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Plants with Increased Expression of *ent*-Kaurene Oxidase are Resistant to Chemical Inhibitors of this Gibberellin Biosynthesis Enzyme

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The gibberellin (GA) biosynthetic pathway includes the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid, catalyzed by the enzyme *ent*-kaurene oxidase (KO). *Arabidopsis* plants overexpressing the *KO* cDNA under the control of the cauliflower mosaic virus 35S promoter, with or without a translational fusion to a modified green fluorescent protein (GFP), are very similar to wild-type (WT) plants under normal growth conditions. In contrast, when WT and 35S:KO (or 35S:KO–GFP) seeds, seedlings or pollen tubes are grown in the presence of chemical inhibitors of KO, such as paclobutrazol and uniconazole, plants with increased *KO* expression are partially resistant to the effects of these inhibitors. In combination with the observation that decreased KO levels increase the sensitivity to KO inhibitors, the 35S:KO phenotypes demonstrate that the modification of KO enzyme levels could be used to create transgenic crop plants with altered KO inhibitor response. These results also suggest that the *KO* gene could be used as a selectable marker for plant regeneration based on resistance to KO inhibitors. Finally, the observation that pollen tubes expressing 35S:KO or 35S:KO–GFP have decreased sensitivity to KO inhibitors provides further evidence for a physiological role for GAs in pollen tube elongation.

Keywords: *Arabidopsis* — *ent*-kaurene oxidase — Gibberellin — Pollen tubes — Plant growth regulator.

Abbreviations: CPS, *ent*-copalyl diphosphate synthase; GA, gibberellin; GC-SIM, gas chromatography-selected ion monitoring; GFP, green fluorescent protein; KO, *ent*-kaurene oxidase; KS, *ent*-kaurene synthase; WT, wild-type.

Introduction

Endogenous plant hormones influence many aspects of growth and development. One of the best characterized of these hormones, at least in terms of biosynthesis and physiology, are the gibberellins (GAs). In general, GAs act as growth promoters and are required for processes such as seed germination, leaf and stem elongation and various aspects of reproductive development.

The GA biosynthesis pathway has been investigated in depth, and most of the genes encoding biosynthetic and catabolic GA enzymes have now been identified. A simplified version of the GA biosynthesis pathway that occurs in *Arabidopsis* is shown in Fig. 1. Chemical inhibitors of various steps in this pathway have been developed, and have a number of applications in agriculture and plant research. Some of the best known inhibitors include paclobutrazol and uniconazole, which are closely related chemically, and are thought to interact directly with the *ent*-kaurene oxidase (KO) enzyme thereby reducing its activity (Sugavanam 1984, Izumi et al. 1985). KO is a multifunctional cytochrome P450 enzyme that catalyzes the three intermediate steps of the GA pathway from *ent*-kaurene to *ent*-kaurenoic acid. Loss-of-function mutations have been identified in both the *Arabidopsis* (*GA3*) and pea (*LH*) genes encoding KO (Helliwell et al. 1998, Davidson et al. 2004). Mutations in the *Arabidopsis GA3*/*KO* gene lead to a non-germinating, dwarf, male-sterile phenotype. By contrast, in pea the *lh* mutants have normal seed germination, are dwarf, male-fertile, and have reduced seed size and increased seed abortion (Swain et al. 1997). Some of this variation may reflect differing roles for GAs in the two species, for example in seed germination, while some may be due to the existence of other genes that encode proteins with KO activity. The recent molecular identification of the *GA3*/*KO* gene allowed Helliwell et al. (Helliwell et al. 1998, Helliwell et al. 1999) to confirm that the *ga3-1* and the *ga3-2* mutations represent what appears to be the only gene encoding KO in *Arabidopsis*. A genomic clone containing the *GA3* gene and a construct in which the cauliflower mosaic virus 35S promoter drives expression of the *KO* cDNA were both used to complement the *ga3-2* allele. Although this result demonstrates that both transgenes encode a functional KO protein, no phenotypes suggesting an increase in GA levels compared with wild-type (WT) plants were identified (Helliwell et al. 2001).

In addition to the phenotypes described above, the pea *lh-2* mutant also displays altered vegetative sensitivity to paclobutrazol, uniconazole and other KO inhibitors. Significantly, of the three *lh* alleles examined, only *lh-2* has increased sensitivity to paclobutrazol, while *lh-1* and *lh-3* plants, and mutants with defects in other GA biosynthesis enzymes, respond to paclobutrazol in the same way as WT plants (Swain et al. 1997, Davidson et al. 2004). The recent confirmation that

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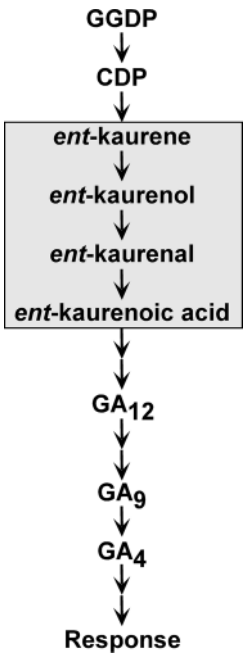

Fig. 1 GA biosynthesis pathway. In *Arabidopsis* seedlings, the non-13 hydroxylation pathway of GA biosynthesis predominates, leading to the formation of the active GA_4. The shaded box indicates the steps catalyzed by KO. These steps are also the target of structurally related GA biosynthesis inhibitors such as paclobutrazol, uniconazole, inabenfide and ancymidol. GGDP, geranyl geranyl diphosphate; CDP, *ent*-copalyl diphosphate.

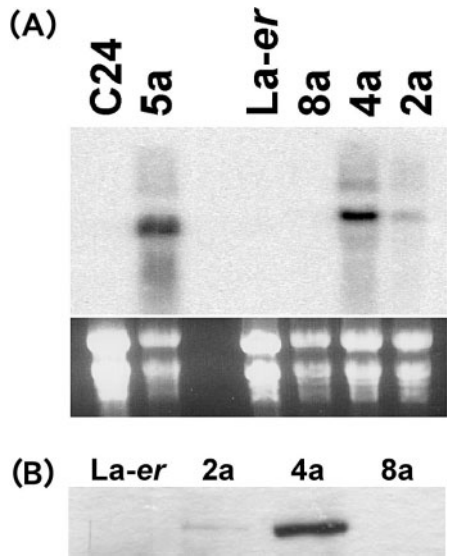

Fig. 2 Northern and Western analysis of lines overexpressing the *ent*-kaurene oxidase gene. (A) Young seedlings were used for Northern analysis with *KO* as a probe. The genotypes (from left to right) are: WT C24, 35S:KO/5a (C24 background), WT La-*er*, 35S:KO–GFP/8a, 35S:KO–GFP/4a and 35S:KO–GFP/2a (all in a La-*er* background). The lower panel shows rRNA for comparison of RNA loading in each well. (B) Western blot using an anti-GFP antibody on protein extracts from WT and 35S:KO–GFP plants.

LH encodes KO (Davidson et al. 2004) suggests that the increased sensitivity of *lh-2* plants to KO inhibitors is the result of reduced KO protein levels. In contrast, this model suggests that although KO activity is reduced in *lh-1* and *lh-3* plants, protein levels are similar to WT. The mutations observed in the *lh* alleles are consistent with this hypothesis: *lh-1* and *lh-3* cause amino acid substitutions, while *lh-2* prevents correct mRNA splicing and is predicted to produce a truncated protein without activity (Davidson et al. 2004). Since *lh-2* is not a null allele (Swain et al. 1993), a reduced amount of WT protein is also thought to be produced, presumably from rare incidents of correct splicing. Given these results, increasing KO protein levels is predicted to result in the opposite phenotype: plants with reduced sensitivity to inhibitors of KO.

In addition to *KO*, the majority of the other genes encoding GA biosynthesis enzymes have been cloned from *Arabidopsis* and from several other species. Use of the 35S promoter to drive expression of *Arabidopsis GA1* and *GA2* and cucumber *CmKS* (Yamaguchi et al. 1996), genes that encode either *ent*-copalyl diphosphate synthase (CPS; *GA1*) or *ent*-kaurene synthase (KS; *GA2* and *CmKS*), has little apparent effect on plant growth or development, although overexpression of these genes can complement mutations in the corresponding *Arabidopsis* gene (Sun and Kamiya 1994, Yamaguchi et al. 1998). Recent analysis of *Arabidopsis* plants containing 35S:KS and/or 35S:CPS has now revealed that dramatically increasing (>1,000-fold) the levels of GA precursors before GA_{12}-aldehyde (Fig. 1) does not have a detectable effect on bioactive GA levels, presumably because of limits in the later part of the GA biosynthesis pathway (Fleet et al. 2003). This result is also consistent with the absence of an obvious phenotype in 35S:KO plants, as described above.

Consistent with the hypothesis that the later steps of the GA pathway are limiting for GA biosynthesis, greater success in manipulating GA content and plant stature has been achieved using various members of the GA 20-oxidase gene family (Hedden et al. 1999, Hedden and Phillips 2000). For example, overexpression of an *Arabidopsis* GA 20-oxidase gene in *Arabidopsis* results in longer hypocotyls, early flowering, increased stem elongation and reduced seed dormancy. These plants also have increased levels of GA_4, the main bioactive GA in *Arabidopsis* (Coles et al. 1999).

In addition to genes involved in GA biosynthesis, a number of genes encoding 2-oxoglutarate-dependent dioxygenases, involved in the irreversible conversion of active GAs and their precursors into inactive forms, have also been identified. These enzymes belong to a class known as GA 2-oxidases, and are encoded by small gene families in *Arabidopsis*, pea and other species (MacMillan et al. 1997, Lester et al. 1999, Martin et al. 1999, Thomas et al. 1999, Sakamoto et al. 2001). Overexpression of various GA 2-oxidases in *Arabidopsis* results in a dwarf phenotype, which can be rescued by applied

Table 1 GA levels in WT La-*er* and 35S:KO–GFP/4a plants

Gibberellin	WT La-*er*	35S:KO–GFP/4a
GA_{53}	0.58	0.95
GA_{44}	0.39	0.46
GA_{19}	7.26	8.25
GA_{20}	1.37	1.30
GA_{29}	1.82	Lost
GA_1 (active)	0.58	0.64
GA_8	4.47	5.79
GA_{15}	5.18	5.18
GA_{24}	10.63	14.66
GA_9	6.71	6.05
GA_4 (active)	3.66	3.66
GA_{34}	14.76	18.49

All values are expressed in ng g dry weight^{-1}.

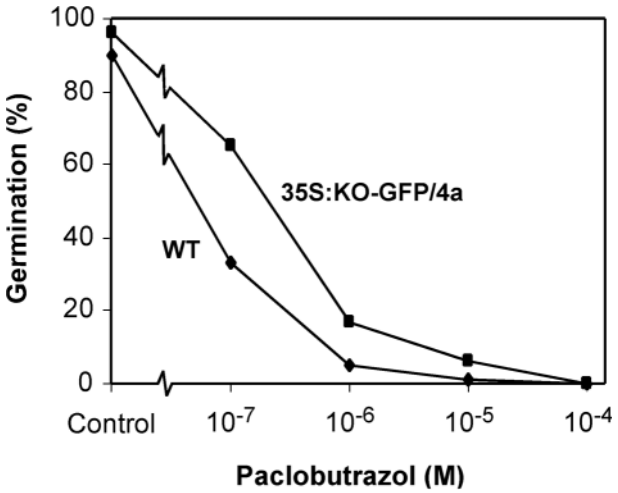

Fig. 3 Paclobutrazol dose–response curve for seed germination. WT and 35S:KO–GFP/4a seeds were harvested from plants grown together, sowed 14 days post-harvest, and germination was scored (n > 136) after 1 week on various paclobutrazol concentrations.

GA, confirming the importance of GAs in vegetative growth (Hedden et al. 1999, Hedden and Phillips 2000, Schomburg et al. 2003). Similar results were obtained when a rice 2-oxidase was ectopically expressed in rice (Sakamoto et al. 2001). In contrast, ectopic expression of a novel pea 2-oxidase (PsGA2ox2) has revealed that GAs are required for normal pollen tube elongation: a presumed reduction in pollen tube GA levels, due to overexpression of PsGA2ox2, or incubation of pollen tubes in vitro in the presence of uniconazole, impairs pollen tube growth (Singh et al. 2002).

In this study, we demonstrate that 35S:KO and 35S:KO–green fluorescent protein (GFP) *Arabidopsis* lines have decreased sensitivity to KO inhibitors, and use these lines to provide further evidence for a physiological role for GAs in pollen tube elongation.

Results

KO overexpression lines contain increased KO mRNA and protein levels

Constructs designed to overexpress the *KO* gene previously have been introduced into *Arabidopsis* and the effects on plant development examined (Helliwell et al. 2000, Helliwell et al. 2001). In this study, four independent lines with varying levels of transgene expression were selected for further analysis. Three lines carry the 35S:KO–GFP translational fusion construct, which has been shown to lead to the production of translated KO–GFP protein (Helliwell et al. 2001). As a control to confirm that the observed phenotypes (see below) were not caused by the presence of the GFP moiety, an additional 35S:KO line lacking GFP was also examined. Northern blot analysis (Fig. 2) confirmed that the 35S:KO–GFP lines contain a range of KO–GFP expression, from low to relatively high, while the 35S:KO line possesses increased expression of KO mRNA. Based on these data, lines 4a and 5a are strong overexpressors, while line 2a is intermediate and line 8a is a relatively weak overexpressor. Western blots confirmed that lines expressing *KO–GFP*, unlike WT plants, possess a protein of the predicted size for KO–GFP recognized by an anti-GFP antibody, and the abundance of this protein is correlated with mRNA levels (Fig. 2). Although a band representing the *KO–GFP* mRNA or protein in line 8a is difficult to detect in the figure, these plants accumulate detectable levels of GFP fusion protein based on GFP fluorescence in 35S:KO–GFP/8a pollen tubes grown in vitro (data not shown).

GA levels are not altered in 35S:KO–GFP seedlings

Endogenous GA levels were compared (see Materials and Methods) between WT La-*er* plants and 35S:KO–GFP/4a seedlings, representing the strongest line in terms of *KO–GFP* expression (Fig. 2). Consistent with the similar growth of the two genotypes under standard conditions (Fig. 4B; Helliwell et al. 2000) and the results of Fleet et al. (2003), GA levels were similar, including the levels of GA_4, the major biologically active GA in *Arabidopsis* (Table 1). We also measured the levels of early GA precursors from WT and 35S:KO–GFP/4a seedlings grown in the presence of paclobutrazol. Both WT (41 ng g FW^{-1}) and 35S:KO–GFP/4a (45 ng g FW^{-1}) seedlings accumulated *ent*-kaurene, demonstrating that the paclobutrazol treatment was effective. Low endogenous levels of *ent*-kaurenoic acid (2.4 ng g FW^{-1}) were observed in 35S:KO–GFP/4a seedlings, while this GA precursor could not be detected in WT seedlings, consistent with increased *ent*-kaurene metabolism in the transgenic line. GA_{12} levels were similar and very low (<1 ng g FW^{-1}) in both genotypes.

Seed germination of KO expressors is resistant to paclobutrazol

In *Arabidopsis*, as in several other species, GAs are usually required for seed germination. Consequently, imbibition of WT seeds in the presence of paclobutrazol prevents germination, and this effect can be overcome with exogenous GA or in mutants with increased GA signaling such as *spy* (Jacobsen and Olszewski 1993). For the strongest line, 35S:KO–GFP/4a, a dose–response curve was constructed to compare the response with a range of paclobutrazol concentrations (Fig. 3). Consistent with increased KO levels, 35S:KO–GFP/4a seeds were more resistant to the effects of the KO inhibitor than WT seeds. To compare the germination of the remaining KO overexpression lines, seeds were imbibed in the presence or absence of paclobutrazol at various times after harvest. As shown in Table 2, KO overexpression lines have a mild paclobutrazol resistance phenotype for seed germination, similar to but weaker than *spy-5* seeds. In contrast, 35S:SPY seeds are more sensitive to paclobutrazol than the other genotypes, consistent with previous results (Swain et al. 2001).

Vegetative growth of KO expressors is resistant to paclobutrazol

Pea seedlings lacking KO previously have been shown to be more sensitive to KO inhibitors (Fig. 4A; Swain et al. 1997), suggesting that changes in vegetative KO protein levels may alter the response to this inhibitor. To examine the effect of increasing KO on vegetative (i.e. rosette) growth in *Arabidopsis*, WT and KO overexpression lines were grown on Growool with or without 5×10^{-7} M paclobutrazol (see Materials and Methods). Consistent with previous analysis of plant growth (Helliwell et al. 2000), endogenous GA levels (Table 1) and the results of Fleet et al. (2003), WT and KO overexpressing line 4a plants are indistinguishable when grown on Growool without paclobutrazol, while WT plants grown on paclobutrazol were clearly smaller than control WT plants (Fig. 4 and data not shown). Consistent with the results for seed germination, KO overexpressing lines were larger than the corresponding WT plants when paclobutrazol was added (Fig. 4). While 35S:KO–GFP/2a and 8a plants are only about 30% larger than WT plants on paclobutrazol (Fig. 4D; $P < 0.001$), this difference is easily detected when typical WT and line 2a plants are visually compared (Fig. 4C). KO overexpressing lines were also resistant to the dwarfing effects of ancymidol (data not shown), another inhibitor of KO (Fig. 1).

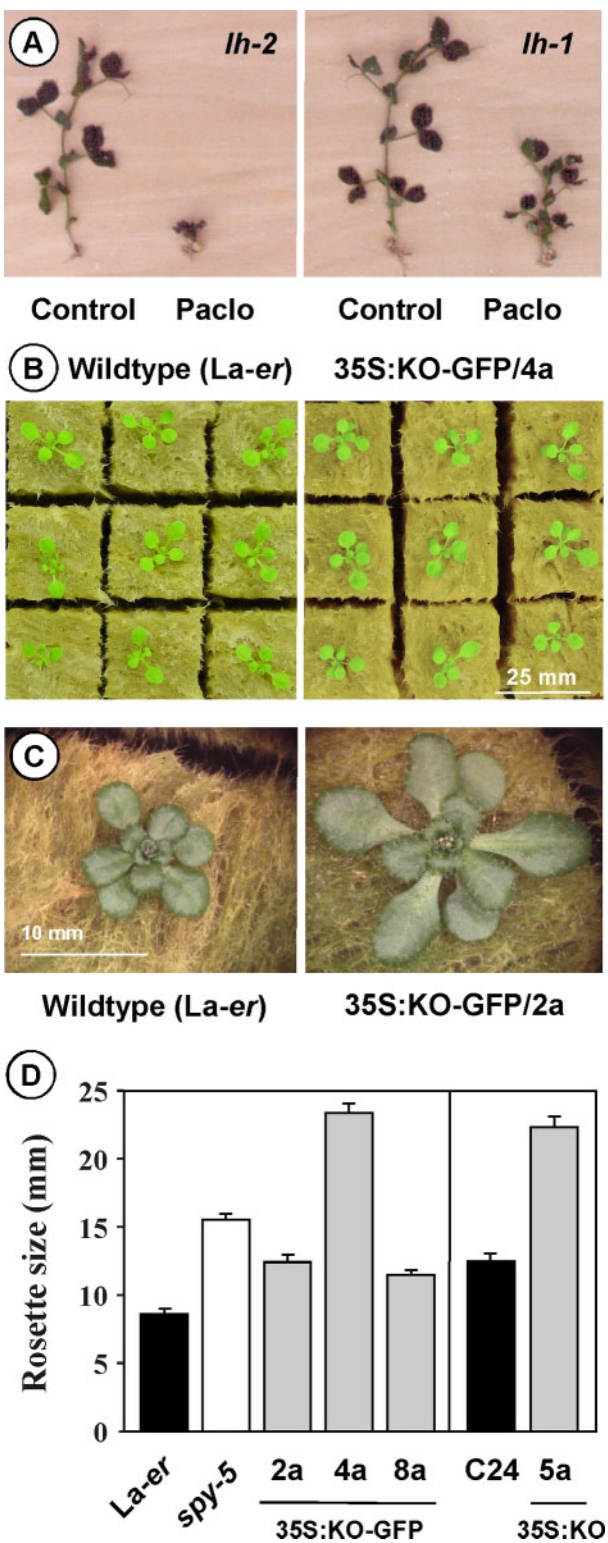

Fig. 4 Vegetative growth of KO overexpression lines is resistant to paclobutrazol. (A) Seedlings of the KO-deficient mutants *lh-1* and *lh-2* germinated with or without 1 μg of paclobutrazol (paclo) applied to the dry seed at imbibition. The *lh-2* mutant is approximately 30 times more sensitive to the dwarfing effects of paclobutrazol (see Swain et al. 1997). Untreated mutant plants (control) are about 25 cm tall. (B) Growth of representative WT La-*er* and 35S:KO–GFP/4a plants on Growool without paclobutrazol. Plants are 18 d old. The scale is the same for both genotypes. (C) Growth of representative WT La-*er* and 35S:KO–GFP/2a plants on Growool containing 5×10^{-7} M paclobutrazol. The scale is the same for both genotypes. (D) Rosette size of WT plants, *spy-5*, various 35S:KO–GFP lines and the 35S:KO line on 5×10^{-7} M paclobutrazol at 42 days of age.

Table 2 35S:KO and 35S:KO–GFP seeds are paclobutrazol resistant at germination

Genotype [a]	1×10^{-5} M paclobutrazol		1×10^{-4} M paclobutrazol
	8 days [b]	94 days	109 days
Wild-type La-*er*	13%	15%	14%
spy-5	86%	90%	82%
35S:SPY #2	ND [c]	0%	0%
35S:KO–GFP/2a	19%	30%	22%
35S:KO–GFP/8a	26%	47%	36%
Wild-type C24	15%	93%	90%
35S:KO/5a	38%	93%	87%

$n > 98$ seeds for each genotype, harvested from plants grown together. Germination was scored after 1 week on 1×10^{-5} M or 1×10^{-4} M paclobutrazol.
[a] Germination on dH_2O only was >96% for all genotypes.
[b] Days after harvest.
[c] ND, not done.

Pollen tube growth of KO expressors is resistant to uniconazole

Recent results using *Arabidopsis* plants containing a 35S:PsGA2ox2 construct have suggested that GAs are required for normal pollen tube elongation (Singh et al. 2002, Swain et al. 2004). As part of this analysis, WT pollen tubes were incubated in vitro with uniconazole, an inhibitor of GA biosynthesis that has a chemical structure very similar to paclobutrazol (Izumi et al. 1985), in order to reduce *de novo* GA biosynthesis. Uniconazole reduced pollen tube elongation, and this effect could be reversed when the appropriate concentration of GA was also present (Singh et al. 2002). Analysis of GFP expression confirmed that the 35S:KO–GFP construct is expressed in pollen tubes (data not shown). This allowed the 35S:KO and 35S:KO–GFP lines to be used to confirm that uniconazole inhibits WT pollen tube growth via an effect on GA levels rather than by non-specific inhibition. Initially we compared the growth of WT La-*er* and 35S:KO–GFP/4a pollen tubes. In contrast to its effect on WT pollen tubes, uniconazole increased the growth of pollen tubes carrying the 35S:KO–GFP/4a transgene (data not shown). There are two possible explanations for this result. First, it has been demonstrated that excessively high GA levels can inhibit pollen tube growth in vitro (Singh et al. 2002 and references therein). It is therefore possible that because of the relatively high *KO–GFP* expression in this line (Fig. 2), endogenous GA levels are superoptimal in untreated 35S:KO–GFP/4a pollen tubes and reduced to more optimal levels, more similar to control WT, by uniconazole treatment. Alternatively, since the other 35S:KO–GFP and 35S:KO lines did not behave in this manner (see below), the unusual behavior of line 4a may reflect other, unknown, genetic changes that alter pollen tube growth. The in vitro pollen tube phenotype of line 4a was not examined further since the inhibitory effect of high GA levels in vitro may not reflect the effects of GAs on in vivo pollen tube growth and hence may not be physiologically meaningful.

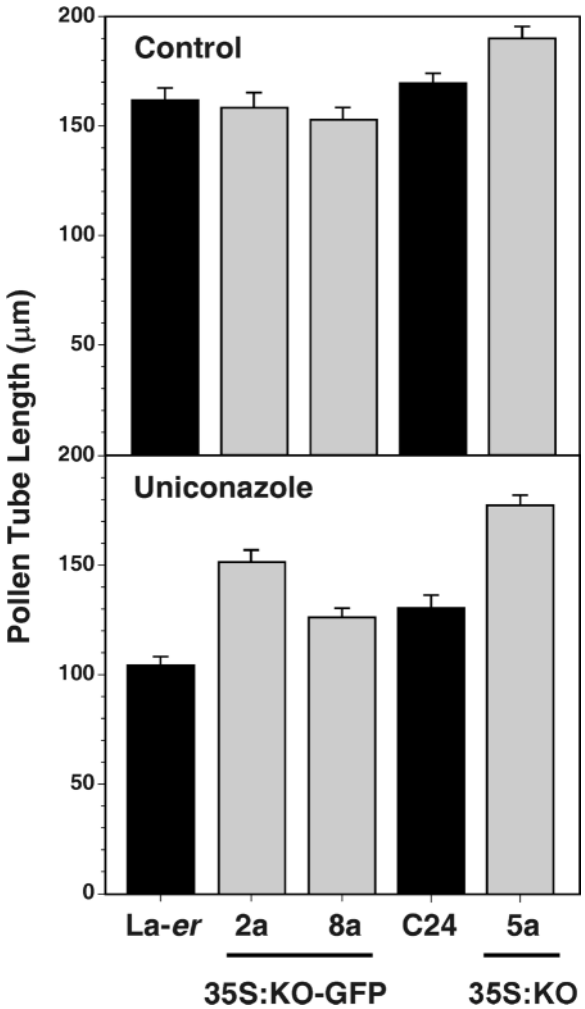

Fig. 5 Pollen tube growth of KO overexpression lines is resistant to uniconazole. Pollen from different genotypes was germinated and grown in vitro either without (Control) or with 1×10^{-5} M uniconazole. Final pollen tube growth was measured after 24 h. At this concentration, the effect of uniconazole can be reversed by exogenous GA (Singh et al. 2002).

Instead, in vitro pollen tube growth of the other 35S:KO–GFP and 35S:KO lines was examined (Fig. 5). Consistent with the results for seed germination and vegetative growth described above, the growth of 35S:KO and 35S:KO–GFP pollen tubes was resistant to uniconazole compared with WT pollen tubes.

Detailed analysis of several 35S:PsGA2ox2 lines has demonstrated that pollen tubes carrying the 35S:PsGA2ox2 transgene, which are thought to be GA deficient, elongate more slowly and are consequently out-competed by WT pollen tubes in the transmitting tract (Singh et al. 2002). A possible implication of this result is that pollen tubes with endogenous GA levels higher than those in WT pollen tubes may elongate faster than normal pollen tubes, although, as mentioned above, high concentrations of exogenous GA can also inhibit WT pollen tube growth. Although the in vitro pollen tube growth assays

Table 3 The 35S:KO–GFP and 35S:KO loci segregate in agreement with the expected 3 : 1 ratio of transgenic : non-transgenic plants based on sensitivity to kanamycin, the marker used for the original selection of transgenic seedlings

Segregating line	Ecotype	Growth on kanamycin		$\chi^2(3:1)$	P
		Resistant	Sensitive		
35S:KO–GFP/4a	La-*er*	641	204	0.33	$0.70 > P > 0.50$
35S:KO–GFP/8a	La-*er*	978	326	0.00	$P > 0.90$
35S:KO/5a	C24	104	22	3.82	$0.10 > P > 0.05$

shown in Fig. 5 suggest that 35S:KO/5a pollen tubes may elongate slightly faster than WT (C24) pollen tubes, a more robust experiment is to compare the effectiveness of pollen tubes with or without the transgene in the same pistil using self-pollinated hemizygous 35S:KO or 35S:KO–GFP plants in which equal numbers of WT and transgenic pollen tubes should be present. For the three independent lines examined, no significant deviation from the expected 3 : 1 ratio of transgenic : non-transgenic progeny was observed (Table 3), suggesting that WT and transgenic pollen tubes are equally likely to fertilize an ovule. Thus, similarly to the situation in vegetative tissues in the absence of GA biosynthesis inhibitors, it appears that increased *KO* expression in pollen tubes does not increase either endogenous GA levels or growth in vivo.

Discussion

Previous analysis of the pea KO-deficient *lh-1* and *lh-2* mutants has revealed interactions between these mutants and chemical inhibitors of GA biosynthesis such as paclobutrazol (Swain et al. 1997). Plants homozygous for *lh-2* are approximately 30-fold more sensitive than *lh-1* to the dwarfing effects of paclobutrazol, although untreated *lh-1* and *lh-2* plants are both around half the height of WT plants (Fig. 4A; Swain et al. 1993). The recent molecular characterization of the *LH* locus suggests that *lh-2* plants contain lower levels of KO, the target of paclobutrazol, compared with *lh-1* and WT plants. Consequently, overexpression of KO might be expected to have the opposite effect: decreased paclobutrazol sensitivity resulting from increased KO protein levels. In this study, we provide evidence that this is indeed the case: although 35S:KO and 35S:KO–GFP plants appear similar to WT under standard growing conditions (Helliwell et al. 2000, Helliwell et al. 2001; Fig. 4B), in the presence of KO inhibitors, they are less affected than WT plants. Although the exact basis of this resistance is not known, a likely explanation is that increased levels of KO (or KO–GFP) reduce the ability of KO inhibitors such as paclobutrazol to inhibit overall KO activity simply by titrating the inhibitor, thereby allowing greater GA biosynthesis in the presence of this inhibitor.

The results described here, in conjunction with the previous characterization of the pea *lh* alleles (Swain et al. 1997, Davidson et al. 2004), suggest several potential novel applications of manipulating KO activity. First, since 35S:KO has no detectable effect on plant development in the absence of KO inhibitors, increased KO expression could be used to make plants, or specific plant organs, resistant to these inhibitors. This may allow the use of KO inhibitors selectively to reduce the growth of competing plants (e.g. weeds) or to reduce the growth of one plant organ while leaving others unaffected. *KO* also has potential to be used as a selectable marker since transgenic plants could be identified on the basis of their resistance to KO inhibitors rather than standard selectable markers such as antibiotics. Inhibitors such as paclobutrazol could also be used routinely in the field to identify transgenic plants for crosses, etc. With regard to this possible use, no co-suppressed 35S:KO lines, in which the endogenous *KO* gene was silenced, have been identified (C. Helliwell, unpublished results). This result suggests that *KO* expression could be used as a stable and reliable selectable marker. Finally, since it is easier to regenerate plants from callus with reduced GA response (Ezura and Harberd 1995), transient silencing of KO in the presence of KO inhibitors could be used to improve plant transformation protocols without an excessive effect on subsequent growth.

While this study describes the effect of increased KO expression on paclobutrazol sensitivity, two other examples of plants with increased resistance to paclobutrazol have been reported. Transgenic *Arabidopsis* with increased levels of one or both of the first two GA biosynthesis enzymes (CPS and KS), required for the conversion of geranyl geranyl diphosphate (GGDP) to the KO substrate *ent*-kaurene (Fig. 1), recently have been characterized in terms of their response to paclobutrazol (Fleet et al. 2003). Some lines contained levels of *ent*-kaurene 1,000 times higher than WT plants, and these high KO substrate levels presumably are responsible for the reduced effect of paclobutrazol on vegetative growth and flowering observed in these lines. This explanation is consistent with the observation that paclobutrazol, and other structurally related inhibitors, may compete with *ent*-kaurene for access to the KO enzyme (Sugavanam 1984).

Another example of altered GA metabolism leading to a reduced effect of paclobutrazol on vegetative growth is provided by the pea *slender* (*sln*) mutant. The pea *SLN* locus encodes a GA 2-oxidase, Ps2ox1 (Lester et al. 1999, Martin et al. 1999), that is required for the conversion of the high levels of GA_{20}, produced during seed development, to GA_{29} and

GA_{29}-catabolite as the seeds mature. Mutant *sln* seeds developing on *sln* maternal plants are unable to remove this GA_{20}, and the dry seeds consequently contain far higher GA_{20} levels than WT seeds. Since this GA_{20} can be converted to the biologically active GA_1 during seed germination and early seedling growth, *de novo* GA_{20} production, and hence KO activity, is not required. As a result, *sln* seedlings are resistant to the dwarfing effects of inhibitors, such as paclobutrazol, that act before GA_{20}, but sensitive to inhibitors, such as prohexadione, that act after GA_{20} (Ross et al. 1993, Ross et al. 1995).

A physiological role for GAs in *Arabidopsis* pollen tube growth has been established recently (Singh et al. 2002, Swain et al. 2004). As an important part of this work, uniconazole was used to inhibit the elongation of WT La-*er* pollen tubes growing in vitro. The ability to rescue this inhibition with an appropriate GA concentration suggested that uniconazole was inhibiting pollen tube growth solely via its effect on GA biosynthesis. However, the formal possibility remains that uniconazole is not specific for KO in this system, and inhibits pollen tube growth independently of GAs. The results presented here provide further evidence that the effect of uniconazole on pollen tube growth is due to reduced KO activity. The reduced ability of uniconazole to inhibit the growth of 35S:KO and 35S:KO–GFP pollen tubes strongly supports the conclusion that uniconazole acts specifically on KO. Thus, the 35S:KO and 35S:KO–GFP lines described here provide additional evidence for a physiologically significant role for GAs in pollen tube growth.

Materials and Methods

Plant material and growth conditions

Several independent 35S:KO–GFP lines were isolated previously in the *Arabidopsis thaliana* Landsberg *erecta* (La-*er*) ecotype, and an additional 35S:KO line was identified in the C24 ecotype (Helliwell et al. 2001). Lines containing a single transgene locus were selected based on the segregation of the kanamycin resistance gene that serves as a selectable marker for the T-DNA. Seeds used for germination assays were harvested from parent plants grown together under identical conditions. All seeds were stratified for 3 days at 4°C under dim light to aid germination. Plants were grown in growth rooms with 18 h of white fluorescent light (22°C, 60–70 μmol photons m^{-2} s^{-1} at pot top) and 6 h of dark (20°C) on either Growool (GroWool Horticultural Systems, NSW, Australia), 0.8% agar (Spectrum, CA, U.S.A.) containing Murashige and Skoog salts (2.3 g l^{-1}, Sigma-Aldrich, MO, U.S.A.) and 1% (w/v) sucrose, or individually in a peat-based soil mixture of CSIRO-Plant Industry (Canberra, Australia) in the Arasystem (BetaTech, Gent, Belgium).

Seed germination on paclobutrazol was performed as described in Jacobsen and Olszewski (1993). For assaying vegetative resistance to paclobutrazol, seeds were germinated on Growool, and after 1 week paclobutrazol was added to a final concentration of 5×10^{-7} M.

Pea seeds were grown and treated with paclobutrazol as previously reported (Swain et al. 1997).

RNA gel blot analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. A 10 μg aliquot of each sample was separated on a 1.2% agarose–formaldehyde gel and blotted onto a Hybond-N membrane (Amersham, U.K.). The blot was probed using the *GA3* RNA probe described in Helliwell et al. (1998) and visualized by phosphorimager.

Protein gel blot analysis

Protein was extracted from approximately 0.25 g of 15-day-old leaf material by grinding with acid-washed sand followed by the addition of 1 ml of sample buffer (80 mM Tris–HCl pH 8.8, 10% glycerol, 10% SDS, 0.002% bromophenol blue, 4% 2-mercaptoethanol). Samples were boiled for 5 min then centrifuged for 5 min at 16,000×*g*. The supernatant was used for SDS–PAGE (Laemmli 1970) with a 12% resolving gel. Duplicate gels were run; one gel was transferred to PVDF membrane for Western blot analysis and the other was stained with Coomassie blue to confirm equal protein loading. Protein blot analysis was carried out using an anti-GFP peptide antibody conjugated with horseradish peroxidase (Clontech, Palo Alto, CA, U.S.A.) at a 1 : 500 dilution.

GA determinations

GAs were quantified using gas chromatography-selected ion monitoring (GC-SIM) with di-deuterated internal standards. Following harvest and lyophilization, samples were extracted in 100 ml of 80% methanol for 24 h along with a range of $[^2H_2]$GA standards (provided by Professor L.N. Mander, Australian National University). Purification by ethyl acetate partitioning, QAE Sephadex and C_{18} Sep-Pak followed Green et al. (1997). HPLC separation was the same as the initial HPLC step in Gocal et al. (1999) with appropriate fractions pooled, dried and derivatized for analysis by GC-SIM as before (Gocal et al. 1999).

Pollen tube growth assays

Pollen tube growth assays were carried out according to the method described by Taylor et al. (1998). Uniconazole was dissolved in an appropriate volume of pollen germination medium to obtain the desired concentration (Singh et al. 2002).

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