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Induction and Analysis of Gibberellin Sensitive Mutants in *Arabidopsis thaliana* (L.) Heynh.

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Summary. In *Arabidopsis thaliana* 37 independent irradiation or EMS induced mutants were isolated which have an absolute or almost absolute gibberellin (GA) requirement for germination and successive elongation growth. These are called 'non-germinating GA-dwarfs', since without further addition of GA they develop into typical GA-dwarfs, being dark green, stunted and sterile. However, with repeated GA-treatment they develop into fertile plants with a completely wild type phenotype, or nearly so. In addition, 19 independently induced 'germinating GA-dwarfs' were obtained, i.e. mutants which do germinate without GA but develop into typical GA-dwarfs. With repeated GA-treatment these too grow to become completely wild type phenotypes, or nearly so. 'Germinating dwarfs' have been found by previous authors in a number of other plant species. The 'non-germinating dwarfs' form a new class of mutants. The system of non-germinating mutants offers a resolving power unique in higher plants, so that self-detecting rare events like induced revertants or intragenic recombinants can be efficiently screened for.

The 56 GA-sensitive mutants represent mutations at 5 loci, located on three of five *Arabidopsis* chromosomes. At three of the five loci both mutant classes were represented in similar frequency ratio's, whilst at the other two loci only germinating dwarfs were found.

Key words: *Arabidopsis thaliana* – Gibberellin – Gibberellin sensitive mutants – Dwarf mutants – Non-germinating mutants – Gene localization

Abbreviations

GA gibberellin
EMS ethylmethanesulfonate
NG non-germinating
G germinating

Introduction

Since plant hormones play an important role in the regulation of plant life, the isolation of plant hormone deficient mutants is of interest for both plant genetics and plant physiology.

Mutants with a reduced level of abscisic acid (ABA) were found in tomato (the *flacca* mutant; Tal and Nevo 1973) and in *Arabidopsis* (Koornneef et al. 1980). In certain apple-dwarfs the IAA (indol-acetic acid) levels were found to be reduced (Jindall et al. 1974). The gibberellin (GA) sensitive dwarf mutants, isolated in several plant species (for review see Pelton 1964) form the largest and best known group of plant hormone deficient mutants. In this group the GA sensitive mutants in maize (Phinney 1960; 5 different loci) and in rice (Murakami 1970; 2 loci) have been characterized into some detail.

These mutant genes very probably regulate the synthesis of endogenous GA's, as could be concluded from the pronounced response to exogenous GA's, the absence or changed composition of endogenous GA's (Phinney 1960; Murakami 1970; Suge 1978), a response to specific precursors and to different GA's depending on the locus mutated (Katsumi et al. 1964; Murakami 1970), and in one case from feeding experiments with labelled precursors (Hedden and Phinney 1976).

Upon mutagenic treatment, M_2 lines segregating non-germinating, but otherwise well-developed seeds, are not uncommon in *Arabidopsis*. Far more frequent of course are non-germinating shrunken underdeveloped seeds which are classified as embryonic lethals (Müller 1963). Since endogenous growth regulators play an important role in the control of seed germination (Mayer and Shain 1974), it occurred to us, that at least some of the well developed non-germinating seed mutants might represent mutations in genes regulating plant hormone synthesis or function, e.g. GA synthesis. Therefore, we systematically started screening M_2 lines for GA responsive non-germi-

nators. This entirely new class of GA sensitive mutants we call 'non-germinating GA dwarfs', since without further GA treatment (after germination induction) they were found to develop into typical GA dwarfs. The same material was also screened for GA sensitive mutants among dwarfs, which grew from germinating seeds, in order to find 'germinating GA dwarfs', in analogy to the dwarfs in other species. For preliminary reports see Koornneef et al. 1977; Koornneef 1978. The mutation frequencies of the mutants described are given into detail by Koornneef and Dellaert 1981. The gene symbol *ga* is proposed for all GA sensitive mutants in *Arabidopsis* (Koornneef 1978). These have not been described before in this species. We do not place the *ca* mutant (Bose 1971, 1974) and the *le* mutant (Napp-Zinn and Bonzi 1970) into this class as *ca* reacts to a high concentration of GA_3 by a length increase from 2 cm to only 5 cm (Bose 1974). The reaction of *le* is also weak as stalks never get more than a few cm of length at high concentrations of GA. This differs greatly from the wild type length which is over 20 cm (Napp-Zinn pers. comm.). Our criterium is that only those mutants which by (repeated) GA treatment can be made to develop completely into wild type phenotype, or nearly so, should be called GA sensitive mutants.

Material and Methods

Plant Material and Conditions of Culture

Arabidopsis thaliana (L.) Heynh. (2n = 10) is a small fast growing, self fertilizing crucifer. Seed stocks used in the experiments were derived from the pure line 'Landsberg-*erecta*' (Redei 1962). The seeds were sown in 9 cm petri dishes (25, 30 or 36 per dish), equally spaced on perlite saturated with a standard mineral solution, the composition of which was as described by Oostindiër-Braaksma and Feenstra (1973). To break seed dormancy the dishes were kept at 2-4°C for 4-6 days. Germination was at ± 24°C under continuous illumination by fluorescent light tubes (Philips TL 57) at roughly 8 W m^{-2}. After 8 days the seedlings were transplanted into soil (pots or pans) and cultivated in an air-conditioned greenhouse, where additional continuous light was given in the winter (October to April) by frames of TL 57 tubes. For the purpose of testing germination, seeds were sown in plastic petri dishes (ϕ 8.5 cm), with two layers of filter paper (ederol no. 261) saturated with two ml of distilled sterile water. To avoid rapid evaporation, each dish was wrapped in a small polythene bag. Germination was determined 7 days after the end of cold treatment.

The Induction and Isolation of GA Sensitive Mutants

To induce mutants, seeds were preimbibed at 2-4°C during 5 days on filter paper and redried at 24°C during 24 hours on filter paper. The seeds were then treated with ethylmethanesulfonate (EMS, 10 mM, 24 h, 24°C) or irradiated after 4 hours submersion in tap water, with X-rays or fast neutrons (Dellaert 1980; Koornneef and Dellaert 1981).

The resulting M_1 plants were cultivated in soil and individually harvested. In the case of EMS experiments, per M_1 plant, a number of siliquae in general from the top of the main stem were harvested; in the case of the radiation experiments, only one well-filled silique from the top of the main stem was harvested (Dellaert 1980). It should be noted that the top of the main stem is predominantly non-chimeric due to progressive loss of chimerism (Balkema 1972; van der Veen unpublished).

To isolate non-germinating GA sensitive mutants, M_2 lines, separately sown on standard mineral medium, were screened at day 8 after the end of cold treatment. All well developed seeds that had not germinated were transferred with a small brush to petri dishes containing 10^{-5} M GA_{4+7} in the medium. These dishes were placed back into the climate room, and after another 8 days all seedlings were transplanted into soil. Those that developed into dwarfs were sprayed with a solution of 10^{-4} M GA_{4+7} to restore normal growth and ensure fertility. Seeds from the resulting M_3 lines were tested for germination behaviour and GA sensitivity. From most of the M_2 lines used for screening, the seedlings that were obtained without GA were planted out and scored for dwarf and compacta mutants. The GA sensitivity of these dwarfs was tested in the M_3 and sometimes already in the M_2 generation. The criterium for GA sensitivity was that by spraying the mutants weekly for three weeks with a solution of 10^{-4} M. GA_{4+7} the wild type phenotype could be restored completely, or nearly so.

Genetic Characterization

The different mutants obtained in successive experiments were tested *with a gradually built up representative set of tester mutants* for allelism vs. non-allelism on the basis of non-complementation vs. complementation to wild type in their F_1's. Mutants that showed non-complementation with a particular tester were in general retested with a second mutant at the same locus. Gene localization was done by trisomic analysis (Koornneef and van der Veen 1978) and by linkage analysis of F_2 populations. The recombinant fraction was calculated by the Product Ratio Method, using the tables of Stevens (1939). The chromosome denotation was as proposed by Koornneef and den Besten (1979). Segregation frequencies of the *ga-1*, *ga-2* and *ga-3* mutants were determined upon crossing with wild type, in most cases already in the M_2 generation. The F_2 progenies (size 75-150 plants) were sown in petri dishes with 10^{-5} M GA_{4+7} in the medium and after transplanting into soil the fraction of dwarf mutants was determined when the plants were about 5 weeks old.

Results

The Isolation and Description of GA Sensitive Mutants

Up to now 37 independently induced non-germinating GA sensitive mutants have been isolated. In the EMS experiments their frequency was about 6 per 1000 M_2 lines tested. All are very similar in overall morphology. Germination can be restored completely by GA (Fig. 1). All GA's tested, viz. GA_3, GA_{4+7}, GA_7 and GA_9, have this effect, GA_{4+7} being the most effective. Without further GA spray, the initially completely normal looking seedlings, upon transfer to soil, develop into dark green dwarfs, which later develop a bushy appearance (Fig. 2). Petals

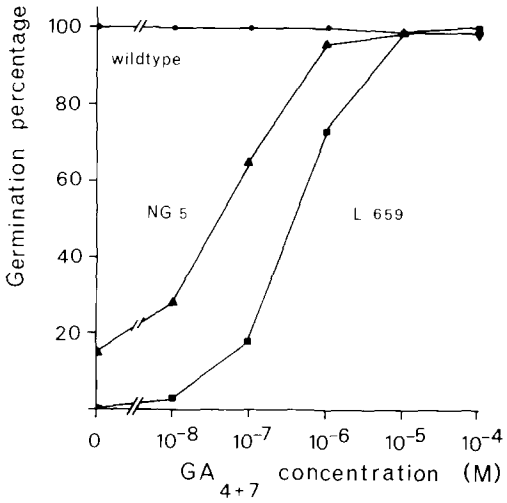

Fig. 1. The germination of two typical non-germinating mutants at the *ga-1* locus and wild type at different concentrations of GA_{4+7}

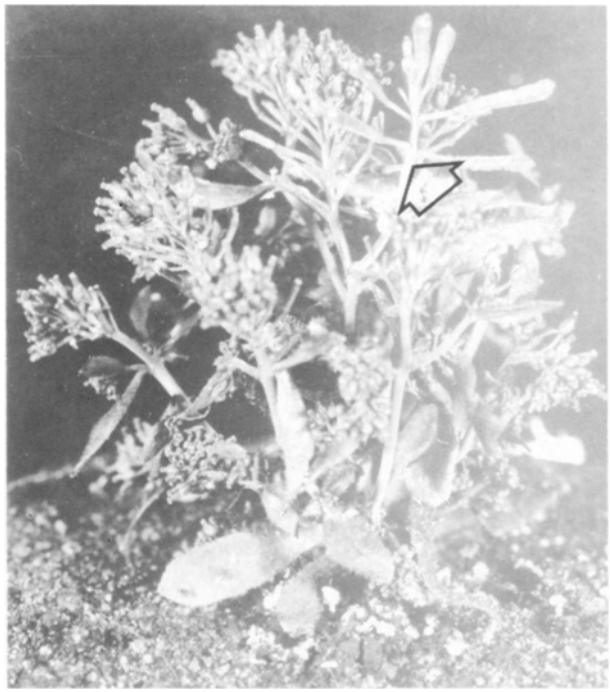

Fig. 2. Non-germinating mutant (NG5), germinated by GA_3. Arrow indicates an inflorescence pollinated with pollen from a normal plant

and stamens are very poorly developed; pistils and sepals are almost normal (Fig. 3). No selfed seed is formed, but seed set can be obtained by pollen from a normal plant. Flowering time was not markedly affected.

By spraying these dwarfs weekly for at least three weeks with 10^{-4} M GA_{4+7}, starting 2-3 weeks after germination, the phenotype of the wild type, including plant length, flower morphology and fertility, can be restored completely, or nearly so (Fig. 4). In old dwarfs (over 4

Fig. 3. Flower morphology of NG5 (*ga-1*) after germination induction by GA_{4+7} (a) and of wild type (b)

weeks old) length growth cannot be restored completely but flower morphology and fertility can be restored even in relatively very old dwarfs. Upon termination of GA spraying, symptoms of GA deficiency will develop progressively, mainly at the top of the inflorescences.

As mentioned above dwarfs were also selected from normal germinating lines. Those which responded well to spraying with GA_{4+7} could be divided into two classes:

1 Mutants with a phenotype similar to that of non-germinating GA sensitive dwarfs. These dwarfs all appeared to be alleles at the loci *ga-1*, *ga-2* and *ga-3* (see next section).

2 Dwarf mutants that were in general less extreme than the former; flower morphology and fertility in particular were almost completely normal in this group (Fig. 4). This type of dwarf represented mutants at the *ga-4* and *ga-5* loci.

It is difficult to say which proportion of dwarf mutants is GA sensitive, as dwarf and compact types form a very large and diverse group of mutants, many of them having a reduced fertility. Among dwarfs that are reasonably fertile, GA sensitive dwarfs (class 2) represent only a small minority. Many sterile dwarf plants were sprayed with GA_{4+7} in the M_2. A small proportion of these mutants, in which fertility could be restored, form the class 1 GA sensitive dwarfs. In the EMS experiments about 5 GA sensitive dwarfs of both classes could be found per 1000 M_2 lines tested.

To compare the germination behaviour of the different mutants isolated at the *ga-1*, *ga-2* and *ga-3* loci, homozy-

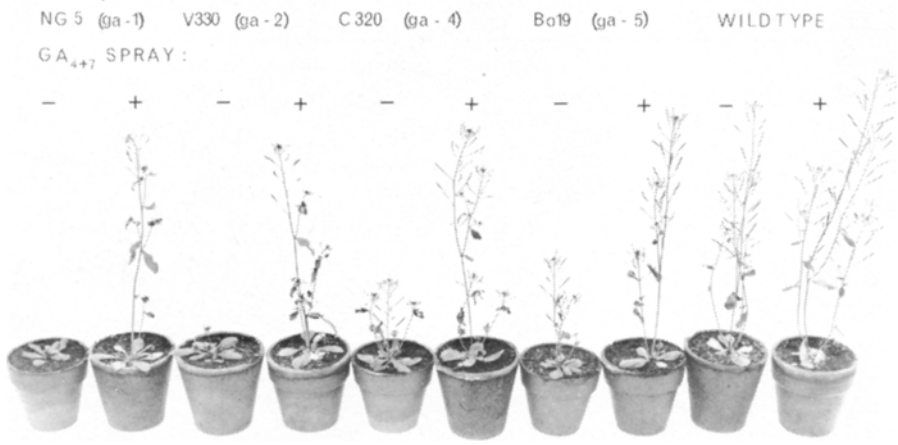

Fig. 4. The response of several *ga*-mutants and wild type *Arabidopsis* to two sprays with 10^{-4} M GA_{4+7} about two and three weeks after germination. All plants germinated without GA, which is rare for NG5 but normal for the others. Note that in the *ga-1* and *ga-2* mutants symptoms of GA deficiency appear again in the top of the inflorescence

gous lines of each independently induced mutant were obtained as follows: Plants were selected from F_3 lines derived from mutant × wild type crosses and in a few cases from lines that were obtained after several generations of line selection of the original mutants. Thus, all mutants have a genetic background, undisturbed as much as possible by other mutations. The selected lines were grown together and harvested on the same day. All seed parents were given 10^{-5} M GA_{4+7} to initiate germination and sprayed two times, i.e. two and three weeks after germination. The germination of two month-old seeds from six individual parent plants per mutant line was tested (50-100 seeds/plant). The frequency distribution of the average germination percentage per mutant is shown in Fig. 5. No clear differences exist between the loci but two different allele groups appear within the loci, depending on the selection criterium used. Among the non-germinating mutants, lines are present which show a certain amount of germination; most germinating mutants show some reduction of germination. After transfer to soil 'spontaneous germinators' always develop into typical GA dwarfs. Germination of these mutants without GA has been found to be a character depending greatly on the harvest period and other factors known to affect the germination of dormant seeds: storage, cold treatment after sowing, KNO_3, light quality and intensity (to be published elsewhere). It should be stated that some lines never showed any germination without GA. So among the mutants at the *ga-1*, *ga-2* and *ga-3* loci, a large range from absolute to no GA requirement for germination is available.

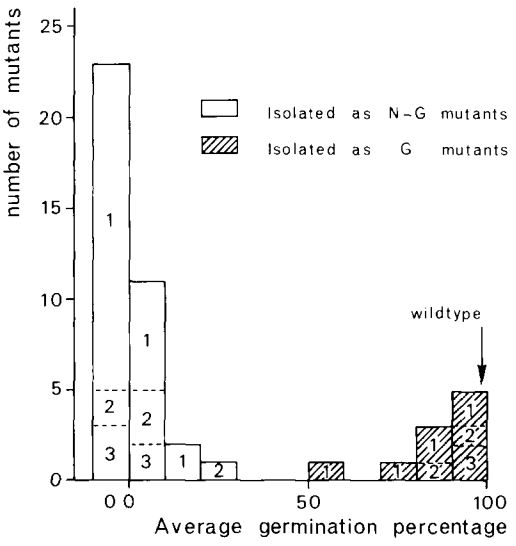

Fig. 5. The distribution of the average germination percentage of 47 independently induced mutants at the *ga-1* (1), *ga-2* (2) and *ga-3* (3) loci

Genetical Analysis of the Mutants

Complementation tests between the mutants revealed that the non-germinating GA mutants represent mutations at three different loci. Among the 19 germinating GA sensitive dwarfs 10 were at the same three loci, the other 9 at a fourth and fifth locus (Table 1). Among the morphologically identical *ga-1*, *ga-2* and *ga-3* mutants, *ga-1* mutants predominate among all groups (viz. non-germinating, germinating dwarfs, EMS and radiation). This locus specificity within these loci is significant $\chi^2_2 = 25.58$ ($p < 0.01$).

No clear indication was obtained for intragenic partial complementation between non-germinating mutants. Crosses between non-germinating and germinating alleles of the *ga-1*, *ga-2* and *ga-3* loci mostly germinated, germination thus behaving as dominant. The resulting dwarfs

Table 1. Results of complementation tests with GA sensitive mutants

Type of dwarf	Mutagen	Locus					Total
		ga-1	*ga-2*	*ga-3*	*ga-4*	*ga-5*	
Non-germinating	EMS	21	4	5	–	–	30
	Fast neutrons	4	1	–	–	–	5
	X-rays	1	1	–	–	–	2
	sub total	26	6	5	–	–	37
Germinating	EMS	5	2	2	7	1	17
	Fast neutrons	1	–	–	–	–	1
	X-rays	–	–	–	1	–	1
	sub total	6	2	2	8	1	19
Total per locus		32	8	7	8	1	56

Table 2. Chromosome location and recombinant fractions between *ga*-loci and representative marker genes of the chromosomes involved

Locus	Chrom./ chrom.arm	Marker tested	Recombinant fraction
ga-4	1A	*an*	0.31 ± 0.05
		dis-1	0.11 ± 0.05
ga-2	1B	*ch*	0.41 ± 0.05
		ap-1	0.18 ± 0.02
ga-1	4	*cer-2(=vc-2)*	0.33 ± 0.04
		ag	0.31 ± 0.02
ga-5	4	*ag*	0.08 ± 0.01
ga-3	5	*ms*	0.11 ± 0.02
		tz	0.40 ± 0.02

References for markers and their location: *an, dis-1, ch*: Feenstra 1978; *ap-1, cer-2, ag, ms*: Koornneef and Den Besten 1979; *tz*: Lee-Chen and Steinitz-Sears 1967

were never taller than the germinating dwarf parent.

From the gene mapping experiments it appears that the *ga* loci are distributed at random over the *Arabidopsis* genome (Table 2). No close linkage between any pair of *ga* loci was detected.

All *ga* mutants behave as monogenic recessives to wild type. Estimates of the segregation frequencies for the *ga-1*, *ga-2* and *ga-3* locus have been given in Table 3. By using a χ^2 test according to Brandt and Snedecor it appeared that no significant differences exist between respective loci, mutagens and types of mutant when testing within remaining groups.

It may be of interest that the average segregation frequency for non-germinating mutants (20.7%) is significantly lower than the expected 25%. However, the fre-

Table 3. Segregation ratio's in F_2 with wild type mutants at the *ga-1*, *ga-2* and *ga-3* loci. Number of seeds tested per F_2 is approximately 65-150

Type of dwarf mutant	Locus	Mutagen	Segregation wild types	Ratio : mutants	Mutant %	χ^2 (3:1)	No. of mutant lines tested	No. of mutant lines sign. deviating from 3:1 at $p < 0.05$
Non-germinating	*ga-1*	EMS	1810	: 489	21.3 ± 0.8	17.06[a]	20[b]	2
	ga-1	Radiation	463	: 113	19.6 ± 1.6	8.90[a]	5	2
	ga-2	EMS	401	: 92	18.7 ± 1.7	10.56[a]	4	2
	ga-2	Radiation	225	: 49	17.8 ± 2.3	7.40[a]	2	1
	ga-3	EMS	443	: 130	22.7 ± 1.7	1.63	5	0
			3342	: 873	20.7 ± 0.6	41.34[a]	36	7
Germinating	*ga-1*	EMS	430	: 126	22.7 ± 1.8	1.62	5	0
	ga-1	Radiation	31	: 12	27.9 ± 6.8	0.19	1	0
	ga-2	EMS	119	: 32	21.2 ± 3.3	1.17	2	0
	ga-3	EMS	211	: 81	27.7 ± 2.6	1.17	2	0
			791	: 251	24.1 ± 1.3	0.46	10	0

[a] $p < 0.01$
[b] one NG EMS mutant was accidentally not tested

quency for germinating alleles of the same loci (24.1%) does not differ from this percentage. In itself recessive deficits for induced mutants are by no means uncommon.

Discussion

Of the GA sensitive dwarfs described in a number of higher plant species, only in the case of maize (Phinney 1960; Hedden and Phinney 1976) and rice (Murakami 1970) are clear indications available that genes regulating GA synthesis are mutated in these genotypes.

Because the biosynthetic pathway of gibberellins is rather complex (Barendse 1975; Hedden et al. 1978) it is likely that many loci are involved. The later part of the pathway consists of interconversions between the different GA's (up to 50 different GA's have been isolated up to now and many of these in higher plants). It might be possible that some mutations in genes regulating these interconversions escape detection because 'escape routes' are available.

In maize five loci have been identified (Phinney 1961) and in rice at least two (Murakami 1970). It should be pointed out that because of their high sterility without GA spray (Cooper 1957), GA dwarfs are not easy to maintain in mutant collections. Non-germinating GA responsive mutants seem to have passed unnoticed.

As the physiological characterization of the mutants is not yet completed, the exact nature of the *ga* loci in *Arabidopsis* cannot yet be established. However, the most plausible explanation appears to be that they control steps in gibberellin biosynthesis. The finding of mutants at a same locus that have a different degree of GA requirement indicates that 'leaky' alleles are rather frequent, because, apart from the germinating dwarfs, some non-germinating mutants that also show partial germination under particular circumstances should be considered as 'leaky'. The apparent discontinuity between mutants selected as dwarfs and mutants selected as non-germinators might be caused by the selection criterium, although the same material has been screened for both types. A probable reason for the discontinuity could be the steepness of the GA dose response curve for germination.

Since in the dwarfs germination can be perfect while length growth is far from normal, the GA requirement for germination is probably much lower than for elongation growth and normal flower development.

The nature of the *ga-4* and *ga-5* loci is still under speculation. For *ga-4* there are indications from tests with different GA's (Koornneef unpublished), that it controls interconversion between some GA's.

An explanation for the somewhat reduced segregation frequencies might be a reduced viability or an incomplete 'rescue' by 10^{-5} M GA_{4+7}. However, it seems that these factors are of minor interest, as the viability of the mutant seedlings recovered by GA application to the seeds normally is very good; these plants do not differ from wild type in the most important period for survival in a greenhouse. The possibility of incomplete rescue seems to be ruled out by Figure 1. Reduced transmission, by the male gametophyte especially, might be a more important factor.

Except for the use of *ga*-mutants in illucidating the genetics of the gibberellin synthesis, these mutants, especially the 'non-germinators', are of particular interest as they provide an example of auxotrophic mutants which are so rare in higher mutants (Redei 1975). In *Arabidopsis* thiamine deficient mutants (Feenstra 1964; Redei 1965) are so rare in higher plants (Redei 1975). In *Arabidopsis* expresses itself already at the level of germination, they can be used much more efficiently than the seedling thiamine auxotrophics in experiments for the research of e.g. intragenic recombination and reverse mutations. The dwarf vs. non-dwarf phenotype provides a welcome check of the non-germinating vs. wild type phenotype in cases when germination occurs due to leakiness. For the use of *ga*-mutants for the study of intragenic recombination and of reversion see Koornneef (1979) and Koornneef et al. (1980), respectively.

Another application of *ga*-mutants might be in plant cell genetics, where e.g. complementing auxotrophic mutants can be used to select fusion products of lines mutated at different *ga*-loci. Non-germinating GA sensitive mutants are not restricted to *Arabidopsis* but can also be found in other plant species (e.g. tomato; van der Veen unpublished).

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