_2 seeds were collected and sown in pots. Novel phenotypes with characteristics of both the parent mutants were identified from the F_2 population. Other phenotypes in the F_2 population were also scored. Chi-square analysis was used to determine the significance of the dihybrid ratio (9:3:3:1).

Application of GA_4

A 10^{-4} M GA_4 solution containing 0.02% Tween-20 (v/v) was sprayed to drip off, twice a week for 2 weeks, onto the *ms33* plants, beginning ca. 1 week prior to anthesis.

Results

Vegetative growth and flowering in *ms33* and WT

WT seeds sown under normal growth conditions (22°C/18°C and 16h/8 h photoperiod, day/night) began to germinate after 2 days (see below). After 3 weeks of vegetative growth, when the ninth leaf had emerged, plants began to bolt and differentiate flowers. Each plant produced a primary inflorescence and 4–5 secondary inflorescences. Subsequently a number of siliques were developed. The major contributor to plant height was the peduncle.

For the *ms33* mutant seeds (obtained from plants grown under the low-temperature treatment) there was a delay in germination, which led to a delay in vegetative growth and flowering by approximately 5 days. However, if *ms33* seeds were stratified at 4°C for 3 days, the vegetative growth and flowering time of *ms33* plants were similar to WT plants. There were no gross phenotypic differences between the vegetative organs of *ms33* and WT plants that developed from seeds exposed, or not exposed, to 4°C prior to germination.

Morphology of *ms33* and WT flowers

In *ms33* flowers all the floral organs were present, and in the same order, as in WT flowers. Before anthesis, the petal and stamen lengths of *ms33* floral buds were similar to WT, but their sepals and gynoecium were longer than in WT and the gynoecium protruded through the buds (Table 1, Fig. 1a, b). Immediately before anthesis, there was rapid growth of petals in WT flowers and the long stamens extended beyond the level of the stigma at anthesis (Fig. 1c). However, in *ms33* floral buds petal elongation was delayed by approximately 7 days and growth of stamens was inhibited such that at maturity

Table 1 The length (cm) of floral organs in wild type (WT) and *ms33* buds before opening, and in mature flowers at anthesis. Each value is a mean ± SE ($n = 20$) for each organ type

Growth stage	Organ type	WT	*ms33*
Unopened Floral bud	Sepal	2.01 ± 0.02	2.14 ± 0.02*
	Petal	1.81 ± 0.03	1.75 ± 0.02
	Long median stamen	1.36 ± 0.02	1.39 ± 0.02
	Gynoecium	1.89 ± 0.02	2.02 ± 0.02*
Mature flower	Sepal	2.04 ± 0.03	2.18 ± 0.02*
	Petal	3.39 ± 0.04	3.35 ± 0.04
	Long median stamen	2.87 ± 0.02	1.72 ± 0.02*
	Gynoecium	2.44 ± 0.05	3.32 ± 0.06*

*Significantly different from WT at $P < 0.01$

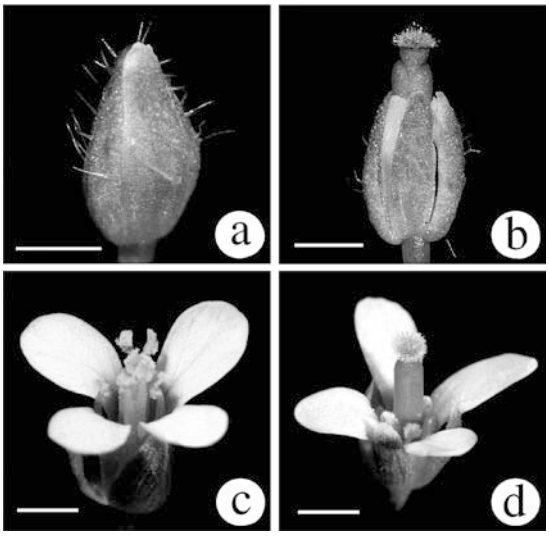

Fig. 1a–d Wild type (WT) and *ms33* floral buds and mature flowers. **a** WT floral bud just before anthesis. **b** *ms33* floral bud showing gynoecium growth before opening. **c** Mature WT flower with long stamens. **d** Mature *ms33* flower with short stamens. *Bar* 1 mm

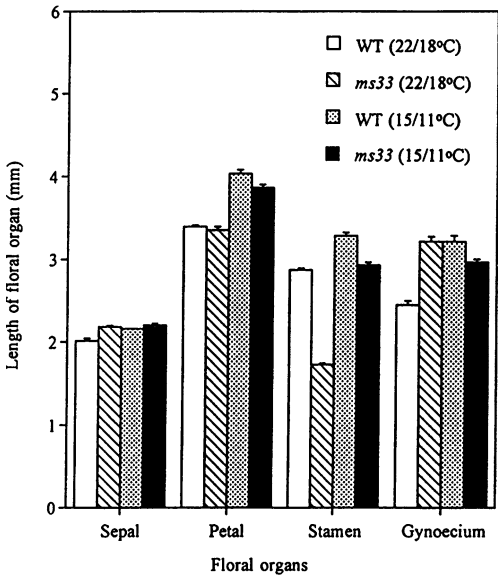

Fig. 2 Lengths of floral organs of WT and *ms33* plants grown at 22°C/18°C or 15°C/11°C (day/night). Each value is a mean of 20 floral organs from as many flowers. *Bars* SE

the stamens barely reached the mid-position of the gynoecium (Fig. 1d). A comparison of floral organs at anthesis showed no difference in final petal lengths between *ms33* and WT flowers. However, the mutant stamens were significantly shorter, and sepals and gynoecium longer, than the corresponding WT organs (Table 1, see also Fei and Sawhney 1999).

The low temperature treatment, i.e., 15°C/11°C, had different effects on the growth of floral organs in *ms33* and WT. Although the growth of sepals was unaffected by low temperature for both types of flowers, the petal length increased in both *ms33* and WT by ca. 15% and 18%, respectively (Fig. 2). For stamen length there was a much greater increase in the mutant (74%), compared to only a 10% increase for the WT, and the mutant stamen length at low temperature was similar to that of WT at normal temperatures (see also Fei and Sawhney 1999). In contrast, carpel length was reduced in *ms33* flowers by about 8% and increased in WT flowers by 30% (Fig. 2).

Seed germination

In light, WT seeds showed approximately 10% germination after 2 days at 24°C, with maximum (100%) germination at 4 days. However, the germination of *ms33* seeds was delayed for 3 days and maximum germination (98% average) was reached at day 7 (Fig. 3a). In the dark, germination was delayed for both WT and *ms33* seed, e.g., after 7 days maximum germination in WT was, on average, 66% and in the mutant 28% (Fig. 3a).

Pretreatment of WT and *ms33* seeds at either 15°C or 4°C under moist conditions for 3 days followed by 24°C in the dark resulted in enhanced germination in both genotypes. WT seeds exposed to 15°C showed approximately 75% germination at 2 days in the dark in comparison to no germination in the controls, i.e., 24°C (Fig. 3b). Similarly, *ms33* seeds exposed to 15°C showed approximately 55% germination after 2 days versus no germination under 24°C. However, after 7 days the germination percentage for *ms33* seeds pretreated at 15°C increased to 63% compared to 28% in the control (24°C) seeds. Seeds exposed to 4°C showed reduced germination relative to the 15°C treatment for both WT and *ms33* seeds, but germination was still higher than that at normal (24°C) temperatures (Fig. 3b).

As expected, applied GAs stimulated seed germination in the dark for both WT and *ms33* seeds. WT seeds treated with 10^{-4} M GA_3showed a small increase in germination compared to the control after 1 week (Fig. 3c), but at 10^{-3} M GA_3 germination was enhanced to 100% at day 3. However, GA_4 was much more effective in stimulating germination than GA_3 at the same concentration, e.g., with 10^{-3} M or 10^{-4} M GA_4, 100% germination was obtained in WT seed after 2 and 3 days, respectively (Fig. 3c).

GAs also stimulated the germination of *ms33* seeds in the dark. After 1 week, 10^{-4} M GA_3 increased the germination to approximately 40%, and 10^{-3} M GA_3 to 90%, compared to 28% for the dark control (Fig. 3d). In contrast, with 10^{-3} M or 10^{-4} M GA_4, *ms33* seeds showed 95% germination at 3 and 5 days, respectively (Fig. 3d). Thus, GA_4, even at a low concentration, both enhanced the rate and increased the final percent germination compared to GA_3, for both WT and *ms33* seeds.

The germination of both the WT and *ms33* seeds was totally inhibited by treatment with 10^{-4} M PP333, an

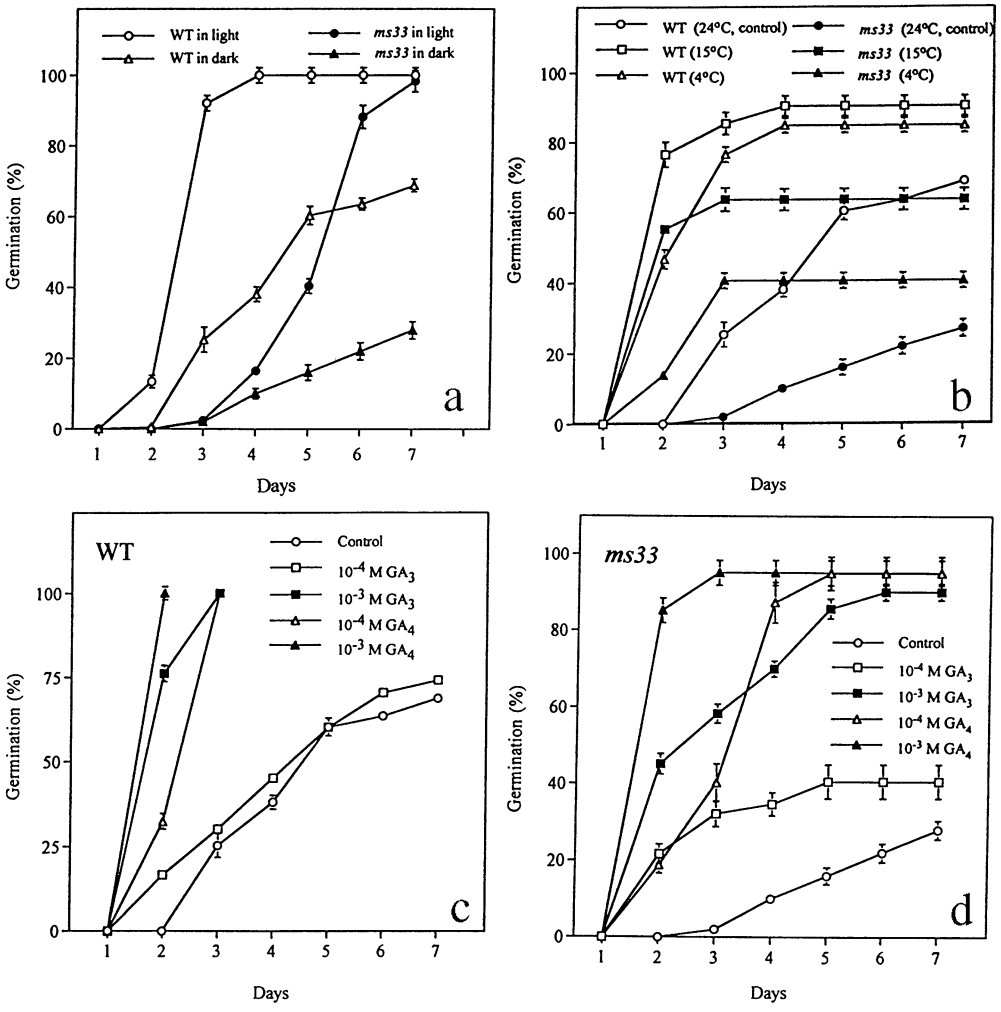

Fig. 3a–d Germination of WT and *ms33* seeds with different treatments at 24°C for 7 days. **a** WT and *ms33* seeds germinated in white light and in the dark. **b** WT and *ms33* seeds were stratified either at 15°C or 4°C for 3 days, and then germinated in the dark. **c** WT seeds germinated in the presence of different concentrations of gibberellin A3 (GA_3) or GA_4 in the dark. **d** *ms33* seeds germinated in the presence of different concentrations of GA_3 or GA_4 in the dark. Fifty seeds were sown for each treatment. Each value is a mean of three replicates. *Bars* SE

inhibitor of GA biosynthesis. This inhibition could be entirely overcome by the addition of 10^{-4} M GA_4, but only partially by 10^{-4} M GA_3 (Fig. 4).

Endogenous GAs in *ms33* and WT flowers

A profile of endogenous GAs in WT and *ms33* flowers for plants grown at normal temperatures included gibberellins $A_{1,4,8,9,15,20,24}$(GA_{44} and GA_{53} were undetectable in both WT and *ms33* flowers). In WT flowers, GA_4 was the major GA followed by GA_{24}, GA_{15}, GA_8, GA_9 and GA_1 (Table 2). In *ms 33* flowers most of the GAs were reduced in amount, relative to WT, except for GA_8, an inactive metabolite of GA_1. In particular, GA_4 levels were 12-fold lower in *ms33* than in WT and GA_9 and GA_{15}, two GA_4 precursors, were 6- and 8-fold lower in *ms33* than in WT (Table 2).

In low-temperature-grown WT flowers, GA levels, especially GA_1, GA_4 and GA_{20}, declined relative to GAs in WT flowers grown at normal temperature (Table 2). In contrast, in *ms33* flowers grown at low temperature, GA_4 levels increased relative to those in *ms33* flowers from normal temperatures. The levels of other GAs in

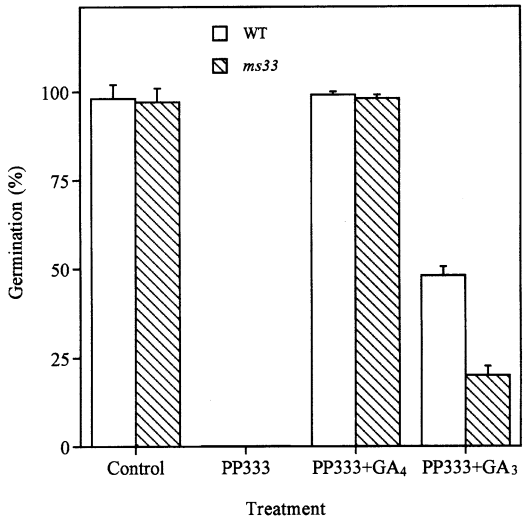

Fig. 4 Germination percent of WT and *ms33* seeds in H_2O (control), 10^{-4} M paclobutrazol (PP333) plus 10^{-4} M GA_4, or 10^{-4} M PP333 plus 10^{-4} M GA_3 in light at 24°C for 7 days. Fifty seeds were germinated for each treatment. Each value is a mean of three replicates. *Bars* SE

Table 2 Levels (ng/gDW) of endogenous gibberellins (GA) in mature flowers from WT and from the *ms33* mutant. Plants were grown at normal (22°C/18°C) or low (15°C/11°C) temperatures. Standard errors of the mean (where shown) are from two* or three replicates. *GC-MS-SIM* Gas chromatography-mass spectrometry-selected ion monitoring, *HPLC* high performance liquid chromatography

GA	WT		*ms33*	
	22°C/18°C	15°C/11°C	22°C/18°C	15°C/11°C
GA_1	4.87 ± 3.22	1.14 ± 0.26	1.34 ± 0.19	0.80 ± 0.07
GA_4	21.36 ± 6.91	8.87 ± 2.02	1.87 ± 0.09	2.96 ± 1.03
GA_7	nd[a]	nd	nd	nd
GA_8	7.95 ± 2.05*	–[c]	8.1	–
GA_9	6.56 ± 0.35	5.44 ± 2.39	0.96 ± 0.11	0.75 ± 0.47
GA_{15}	8.45 ± 1.45*	6.07 ± 1.45*	1.00 ± 0.9*	0.51 ± 0.51*
GA_{19}	Lost[b]	–	Lost	–
GA_{20}	1.6	0.35 ± 0.05	nd*	0.25 ± 0.15
GA_{24}	9.7	–	3.2	–
GA_{44}	nd	–	nd	–
GA_{53}	nd	–	nd	–

[a]Endogenous GA not detected by GC-MS-SIM, despite ready detection of [17,17-$^2H_2$]-labeled GA internal standard
[b]GA_{19} fraction was lost at HPLC stage of workup
[c]Endogenous GAs not assessed in low-temperature-grown samples

ms33 flowers at low temperature tended to be reduced, or were no different, than in flowers grown at normal temperature. Thus, low temperature severely reduced GA levels in WT flowers, but had mixed effects on GA levels in *ms33* flowers.

Endogenous IAA and ABA in *ms33* and WT flowers

WT flowers from plants grown at normal temperatures contained strikingly higher IAA levels, approximately 6-fold higher, than *ms33* flowers from plants grown at normal temperatures (Fig. 5a). However, at low temperatures the IAA content of WT flowers was reduced to less than one-half of that found in WT flowers grown under normal temperatures (Fig. 5a). Even so, IAA content in WT flowers of low temperature plants was still two times higher than that in *ms33* flowers grown at normal or low temperatures (Fig. 5a). In *ms33* flowers, IAA levels were not affected by low temperature (Fig. 5a).

The relative content of ABA was lower in WT than in *ms33* flowers at normal temperatures (Fig. 5b). At low temperature the levels of ABA in WT flowers increased by approximately 55%, but there was little effect of low temperature on ABA levels in *ms33* flowers, thus resulting in near equal levels for the mutant and WT flowers grown at low temperature (Fig. 5b).

Double mutants

The differences in endogenous hormones levels in WT and *ms33* mutant flowers grown at normal and low temperatures suggested that the *ms33* mutation affects

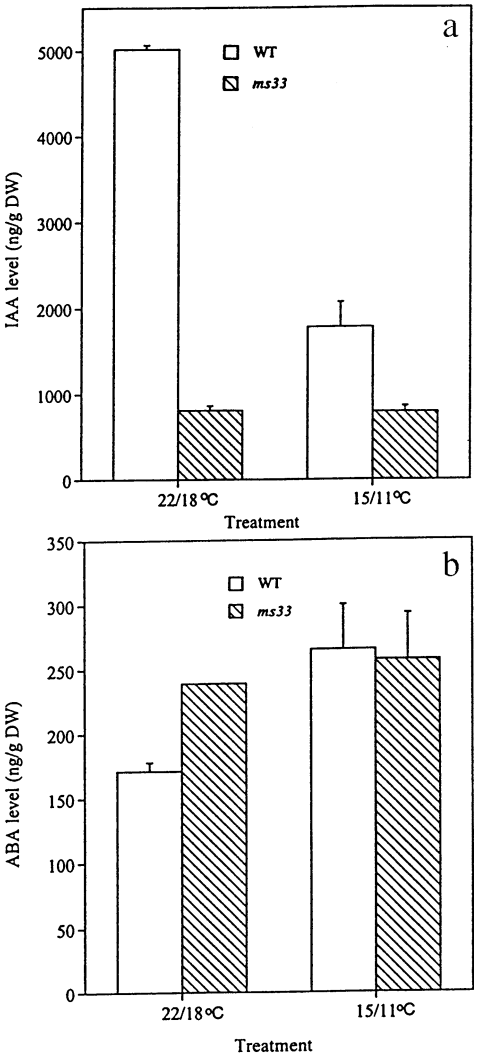

Fig. 5a,b The relative levels of endogenous indole-3-acetic acid (IAA) and abscisic acid (ABA) in mature flowers of WT and *ms33* plants grown at different temperatures. **a** IAA,**b** ABA. Each value is a mean of two or three replicate samples. *Bars* SE

either the biosynthetic (or catabolic) or signal transduction pathways of hormones, especially for GAs. A genetic approach was used to further investigate the role of hormones by constructing double mutants of *ms33* with the following *Arabidopsis* mutants, *spindly-3* (*spy-3*), a GA-signal transduction mutant (Jacobsen and Olszewski 1993), and *aba1-1*, an ABA-deficient mutant (Koornneef et al. 1982).

ms33 aba1-1

The *aba1-1* (Landsberg ecotype background) mutant has low ABA content and is characterized by reduced peduncle length and plant height, an increase in transpiration rate and wilting of plants even under normal temperatures (Koornneef et al. 1982). Under our growth conditions the mean height of *aba1-1* plants was 7.6 ± 0.5 cm ($n = 30$ plants), compared to 23.0 ± 0.2 cm

($n = 30$ plants) for WT (see also Fig. 6a). However, the floral phenotype of the *aba1-1* mutant was similar to WT, and the flowers were both male- and female-fertile (data not shown). As described earlier, the height (primarily peduncle length) of the *ms33* mutant is similar to WT, but the flowers have short stamens, they produce non-viable pollen, and have a higher level of ABA at normal temperatures than the WT.

The double mutant *ms33 aba1-1* was identified in the F_2 progeny. In a total of 923 F_2 plants, four phenotypes were identified: 543 tall plants which had long stamens in their flowers and normal silique development with seeds (WT), 172 tall plants with short stamens and no silique development (*ms33*), 154 short plants which had long stamens in their flowers, normal silique development, but were wilty (*aba1-1*), and 54 plants of a novel additive phenotype that were short, wilty, had flowers with short stamens (Fig. 6b) and showed no silique development (*ms33 aba1-1*) (Fig. 6a). The ratio of these phenotypes was 9.8:3.1:2.8:1 ($X^2 = 3.43$, $P > 0.25$).

ms33 spy-3

The *spy-3* (Columbia ecotype) is a GA signal transduction mutant and the phenotype of a homozygous recessive *spy-3* plant is similar to WT plants treated with GAs, i.e., plants exhibit long hypocotyls, elongated peduncles, light green leaves and early flowering (Jacobsen and Olszewski 1993). Under our normal temperature conditions, the mean height of *spy-3* plants was 52.2 ± 0.4 cm ($n = 30$ plants), compared to 32.9 ± 0.65 ($n = 30$ plants) in the WT (Columbia) (see also Fig. 6c). The height of *ms33* was 24.6 ± 0.3 cm ($n = 30$ plants) at maturity.

In 1,472 plants of the F_2 progeny from a cross of *ms33* and *spy-3*, four different phenotypes were identified: 844 plants were of normal height and produced siliques with seeds (WT), 257 plants were of normal height, but their flowers had shortened stamens and were male sterile (*ms33*), 286 plants were tall and produced siliques and seeds (*spy-3*), and 85 plants were tall and male sterile with shortened stamens in their flowers (Fig. 6d). The last category was novel and showed the additive phenotype of *ms33* and *spy-3* mutations (Fig. 6c, d). The ratio of phenotypes of these plants was 9.8:3.0:3.3:1.0 ($X^2 = 2.36$, $P > 0.25$).

Restoration of male fertility in *ms33*

The *ms33* plants were grown at the following temperatures [(day/night (°C)]: 12/10, 15/11, 18/15, 30/24 and 22/18 (control) with a photoperiod of 16/8 h (day/night). Only under 15°C/11°C was there a partial reversion to male fertility in *ms33* flowers, as evidenced by the development of some siliques with seeds. However, the number of siliques developed on *ms33* plants varied and approximately 43% of the mutant plants (in a population of 180 plants) produced seeds. WT plants were unaffected, in terms of silique and seed development, by these low (15°C/11°C) temperatures. A partial restoration of male fertility was also obtained when GA_4 (10^{-4} M) was applied to *ms33* plants under normal (22°C/18°C) temperature conditions. The seeds produced from both these treatments were sown under normal temperature conditions, and all plants that developed showed the *ms33* phenotype. Thus, both applied GA_4 and low temperatures could give a partial restoration of male fertility in the *ms33* mutant.

Discussion

The phenotype of the *ms33* mutant in *Arabidopsis* was described earlier as exhibiting inhibition of stamen filament growth and abnormalities in pollen development (Dawson et al. 1993; Fei and Sawhney 1999, 2001). Here we show that the *ms33* mutant has other pleiotropic effects i.e., aberrant growth of all floral organs, and delayed seed germination and flowering time. However, there were no apparent differences in the morphology of vegetative parts, i.e., leaves and stem, of the WT and *ms33*. Although the growth of all floral organs was affected in *ms33*, the effects were different, i.e., delayed petal expansion, inhibition of stamen filament growth, but enhanced sepal and gynoecium growth. Thus, the *MS33* gene function is required for normal growth of all floral organs, not just for stamens. As reported elsewhere, the inhibition of stamen filament growth in *ms33* was caused by inhibition of cell elongation prior to anthesis, and this effect could be rescued by low temperature or exogenous GA, suggesting that the mutant may have a defect in GA biosynthesis or signaling (Fei and Sawhney 1999). Similarly, in the *ga1-1* mutant in *Arabidopsis*, the stunted growth of the stamen filament could be restored by exogenous GA (Goto and Pharis 1999). The delay in corolla expansion in *ms33* may also be due to a delay in cell elongation caused by a defect in endogenous GAs since in other systems corolla expansion is known to involve rapid cell elongation immediately before anthesis and this process is regulated by GAs (reviewed in Greyson 1994; Goto and Pharis 1999). At low temperature, the growth of all mutant floral organs was comparable to the WT; thus the effects of low temperature may also be mediated through changes in endogenous GAs (discussed below).

The *ms33* mutation also delayed seed germination and flowering time for plants grown at normal temperatures. Kinetic studies showed that both in light and dark seed germination was delayed in *ms33*, relative to WT. In the dark, both the rate of germination and percentage germination were reduced for both WT and *ms33*, compared to in the light. Exposing seeds to low temperature before germination enhanced the rate of germination as well as the final percentage germination in both WT and *ms33* seeds in the dark, with the 15°C treatment yielding a better response than 4°C. In *Ara-*

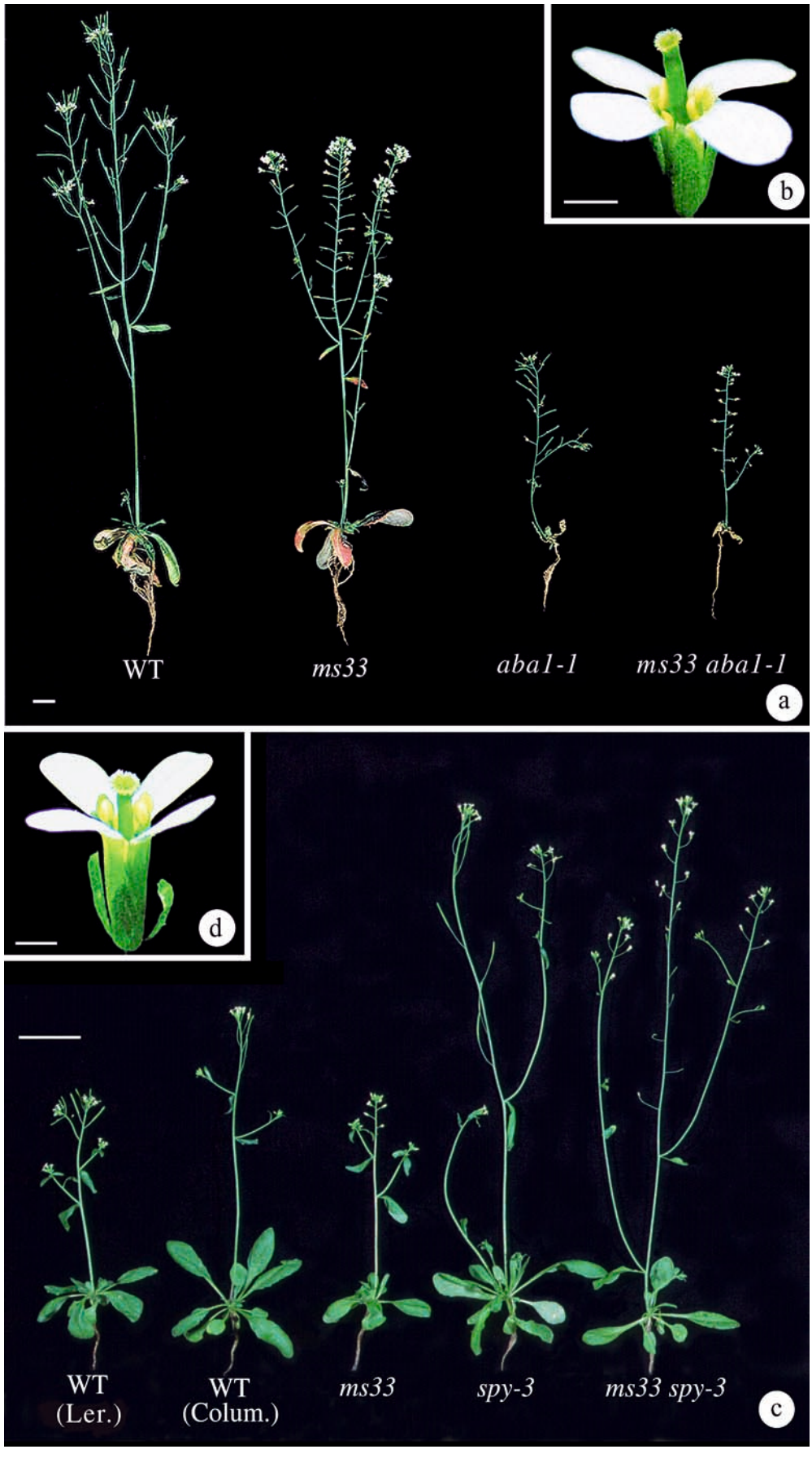

Fig. 6 a Representative plants of WT, *ms33*, *aba1-1* and *ms33 aba1-1* identified from the F_2 generation after 6 weeks of growth. **b** A double mutant *ms33 aba1-1* flower with shortened stamens. **c** Representative plants of WT (Landsberg), WT (Columbia), *ms33*, *spy-3* and *ms33 spy-3* identified in the F_2 generation after 30 days of growth at normal conditions. **d** Double mutant *ms33 spy-3* flower showing shortened stamens *Bars* **a** 1 cm; **b**, **d** 1 mm; **c** 5 cm

bidopsis, it is common to stratify seeds with 4°C for increased germination (Weigel and Glazebrook 2002) but, as our results show, 15°C treatment is better than 4°C. Low temperature is known to increase endogenous levels of GAs (Hazebroek et al. 1993; Ma et al. 1996) and it also enhances the responsiveness of *Arabidopsis* seeds to applied GA (Derkx and Karssen 1993). Thus, in the *ms33* mutant the increase in germination at low temperature may be due to an increase in endogenous GAs and/or enhanced GA signaling.

Exogenous GAs can often replace the need for environmental stimuli, e.g., temperature pretreatment or the requirement of light for germination. This suggests that endogenous GAs are important intermediates in the environment-induced stimulation of germination. The absolute dependence on applied GA for germination of both the *ga1-1*mutant of *Arabidopsis*, and the *gib-1* mutant of tomato also strongly favors a key role of GA in the control of germination (Karssen et al. 1989). In WT and *ms33* seed, we found that GA_4 was more effective than GA_3 in promoting seed germination (Fig. 3c, d). Treatment of seed with PP333 (Fig. 4), an inhibitor of GA biosynthesis, further supports the suggestion that $GA_4$is a more effective promoter of seed germination than GA_3 in both WT and *ms33* seed.

Analysis of endogenous GAs showed that the major GA in WT flowers was GA_4, which is also a major GA in *Arabidopsis* leaves (Talon et al. 1990), seeds (Derkx et al. 1994) and germinating seeds (Ogawa et al. 2003). WT flowers contained 12 times more GA_4 and 4 times more GA_1 than *ms33* flowers. Relatively high levels of GAs have been associated with normal flower development, in particular stamen and petal development (e.g., Murakami 1975; Dathe and Sembdner 1980). Conversely, some male-sterile mutants are known to contain relatively low levels of GAs compared to their WT counterparts (Sawhney 1974; Nakajima et al. 1991), and in male-sterile anthers of rice, GA_4 was specifically low (Nakajima et al. 1991). Further, applied GAs are known to induce fertility in some male-sterile mutants of tomato, barley, and *Cosmos*, and in GA-deficient, male-sterile mutants in tomato (reviewed in Sawhney and Shukla 1994). Thus, there is a good correlation of low endogenous GAs with male sterility. Since exogenous GA_4 can partially restore fertility in the *ms33* mutant and since low temperature also partially restores fertility and increases GA_4 levels in *ms33*, these findings lend support to the view that low levels of GA_4 are at least the partial cause of male sterility in *ms33*.

The *ms33* flowers also contained low levels of IAA and high level of ABA compared to WT. Auxins have been shown to have a role in flower development and in petal growth (Moe 1971; Kopecewicz et al. 1979). Applied ABA is known to induce male sterility in some species (reviewed in Sawhney and Shukla 1994) and a male-sterile mutant in tomato contains high ABA content (Singh and Sawhney 1998). Thus, a low level of IAA and high ABA could be possible factors in the delay of petal expansion, pollen abortion and inhibition of stamen filament growth in *ms33* flowers. However, relative to WT, the mutant had only a modest increase (25%) in ABA levels. Hence, it does not seem likely that elevated ABA levels is causal for the phenotypic lesions in mutant flowers. Further, the double mutant *ms33 aba1-1* showed the male-sterile phenotype suggesting that reduction in ABA content alone is not sufficient for the restoration of fertility in *ms33*.

The low level of GA_4 and its precursors in *ms33* flowers suggests that the *ms33* mutation may alter GA biosynthesis. In *Arabidopsis*, there are at least two major GA biosynthetic pathways from GA_{12}, the first GA in GA biosynthesis in plants (Talon et al. 1990; Finkelstein and Zeevaart 1994; Sponsel 1995): (1) the early 13-hydroxylation of GA_{12} that leads to GA_{53}, GA_{44}, GA_{19}, GA_{20}, and GA_1, and (2) the early non-hydroxylation of GA_{12} leading to GA_{15}, GA_{24}, GA_9 and GA_4 (GA_{20} can also originate from GA_9, and GA_1 from either GA_{20} or GA_4). It thus appears that in both WT and mutant flowers only the early non-13-hydroxylation pathway is operative. Further, since in the *ms33* mutant GA_4 level is severely reduced (Table 2), we conclude that a product of the *MS33* gene allows GA biosynthesis within this pathway to be maintained at a high level, thereby maintaining high levels of GA_1 and especially GA_4.

While low temperature reduced levels of GA_4 by 2.5-fold in WT flowers, these lowered levels are still relatively high (almost 10 ng/g DW), and WT plants grown under low temperature produce normal flowers. Hence, levels of 10–23 ng/g DW of GA_4 in WT must be sufficient to support normal floral organ growth. These results are consistent with the conclusion that GA_4 is an important native GA in a wide range of developmental processes in *Arabidopsis*, especially floral organ growth (Goto and Pharis 1999).

The question of whether GA signaling rather than GA biosynthesis is affected in the *ms33* mutant was addressed by constructing a double mutant of *ms33* with a GA signal transduction mutant *spy-3*. The *spy-3* mutant has the same phenotype as WT plants treated with GA, i.e., tall plants and early flowering (Jacobsen and Olszewski 1993). In the double mutant *ms33 spy-3*, the phenotype of plants was similar to *spy-3*, i.e., long peduncles and early flowering. Additionally, the phenotype of the double mutant flowers was similar to that of *ms33*, i.e., flowers were male-sterile and stamen length was inhibited. These results show that the stimulation of the GA signaling pathway by *spy-3* does not overcome the inhibition of stamen filament growth and the abortion of pollen development. Thus, the expression of male sterility in *ms33* seems likely not to be related to a blockage in the GA signal transduction pathway.

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