THE GENETICS OF SOME PLANTHORMONES AND PHOTORECEPTORS IN *ARABIDOPSIS THALIANA* (L.) HEYNH.

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THE GENETICS OF SOME PLANTHORMONES AND PHOTORECEPTORS IN *ARABIDOPSIS THALIANA* (L.) HEYNH.

Proefschrift

ter verkrijging van de graad van

doctor in de landbouwwetenschappen,

op gezag van de rector magnificus,

dr. C.C. Oosterlee,

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VOORWOORD

Als van een onderzoek gezegd kan worden dat het geen éénmanszaak is, dan geldt dat zeker voor datgene waarvan de resultaten in dit proefschrift beschreven zijn. Een groot aantal personen wil ik dan ook voor hun bijdrage bedanken. In de eerste plaats mijn promotor, Prof. J.H. van der Veen. Zijn idee, dat niet-kiemende mutanten groeistofmutanten zouden kunnen zijn was de basis van het onderzoek. Ook de door Prof. van der Veen in de loop der jaren opgebouwde collectie mutanten (waaronder reeds 2 niet-kiemende GA mutanten) en trisomen en zijn grote ervaring op het gebied van mutatie-onderzoek zijn van essentieel belang geweest bij de start van het onderzoek. Bijzonder dankbaar ben ik voor zijn stimulerende interesse en vele suggesties gedurende het gehele onderzoek. Ook zijn hulp bij het leesbaar maken van de verschillende artikelen is onmisbaar geweest.

De vakgroepen Plantenfysiologie en Plantenfysiologisch Onderzoek ben ik zeer erkentelijk voor de medewerking die zij hebben verleend zowel bij het beschikbaar stellen van accomodatie als bij het meewerken aan- en meedenken over de experimenten. Dit ondanks enkele voor fysiologen onaangename eigenschappen van het proefplantje. Dr. C.J.P. Spruit, die mij heeft ingewijd in enige geheimen van de fotomorfogenese heeft met zijn organisatorische en wetenschappelijke kwaliteiten een essentiële bijdrage geleverd aan het karakteriseren van de lichtreceptormutanten. Van groot belang is ook geweest de samenwerking met Dr.C.M.Karssen in het bijzonder met betrekking tot de ABA mutant. Deze samenwerking, begonnen als burenhulp, maar intensief voort-

STELLINGEN

1. Het ongewijzigde verloop van de zaadkieming bij gibberelline-deficiente mutanten van gerst en mais en bij gibberelline-ongevoelige mutanten van gerst en tarwe, wijst op een niet wezenlijke rol van gibberellinen bij de kieming van deze soorten.

2. De fysiologische betekenis van het transport van gibberellinen en abscisinezuur is tamelijk gering.

3. Alleen het door het embryo-genotype bepaalde abscisine-zuur induceert kiemrust in zaden van *Arabidopsis thaliana*.
   Dit proefschrift.

4. Naast fytochroom spelen ook andere fotoreceptoren een rol bij de door licht gereguleerde remming van de hypocotylgroei in *Arabidopsis thaliana*.
   Dit proefschrift.

5. Hormoon-deficiente mutanten kunnen niet geisoleerd worden door te selecteren op tolerantie voor concentraties van het betrokken hormoon die bij het wildtype remmend werken.
   Dit proefschrift.

6. Plantenhormoonmutanten verdienen meer aandacht in de plantencelgenetica, gezien de essentiële rol van deze stoffen bij de celdifferentiatie.

7. Het biochemisch en fysiologisch vergelijken van rassen, die naast het te bestuderen gen-contrast ook andere genotypische verschillen vertonen, draagt risico's met zich mee.

8. De lineaire relatie tussen de mutatiefrequentie per locus en de hoeveelheid DNA per genoom van een organisme, zoals deze door sommige auteurs is afgeleid uit literatuurgegevens, lijkt meer gebaseerd op "wishful thinking" dan op een theoretisch goed onderbouwd literatuuronderzoek.
   Abrahamson et al., Nature 245: 460-462 (1973)
   Heddle and Anthanasion, Nature 258: 359-361 (1975.

9. Het model van Reeves et al. om telotrisome uitsplitsingen te beschrijven houdt onvoldoende rekening met het verloop van de meiose bij telotrisomen.
   Reeves et al., Can.J.Genet.Cytol. 10: 937-940 (1968).

10. Zaadkiemingsloci zijn bij veel planten geschikter voor het uitvoeren van genetische fijnstruktuur-analyses dan loci voor pollenkenmerken.
   Dit proefschrift.

11. Eenink en Garretsen waarschuwen er terecht voor dat een gefascieerde bloeistengel bij sla ongewenst is vanwege het negatieve effect op de zaadproduktie. Fasciatie is echter ook een gunstige eigenschap, omdat hierdoor te snel schieten onder lange dag-omstandigheden kan worden voorkomen.
   Eenink en Garretsen, *Euphytica 29: 653-660 (1980)*.

12. De negatieve karaktertrekken, die Verbraeck toeschrijft aan de bewoners van het Westland omstreeks 1930, worden door hem ten onrechte streekgebonden genoemd.
   A.A.A. Verbraeck, Het Westland, Dissertatie Amsterdam, 1933.

Proefschrift van M. Koornneef
The genetics of some planthormones and photoreceptors
in *Arabidopsis thaliana* (L.) Heynh.
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gezet omdat Karssen de mogelijkheden van dit soort mutanten voor zaadfysiologisch onderzoek duidelijk onderkende, heeft geleid tot een zinvolle integratie van de plantenfysiologie en de erfelijkheidsleer. Met Kees Karssen bedank ik ook Rob van Beek voor het uitvoeren van de vele ABA bepalingen.

Dr. G.W.M. Barendse uit Nijmegen ben ik zeer erkentelijk voor zijn onderzoek naar de biochemische achtergrond van de *ga* mutaties. Zeer waardevol heb ik ook altijd de contacten gevonden met de Arabidopsisgroep in Groningen onder leiding van Prof. W.J. Feenstra. Behalve dat deze geleid hebben tot een gezamenlijke publicatie, heb ik ook hun benadering van de biochemische genetica van hogere planten (in het bijzonder van ons gemeenschappelijke object Arabidopsis) als zeer inspirerend ervaren.

Het lijkt misschien alsof alleen buiten de vakgroep hulpvaardige personen aanwezig waren. Niets is minder waar, want dankzij de bekwame assistentie van Margie Conquet, Sandra de Jongh, Corrie Hanhart en Jannie van Eden was het mogelijk de miljoenen zaden uit te zaaien (vaak zaad voor zaad,soms met duizenden tegelijk) en de honderdduizenden planten op te kweken en te beoordelen. Daarbij komen nog de talloze diverse proeven en proefjes die zij met veel inzet en nauwkeurigheid hebben uitgevoerd. De goede sfeer en samenwerking binnen ons Arabidopsisgroepje, waarbij ik ook de stagiaires en doctoraalstudenten reken, is voor het belangrijkste deel aan hen te danken.

Ook aan de plezierige medewerking van de heren G. van IJmeren, J. Beekman, A. Arends, W.H. van Blijderveen en B. Weijman, die het "onkruid" in de kassen verzorgd hebben, denk ik met veel genoegen terug.

Dat Lidwine Dellaert de eerste 3½ jaar tegelijk met mij op de vakgroep aan Arabidopsis werkte, was een waardevolle ervaring. Van haar heb ik geleerd dat je mutatieproefjes niet al te kinderachtig moet aanpakken. Piet Stam wil ik

hartelijk bedanken voor zijn altijd aanwezige bereidheid mij voor te lichten en te helpen met de wiskundige problemen in het onderzoek. Soms tijdens de koffie met wat formules op een papiertje, maar vaak ook door het schrijven van gecompliceerde computerprogramma's, wanneer het rekenen al te lastig werd.

Een andere belangrijke groep die ik niet gauw zal vergeten is het "losse volk", de STOVA stagiaires Hans Denissen, Olga Clei, Frieda van Dreven, Linda Putman, Kees van de Ka en Patty van Loenen Martinet en last but not least de 31 studenten (echt te veel om allemaal te noemen) die 3 en soms 6 maanden lang hebben meegewerkt aan het onderzoek als onderdeel van hun doctoraalstudie. Een groot deel van hun werk is terug te vinden in de hoofdstukken 6 en 7. Het begeleiden van deze bijna altijd enthousiaste studenten is een zeer plezierig deel van mijn onderwijstaak geweest.

Het vele typewerk voor dit proefschrift is voor een belangrijk deel uitgevoerd door Trees Makkes, maar ook de bijdragen in dit verband van Henriet Boelema en Marjon van Hunnik mogen niet onvermeld blijven. Dhr. Haasdijk van plantenfysiologie, Jan Maassen en Jannie van Eden hebben het merendeel van de tekeningen verzorgd, terwijl Hans de Vries en dhr. K. Knoop de fotografie voor hun rekening namen. Jan Maassen, Theo van Lent en dhr. K. Knoop hielden de technische outillage op peil en breidden die soms uit. Voor al deze "technische" hulp in de ruime zin van het woord, mijn hartelijke dank. De overige leden van de vakgroep wil ik bedanken voor de goede sfeer en voor datgene wat ik van hen geleerd heb over andere aspecten van het zeer brede gebied van de genetica.

CONTENTS

GENERAL INTRODUCTION

Growth and differentiation of plants are determined by numerous interactions between environmental factors (light, temperature, supply of minerals etc.) and the genetically conditioned ontogenic development.

Different groups of regulatory substances are involved in growth and differentiation, such as: 1) the planthormones: auxins, cytokinins, gibberellins, abscisic acid and ethylene and 2) photoreceptors like phytochrome.

The role of individual planthormones and photoreceptors has been analysed mainly in correlative studies, in which it was attempted to associate changes in the levels and/or in the response of a particular substance with morphogenetic or biochemical changes. It is obvious, however, that such correlations do not prove that hormones are causally involved in the observed response. Therefore, genotypes in which the level of a single regulating compound is drastically changed not only provide information about its genetic determination but also add an important tool to physiological studies. More specifically, with well defined single gene contrasts it is clear that the observed physiological and biochemical differences are causally related. This genetic approach ideally requires the use of spontaneous or induced monogenic mutants in isogenic lines, i.e. in the absence of obscuring effects of other gene contrasts, known or unknown. For this reason comparisons between cultivated varieties are of limited use for fundamental physiological research; moreover, well pronounced differences are not expected among cultivars, for types with drastic changes in the level of an essential substance are usually not maintained,

because of expected adverse effects on yield etc.

In plants several genes affecting biochemically more or less well defined compounds and processes have been described, e.g. genes for storage proteins (Nelson, 1973), secondary plant metabolites like anthocyanins and epicuticular wax (Wettstein-Knowles, 1979), photosynthesis and photorespiration (Vose,1981; Somerville and Ogren, 1982), mineral uptake (Vose, 1981) and a limited number of amino-acid and vitamin auxotrophs (Gebhardt and King, 1981).

Identified genes controlling hormone metabolism or action have been described before in literature. Some of the relevant mutants were found in existing general mutant collections. Examples are the gibberellin (GA) responsive dwarfs in maize (Phinney, 1960) and some other species (Pelton, 1964); the abscisic acid (ABA) deficient mutants (Tal and Nevo, 1973) and an ethylene responsive mutant (Zobel, 1973) in tomato, and the photosystem I mutants found among the chlorophyll mutants of barley (Hiller et al, 1980). However, many interesting mutants may have escaped detection, because they are (conditionally) lethal or because of the inconspicuous change in appearance when a particular blocked metabolic pathway is bypassed.

A fruitful method aimed at collecting specific classes of mutants is the induction of mutations followed by the appropriate detection and selection procedures. Such direct procedures to isolate physiological mutants in plants have been used in studies on nitrate metabolism (Braaksma, 1982), photorespiration (Somerville and Ogren, 1982) both with *Arabidopsis*, hormone sensitivity of barley aleurone layers (Ho et al., 1980), and the selection of auxin resistant mutants, again in *Arabidopsis* (Maher and Martindale, 1980). These mutants were selected at the seed and plant level. Recently auxin and abscisic acid tolerant lines were isolated in cell cultures (see von Siegemund, 1981).

The design of these detection and selection procedures requires an under-

standing of the physiological process under study and a plant system suitable for both genetical and physiological experimentation. It often involves the growth of large numbers of plants under strictly controlled conditions of growth medium or even the composition of the atmosphere (Somerville and Ogren, 1982). In general large scale testing is only feasable with small plants or with seedlings or seeds, though their small bulk can be a disadvantage for physiological and biochemical analysis. Also efficient mutagenic procedures should be available to keep the number of plants to be screened within operational limits. The small fast growing diploid crucifer *Arabidopsis thaliana* (L.) Heynh. fullfills many of the prerequisites for efficient mutant selection and mutant characterization (Rédei, 1975), for it has a very short life cycle (less than two months), takes little space and still produces thousands of small seeds per plant. Moreover it is a selffertilizer, which facilitates mutant detection and the isolation of genetically homozygous lines, its chromosome number is low (2n = 10), the plants can be grown aseptically and callus and cell suspension cultures are possible (Negrutiu et al., 1978). So far a disadvantage compared to e.g. barley, maize, pea and tomato was formed by its rather fragmentary genetic map.

The present thesis describes the isolation and characterization of genes regulating the metabolism and action of plant hormones and photoreceptors in *Arabidopsis thaliana* (L.) Heynh.

The first 4 chapters present the isolation procedures and the genetical and physiological characterization of gibberellin responsive mutants (chapter 1), abscisic acid deficient mutants (chapters 2 and 3) and photoreceptor mutants (chapter 4). The use of such mutants for plant physiological research is demonstrated especially in chapters 3 and 4.

The last 4 chapters give a more detailed genetic description of the mutants.

In chapter 5 the mutation frequencies per locus are calculated for different mutagens. To locate the genes on the *Arabidopsis* genome, the different linkage groups were first assigned to the 5 chromosomes (chapter 6) and subsequently a detailed linkage map was constructed comprising 76 loci (chapter 7). Finally one locus (*ga-1*) gave the opportunity to analyse its genetic fine structure, which led to the first internally consistent linear map for a higher plant gene (chapter 8).

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CHAPTER 1

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

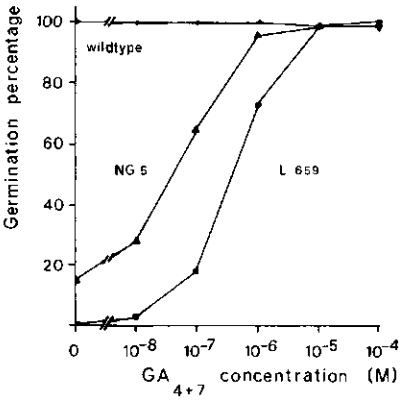

Fig. 1. The germination of two typical non-germinating mutants at the *ga-1* locus and wild type at different concentrations of GA_{4+7}

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-

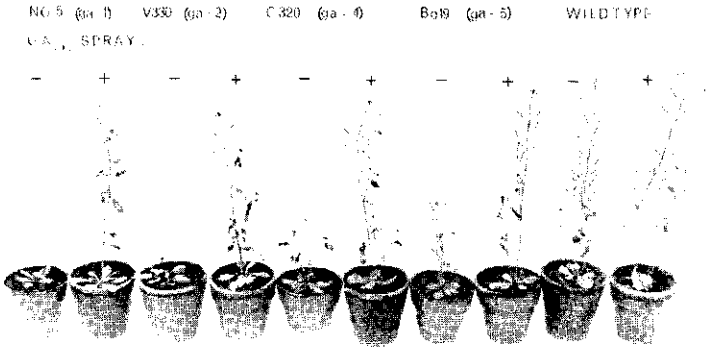

Fig. 4. The response of several *ga*-mutants and wild type *Arabidopsis* to two sprays with 10^{-4} M GA_{4+7} about two and three weeks after germination. All plants germinated without GA, which is rare for NG5 but normal for the others. Note that in the *ga-1* and *ga-2* mutants symptoms of GA deficiency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

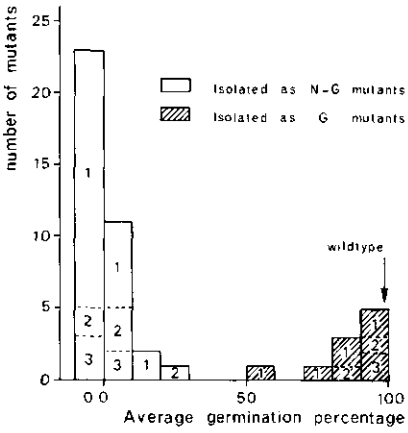

Fig. 5. The distribution of the average germination percentage of 47 independently induced mutants at the *ga-1* (1), *ga-2* (2) and *ga-3* (3) loci

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.

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 plants (Redei, 1975). In *Arabidopsis* thiamine deficient mutant (Feenstra, 1964; Redei, 1965) are the only clear example. However, as the GA deficiency 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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CHAPTER 2

The Isolation of Abscisic Acid (ABA) Deficient Mutants by Selection of Induced Revertants in Non-germinating Gibberellin Sensitive Lines of *Arabidopsis thaliana* (L.) Heynh.

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Summary. By selecting for germinating seeds in the progeny of mutagen-treated non-germinating gibberellin responsive dwarf mutants of the *ga-1* locus in *Arabidopsis thaliana*, germinating lines (revertants) could be isolated. About half of the revertants were homozygous recessive for a gene (*aba*), which probably regulates the presence of abscisic acid (ABA). Arguments for the function of this gene were obtained from lines homozygous recessive for this locus only, obtained by selection from the F_2 progeny of revertant × wild-type crosses. These lines are characterized by a reduced seed dormancy, symptoms of withering, increased transpiration and a lowered ABA content in developing and ripe seeds and leaves.

Key words: *Arabidopsis thaliana* – Abscisic acid – Gibberellin – Physiological mutants – Seeds – Dormancy – Water relations

Abbreviations

ABA	Abscisic acid
GA_{4+7}	Mixture of gibberellin A_4 and A_7
EMS	Ethylmethanesulfonate
NG	Non-germinating
G	Germinating

Introduction

The role of endogenous regulating compounds in the physiology of plants has been studied mainly by correlative studies. The use of genotypes in which the level of one of these compounds is drastically changed adds an important additional tool to plant physiology. This genetic approach ideally requires the use of monogenic mutants or isogenic lines, for only with such single-gene contrasts is it clear that the observed physiological and biochemical differences are causally related.

The hormone abscisic acid (ABA) plays an important regulatory role in a number of physiological processes e.g. the dormancy and germination of seeds, the regulation of water stress, root geotropism and dormancy of buds (Walton 1980). Monogenic mutants with a disturbed ABA metabolism have been studied in tomato by Tal and coworkers (Tal and Nevo 1973). These mutants at the loci *sit*, *not* and *flc*, isolated by Stubbe (1957, 1958, 1959) are characterized by an excessive wilting tendency due to abnormal stomatal behaviour (Tal 1966), which could be reversed by the application of ABA (Imber and Tal 1970). A relation between disturbed ABA metabolism and germination was described in maize by McDaniel et al. (1977) and Smith et al. (1978). They found that precocious germination of viviparous (*vp*) mutants (Robertson 1955) could be attributed to the absence of ABA or to the incapacity to respond to ABA.

In *Arabidopsis* monogenic recessive mutants have been described by the present authors in which germination was reduced or absent under conditions that were suitable for germination of wild-type seeds (Koornneef et al. 1977; Koornneef 1978; Koornneef and van der Veen 1980).

One group of non-germinating mutants, which could be brought to 100% germination by the application of gibberellins (GA_{4+7}, GA_3 and GA_9 were effective), subsequently developed into dwarfs. These dwarfs could be reverted fully or almost fully, to wild-type by GA sprays (Koornneef and van der Veen 1980). These mutants, found in *Arabidopsis* at three loci (*ga-1*, *ga-2* and *ga-3*) probably lack the capacity to synthesize gibberellins. Strictly comparable mutants have been found in tomato, at only two loci so far (Koornneef et al. 1981; van der Veen and Bosma pers. comm.).

Application of gibberellins stimulates the germination of seeds of many plant species (Jones and Stoddart 1977). Seed germination requires in most cases de novo synthesis or activation of these substances. Besides such promoters, also inhibitors like abscisic acid (ABA) may affect the state of dormancy of a seed.

This concept led to the idea that the germination capacity of non-germinating dwarfs might be restored when the level of inhibitors would be reduced by mutating the genes responsible for their production. Selection

for this type of revertants is easy since they are self-detecting: germinating seeds among non-germinating seeds. Apart from mutations in genes regulating the production of inhibitors, also other types of external suppressor mutations may be found as well as intragenic reversions. This paper describes the isolation of ABA deficient mutants in *Arabidopsis* by using this revertant technique. For preliminary reports on this subject see Karssen et al. (1980) and Koornneef et al. (1980).

Material and Methods

Plant Material

Arabidopsis thaliana (L.) Heynh. (2 n = 10) is a small fast growing, self-fertilizing crucifer. Seed stocks used in the present experiments were derived from the pure line Landsberg *erecta* (Redei 1962), which will be referred to as the wild-type. For revertant induction we used the non-germinating, gibberellin-sensitive mutant lines NG5 (EMS induced) and 6.59 (fast-neutron induced). Both lines are mutants induced in the wild-type at the *ga-1* locus on chromosome 4 (Koornneef and van der Veen 1980). To check for seed admixture and unwanted cross-fertilization, both lines were also homozygous recessive for *gl-1* (hairless; chrom. 3), except in the first revertant-induction experiment (see later).

Conditions of Culture

The seeds were sown equally spaced in numbers of 25, 30, or 36 in 9 cm petri dishes on perlite saturated with a standard mineral solution, as described by Oostindiër-Braaksma and Feenstra (1973). The seeds were incubated at 4–6 °C for 4–6 days to break dormancy, and subsequently allowed to germinate at a temperature of approx. 24 °C under continuous light (Philips TL 57) at an intensity of 8 W · m². After 8 days at 24 °C, the seedlings were transplanted into soil and cultivated in an air-conditioned greenhouse, where additional white fluorescent light (Philips TL 57) was given during 24 h per day in the winter (October to April).

Germination Tests

Germination tests were performed in plastic petri dishes (Ø 8.5 cm) on two layers of filter paper (Ederol no. 261) saturated with 2 ml of sterile distilled water. To avoid rapid evaporation, each dish was wrapped in a small polythene bag. Temperature and light conditions were as described above for seeds sown on perlite. Germination was scored 7 days after the start of incubation at 24 °C.

The Induction and Isolation of Revertants

To induce revertants, seeds of NG5 and 6.59 were first redried after dormancy breaking on moist filter paper and then treated with 10 mM ethylmethanesulfonate for 24 hrs at 24 °C in dark (Koornneef et al. 1982). After rinsing with tap water, the seeds were immediately sown in petri dishes containing 10 μM GA_{4+7} in the standard mineral medium and transferred to light as described above. The resulting M_1 seedlings developed into the usual NG5 and 6.59 dark-green, bushy dwarfs. At 4 and 5 weeks after EMS treatment the dwarfs were sprayed with 100 μM GA_{4+7} to stimulate anther development and to provide sufficient seed set from selfing. M_1 plants were individually harvested and the M_2 progenies were sown separately at standard conditions. Screening for germinating seeds (the presumed revertants) was done 8 days after the start of incubation. The seedlings obtained were transplanted into soil. The ultimate selection for revertants was based on the germination behaviour of the M_3 lines.

Genetic Characterization

For genetic analysis the revertants were crossed with wild-type and the parental *ga-1* mutant. Revertant types were scored on their capacity to germinate without GA and on the leaf colour which is slightly different from the darker green colour of non-germinating GA responsive *ga-1* mutants. Presumed ABA-types (recombinant, single recessive to wild-type) were scored on several morphological features (described in detail later in the paper). Allelism versus non-allelism was tested on the basis of non-complementation versus complementation to parental *ga-1* mutant type in F_1's of revertant × revertant or to wild-type in revertant × ABA-type crosses.

Localization on chromosomes was done by trisomic analysis (Koornneef and van der Veen 1978). The position of the *aba* locus on the chromosome was determined by linkage analysis of F_2 populations. The recombination fractions were estimated by the Product Ratio Method using the tables of Stevens (1939).

Measurement of Water Loss in Intact and Cut-off Plants

To determine water loss plants were grown for 2 weeks in plastic pots in the greenhouse and were then transferred to a climate chamber (temperature 22 °C; relative humidity approx. 85%, 12 h fluorescent light at approx. 13 W · m², 12 h dark). From the day of transfer onwards, half of the plants were sprayed with 10 μM ABA every second day, the other half with water. After 8 days in the climate chamber the plastic pots, including the soil surface, were wrapped in aluminium foil to prevent evaporation from the soil surface. Water loss during the third light and dark period after wrapping the pots, was determined by weighing the plants with the pots at the change of dark and light. The leaf surface was measured with an area meter. To measure water loss in cut-off aerial parts, well-watered plants were transferred from the climate room to a laboratory room and kept at 22 °C in white fluorescent light (0.6 W · m²). After an acclimatization period of 1½ h the aerial parts were cut off from the roots and stored in 400 ml glass beakers. Fresh weight was determined every hour.

Determination of ABA Content

Quantitative determinations of endogenous ABA were performed according to techniques described by Knegt et al. (1981) with a few additions. During purification of the extracts of seeds and siliquae the extraction into K_2HPO_4 with subsequent acidification to a final volume of 10 ml diethyl ether was performed three times instead of once. After standard purification the extracts of the leaves were purified additionally by using high pressure liquid chromatography with a re-

verse phase RP8 column operated with a linear gradient from 25% to 75% methanol in water. The solvents were 0.1 M towards acetic acid. ABA and trans-ABA are partially separated in this system, but collected in one fraction. The gas chromatograph was operated with He as carrier gas and 5% CH_4 with Ar as make up gas.

Results

A) Isolation of Revertants and Genetic Characterization

The Isolation of Revertants

From the individual M_2 progenies of 2122 EMS-treated seeds of *ga-1* mutants NG5 and 6.59 (M_2 lines), 31 lines could be isolated that showed 50% (up to 100%) germination under standard germination conditions (Table 1). It appeared that 15 of these lines were germinating, GA-responsive extreme dwarfs with a somewhat weaker appearance and a slightly yellow-brown colour on the leaves compared to the parental *ga-1* mutants. For reasons that will be described in the next sections, this type will be called ABA revertant.

Other isolated revertants were mostly slightly taller and/or had a paler green colour than NG5 and 6.59. The two revertants dominant to the parental lines reached a length of respectively 75% and 50% of the wild-type. These two groups will not be further considered here.

The selection of revertants in NG5 was hampered by the partial germination of this mutant as has been described before (cf. Koornneef 1979; Koornneef and van der Veen 1980), probably due to leakiness of this allele. This led to the decision to transplant only the most conspicuous plants from M_2 lines in which germination occurred as these could be expected to be revertants. Further only the progeny of M_2 plants, which showed some deviating features compared to the parental line, were tested as M_3 lines. Only in the first experiment were M_3 lines from each M_2 line with germinating seeds tested. However, it appeared that all lines ultimately selected as revertants on the basis of a high germination percentage in M_3 had a somewhat deviating morphology.

Genetic Characterization of the Revertants

In crosses between the revertants and the parental *ga-1* mutants, the capacity of the revertant to germinate was found to be monogenic recessive to the inability to germinate of the *ga-1* mutants.

Complementation tests with 14 ABA-revertants revealed that 13 of these independently isolated revertants were allelic. The presence of a second locus needs further confirmation.

In F_2's from revertant × wild-type crosses one expects on the basis of two unlinked loci for germination the ratio 13 germ.: 3 non-germ., and when also taking plant phenotype into account, the ratio 9 wild-type: 3 non-germinating GA dwarf: 3 new (recombinant) phenotype: 1 germinating GA dwarf (revertant). Indeed, a deviating non-dwarf phenotype was observed in all F_2's, which when compared to the wild-type had a reduced vitality (smaller, weaker plant), a slightly yellow-brownish colour, and symptoms of withering, mainly in the influorescence (Fig. 1). The withering symptoms were more pronounced in winter than in summer. These symptoms point to ABA deficiency (see also below). Therefore the new recombinant phenotype was called ABA-type and the mutant allele *aba*. By selfing ABA-type F_2 plants, F_3 lines could be established not segregating for revertant types (expected one among three F_3 lines). Lines homozygous for ABA-type were crossed with a parental *ga-1* mutant to give F_2 and F_3. The segregation of these populations was analysed together with those derived from revertant × wild-type crosses. It should be noted that *aba*^{1} was induced in *ga-1*^{1} (line NG5) and *aba*^{2} in *ga-1*^{2} (line 6.59) background. In the *ga-1* mutant × ABA-type crosses the allelic combinations were interchanged, so all combinations were represented. Since χ^2-test of heterogeneity between the four crosses did not reveal significant dif-

Table 1. Frequencies of independently induced revertants

Experiment	Parental mutant	Number of M_2 progenies tested	Number of revertant lines			Total
			ABA-revertants	Dominant	Others	
I	NG5 (*ga-1*^{1}/*ga-1*^{1})	199	4	0	0	4
II	NG5 (*ga-1*^{1}/*ga-1*^{1})	382	4	1	3	8
II	6.59 (*ga-1*^{2}/*ga-1*^{2})	246	1	0	1	2
III	NG5 (*ga-1*^{1}/*ga-1*^{1})	771	2	0	8	10
III	6.59 (*ga-1*^{2}/*ga-1*^{2})	524	4	1	2	7
		2122	15	2	14	31

Fig. 1 A and B. A BA-type A26 (**A**) showing a withered main stem and withered siliquae compared with wild-type (**B**)

ferences (except one case, see below), the segregation data of the four crosses listed below could be pooled (Table 2).

Crosses in association; (revertant × wild-type):

A) *ga-1*^{1}/*ga-1*^{1}, *aba*^{1}/*aba*^{1} × +/+, +/+

B) *ga-1*^{2}/*ga-1*^{2}, *aba*^{2}/*aba*^{2} × +/+, +/+

Crosses in dispersion; (ga-1 mutant × ABA-type):

C) *ga-1*^{1}/*ga-1*^{1}, +/+ × +/+, *aba*^{2}/*aba*^{2}

D) *ga-1*^{2}/*ga-1*^{2}, +/+ × +/+, *aba*^{1}/*aba*^{1}

These pooled data (Table 2) consistently confirm the segregation at two unlinked loci (*ga-1* and *aba*), in particular the fact that germinating GA dwarfs (revertants) only segregated in F_3 lines when expected.

In F_3 lines from wild-type F_2 plants a significant recessive deficit at the *ga-1* locus was found. With induced mutants a recessive deficit of this magnitude is by no means uncommon and can be ascribed to certation (cf. Koornneef and van der Veen 1980; Koornneef et al. 1982). Since the degree of certation is variable, the single case of significant heterogeneity between crosses can be explained in this way. In addition, the recessive deficit at the *ga-1* locus may also be generated by a reduced survival of non-germinating genotypes when these are induced to germinate by 10 µM GA_{4+7} after 8 days of incubation at 24 °C. This may be partly due to secondary dormancy induced during that period.

With regard to the phenotypes described in the top lines of Table 2 two different aspects have to be considered: 1. Plant phenotype: *aba/aba* leads with a wild-type genetic background (with respect to the *ga-1* locus) to ABA-type plants (slightly yellow-brown colour, withering), whereas with a *ga-1* background (GA dwarf) it leads to GA-responsive dwarfs with a more yellow-brown colour than normal GA dwarfs. 2. Germination: in the presence of *ga-1/ga-1*, which as such leads to a lack of germination, *aba/aba* restores the germination

Table 2. Segregation ratio's in F_2 and F_3 progenies from pooled crosses A, B, C and D (for explanation see text)

		Wild-type G	GA dwarf NG	ABA-type G	GA dwarf (revertant type) G+NG		χ^2_1 (3:1) *ga-1* locus	χ^2_1 (3:1) *aba* locus	χ^2_1 linkage ("2×2 Table")
		+/. +/.	*ga-1/ga-1* +/.	+/. *aba/aba*	*ga-1/ga-1* *aba/aba*				
F_2 generation		555	149	179	56	6[a]	3.6	0.1	2.2
F_3 generation									
F_2 plant wild-type:									
Segregating-digenic	(52)[b]	920	203	284	60		39.1**	1.9	0.1
Segregating NG GA dwarf	(22)	507	122	–	–		10.5**	–	–
Segregating ABA-types	(27)	589	–	167	–		–	3.4	–
Non-segregating	(13)	357	–	–	–	–		–	–
F_2 plant NG GA dwarf:									
Segregating "revertants"	(29)	–	554[c]		175[c]		–	0.4	–
Non-segregating	(10)	–	253[c]		–		–	–	–
F_2 plant ABA type:									
Segregating "revertants"	(29)	–	–	509	133	33[a]	0.2	–	–
Non-segregating	(9)	–	–	213	–		–	–	–
F_2 plant reverant type	(18)	–	–	–	240	59[a]	–	–	–

[a] NG GA dwarfs of the revertant type occurred almost exclusively in the progeny of cross B (see text)

[b] In brackets: Numbers of F_2 plants selfed. These F_2 plants were randomly sampled within each of the four phenotypic classes, but not between classes. Note the good fit within classes (4:2:2:1 and 2:1)

[c] Tested by germination behaviour only

** $P < 0.01$

capacity. This seems to imply that GA is only required for germination if ABA is present.

In a few cases seeds that did not germinate on water, gave, upon addition of GA_{4+7}, dwarfs which showed the leaf colour characteristics of the revertant type. Therefore it is concluded that in these cases the germination capacity in *ga-1/ ga-1, aba/aba* was only partly restored. Such incomplete restoration occurred almost exclusively in cross B (alleles *ga-1*^{2} and *aba*^{2}) and was absent in cross C (alleles *ga-1*^{1} and *aba*^{2}) and in crosses A and D (both *aba*^{1} allele). As a check both G and NG revertant types from cross B were selfed. The seeds obtained gave equal germination percentages, viz. $32.0 \pm 5.4\%$ for the G-parents and $31.1 \pm 3.8\%$ for the NG-parents.

A plausible explanation can be found on the basis of the concept of GA-ABA balance in germination. The *ga-1*^{2} mutant never germinates – this is in contrast to the *ga-1*^{1} mutant which sometimes germinates to some extent. To make the "deeply GA-deficient" *ga-1*^{2} mutant germinate a "strong" (i.e. deeply ABA-deficient) *aba* mutation is necessary. Here *aba*^{2} is not strong enough, whilst *aba*^{1} is.

As mentioned before, the mutant alleles *aba*^{1} and *aba*^{2} were initially induced in *ga-1*^{1}/*ga-1*^{1} (line NG5) and *ga-1*^{2}/*ga-1*^{2} (line 6.59) background respectively. From the crosses C (alleles *ga-1*^{1} and aba^2)and D (alleles *ga-1*^{2} and *aba*^{1}) it follows that a in qualitative sense the germination-restoring effect of *aba* mutants does not depend on the allele at the *ga-1* locus: no allele specificity. Nor does the effect of *aba* alleles seem to be locus-specific with respect to the *ga* loci. This follows from a cross, revertant × *ga-2* mutant:
(*ga-1/ga-1*, + / +, *aba/aba* × + / +, *ga-2/ga-2*, + / +), where germinating dwarfs (*aba/aba*) could be selected which were homozygous recessive at the *ga-2* locus (and wild-type at the *ga-1* locus), as determined by means of test crosses.

In general it appears that *aba* alleles improve the germination of genotypes with a reduced germination.

The *aba* gene could be located on chromosome 5 by trisomic analysis. As said before, *ga-1* was located on chromosome 4, which confirms that the two loci are unlinked. Linkage of *aba* with specific chromosome-5 markers was studied in F_2 populations. The markers involved were *ms* (male-sterile), *tt-2* (transparent testa), *tt-3* (transparent testa, anthocyaninless) and *tz* (thiazole-requiring). The results are summarized in Fig. 2 and show that *aba* is located at the end of chromosome 5, as far as this chromosome has been mapped.

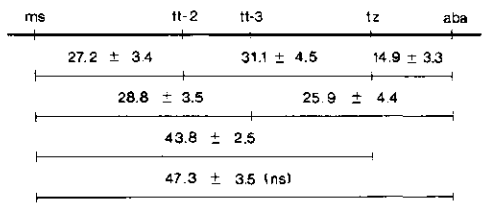

Fig. 2. Provisional linkage map of chromosome 5 and the estimates of recombination percentage between some markers including *aba*. n.s. no significant linkage

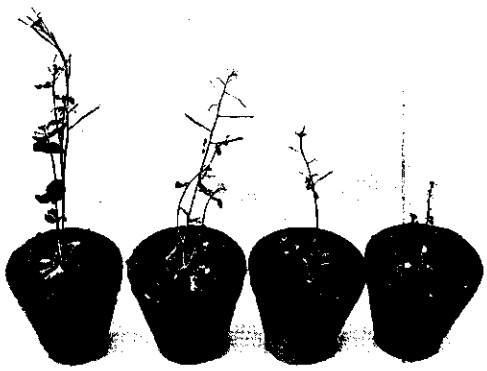

Fig. 3. Adult plants of wild-type and the independently arisen mutants G4 (*aba*^{3}), A26 (*aba*^{1}) and A73 (*aba*^{4})

B) Physiological Characterization

It has been shown in the previous sections that a specific gene (*aba*) with a specific phenotypic expression is able to remove the lack of germination in *ga-1* mutants.

Some phenotypic features of a number of lines the ABA-type (+/+, *aba/aba*) are presented in Table 3 and Fig. 3. All the characteristics show an increasing tendency to deviate from the wild-type in the allelic order *aba*^{3}/*aba*^{3} (G4), *aba*^{1}/*aba*^{1} (A26), *aba*^{4}/*aba*^{4} (A73). The perfect rank correlation between all five parameters implies multiple pleiotropism with specific degrees of expression of the different alleles.

The physiological characterization of the ABA-type was focussed on: 1. The germination behaviour of seeds, because the selection of this genotype was based on this property. 2. water relations of the plant in view of the observation of withering. 3. ABA content, since both previous aspects are related to this compound (Walton 1980).

Germination Behaviour

A comparison of the germination behaviour of seeds from ABA-types (*aba/aba*) with seeds from wild-type

Table 3. Some characteristics of lines of ABA-type as compared with wild-type

	+/+ (wild-type)	*aba*³/*aba*³ (G4)	*aba*¹/*aba*¹ (A26)	*aba*⁴/*aba*⁴ (A73)
Percentage survival after planting	99 ±1[a]	97 ±2[a]	69 ±5[b]	82 ±5[b]
Percentage plants with withered main stem	0	11 ±4[b]	56 ±7[c]	69 ±6[c]
Total plant length (cm)	20.8±0.3[a]	14.8±0.2[b]	8.2±0.3[c]	6.4±0.3[d]
Length of largest rosette leaf (cm)	2.6±0.1[a]	2.1±0.1[b]	1.4±0.1[c]	1.2±0.1[d]
Number of side shoots	4.9±0.1[a]	3.2±0.1[b]	2.7±0.2[c]	2.0±0.1[d]

A different letter indicates a significant difference ($P < 0.05$)

(+/+), harvested the same day and identically stored, showed that seeds of the ABA-type are characterized by a strong reduction of seed dormancy, as judged in line A26 (*aba*¹/*aba*¹) from a reduced requirement for light and cold treatment (Fig. 4). This has been found as well for other *aba* alleles.

The germination of both ABA and wild-type could be completely inhibited by exogenous applied ABA (Fig. 5). The response of the ABA-type is only slightly less than that of the wild-type.

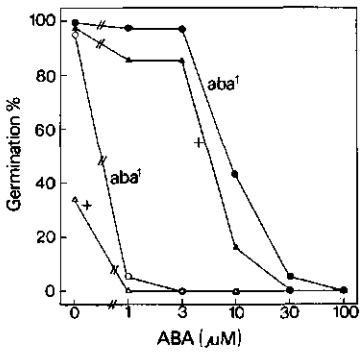

Fig. 5. Germination in white light of wild-type (+) and A26 (*aba*¹) at different concentrations ABA, scored 3 days (open symbols) and 7 days (closed symbols) after incubation

Water Relations

When the plants in the greenhouse were either enclosed in plastic bags, which maintained a high humidity, or were sprayed twice a week with an ABA solution (Table 4), the development of symptoms of withering on ABA-type plants was highly reduced. Plants of ABA-type (*aba*³/*aba*³) grown in a climate room showed an enhanced water loss, which could be considerably reduced by ABA sprays (Table 5).

The enhanced rate of water loss in isolated aerial parts of ABA-types (Fig. 6) can be interpretated as a reduced rate of stomata closure upon water stress caused by the absence of water supply from the roots. This may be caused by a reduced availability of ABA.

Endogenous ABA Content

The level of endogenous ABA was determined in dry, ripe seeds and in siliquae with seeds during de-

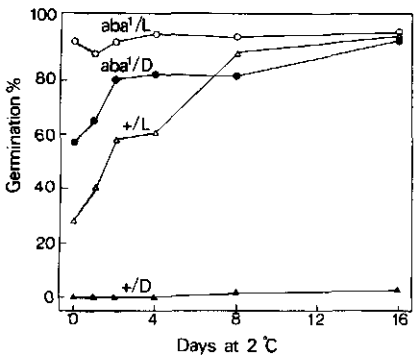

Fig. 4. Germination percentage of ABA type and wild-type in white light (L) and darkness (D) preceeded by different periods of dark-incubation at 2 °C. The seeds were used 4 weeks after harvest

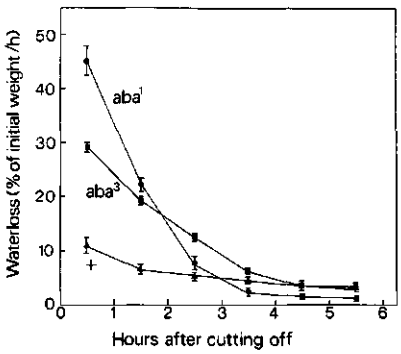

Fig. 6. Water loss by the aerial parts of ABA types and wild-type expressed per hour as percentage of fresh weight at the time of cutting

Table 4. The effect of spraying with ABA and of enclosing the plants in plastic on the percentage of plants with symptoms of withering

	Treatment	Normal	Withered tips of siliquae	Withered siliquae	Withered main stem	
+ / + (wild-type)	Sprayed H_2O	100 (48)	–	–	–	
	1 μM ABA	100 (46)	–	–	–	
	10 μM ABA	100 (48)	–	–	–	
	Enclosed in plastic	100 (25)	–	–	–	
aba^3/*aba*^{3} (G4)	Sprayed H_2O	5 (2)	21 (9)	65 (28)	9 (4)	a
	1 μM ABA	16 (7)	11 (5)	71 (31)	2 (1)	a
	10 μM ABA	83 (38)	11 (5)	7 (3)	–	b
	Enclosed in plastic	83 (35)	–	13 (7)	–	b
aba^1/*aba*^{1} (A26)	Sprayed H_2O	–	8 (3)	22 (8)	70 (26)	a
	1 μM ABA	22 (8)	19 (7)	17 (6)	45 (15)	b
	10 μM ABA	64 (28)	14 (6)	11 (5)	11 (5)	c
	Enclosed in plastic	65 (20)	3 (1)	26 (8)	6 (2)	c

In brackets: the number of plants observed
Within genotypes: a different letter (a, b, c) indicates a significant difference between treatments when testing (χ^2) the normals versus non-normals (withered tips of siliquae, siliquae, and main stem)

Table 5. The effect of the *aba* gene, of ABA sprays and of light on water loss (kg/m^2/h) of *Arabidopsis* plants grown in a climate chamber (plants were cultivated in a 12 h photoperiod or in darkness and sprayed twice a week with water or 10 μM ABA)

	Light period		Darkness	
	H_2O	10 μM ABA	H_2O	10 μM ABA
+ / + (wild-type)	2.5±0.1	1.9±0.1	1.2±0.1	1.0±0.1
aba^3/*aba*^{3} (G4)	6.0±0.5	2.6±0.2	3.2±0.3	0.9±0.1

A 2 × 2 × 2 factorial analysis of variance showed that the main effects, genotype, light and ABA, were highly significant ($P < 0.01$). The three interactions were also significant

velopment (Table 6) and in rosette leaves (Table 7). The ABA content in seeds, in particular during seed development, was found to be much lower in G4 (*aba*^{3}/*aba*^{3}) and reduced below the level of detection in ripe seeds of A26 (*aba*^{1}/*aba*^{1}) and A73 (*aba*^{4}/*aba*^{4}). Rosette leaves contained very low amounts of ABA (Table 7), nevertheless the same rank order wild-type > G4 > A26 appears. In the same order the phenotype of the different alleles was found to deviate from wild-type for a number of characteristics (Table 3).

It appears that ABA-types are deficient in ABA content during various stages of their development, which very probably explains the symptoms of ABA deficiency observed.

Table 6. Endogenous ABA content of ripe seeds and of developing siliquae with seeds. The ripe seeds were extracted within 1 month of harvest, the developing seeds were extracted 10 days after pollination

	Ripe seeds harvested in:				Siliquae with developing seeds harvested in:	
	April 1979		October 1979		January 1980	
	ng/g fresh weight	pg/seed[a]	ng/g fresh weight	pg/seed[a]	ng/g fresh weight	pg/seed[b]
+ / + (wild-type)	71	1.42	27	0.54	117	10.5
aba^3/*aba*^{3} (G4)	8	0.16	7	0.14	12	1.0
aba^1/*aba*^{1} (A26)	nt	nt	< 1	< 0.02	nt	nt
aba^4/*aba*^{4} (A73)	nt	nt	< 1	< 0.02	nt	nt

nt = not tested
[a] seed weight 20 μg/seed
[b] 93% or more of total ABA in siliquae is present in the seeds (Karssen et al., in preparation)

Table 7. Endogenous ABA content of greenhouse grown rosette leaves of four-week-old wild-type and ABA-type

	ABA content (ng/g)
+ / + (wild-type)	6 (8; 3)
aba^3/*aba*^{3} (G4)	2 (2; 2)
aba^1/*aba*^{1} (A26)	1 (2; 0)

In brackets: values of the two replications

Discussion

By means of selection for revertants of non-germinating mutants, interesting physiological mutants with a disturbed ABA metabolism could be isolated. The isolation procedure for ABA-deficient mutants via revertants has the disadvantage of indirectness and inefficiency as it may take three extra generations. However, the short life cycle of *Arabidopsis*, enabled us to remove the parental *ga-1* allele relatively quickly. An alternative would be direct selection for the ABA-type in segregating M_2 populations derived from mutagen-treated wild-type. Here the rather inconspicuous phenotype will be a problem, especially in summer, when the symptoms of withering are almost absent in the greenhouse. Moreover, plants with a slightly deviating colour and a weaker growth occur frequently in M_2 populations of *Arabidopsis*. Selection for non-dormant seeds by sowing these M_2 seeds immediately after harvest may be a more attractive direct selection method. However, the rapid loss of dormancy when the seeds are stored after harvest, also occurring in wild-type, and the large environmental and maternal effects on dormancy may complicate this procedure.

Selection of ABA resistance, which is an attractive procedure especially in cell cultures (Wong and Sussex 1980), probably will not be very efficient either, as the differences between the dose-response curves of mutants and wild-type are relatively small.

The rather unaffected sensitivity of the ABA-type to exogenously applied ABA (Fig. 5) seems to exclude that the ABA-receptor sites are affected in the mutant. Very probably the *aba* gene regulates the biosynthesis of endogenous ABA at all stages of the development of the *Arabidopsis* plant. It cannot be entirely excluded however that the *aba* gene, when homozygous recessive, enhances ABA degradation.

The *aba* mutant may have a similar biochemical background as the *flc*, *not* and *sit* mutants in tomato (Tal and Nevo 1973; Nevo and Tal 1973), and as the background of some of the *vp* loci in maize (Smith et al. 1978). The ABA mutants in *Arabidopsis* seem unique as they combine the characteristics of ABA-deficient mutants in both the tomato (enhanced transpiration) and the maize (reduced seed dormancy). However, it may very well be possible that no attention has been paid to seed germination in tomato nor to the water relations in maize. An indication for a reduced seed dormancy of the tomato mutants comes from the observation of precocious germination in ripe fruits of the *sit* mutant (Koornneef unpublished).

Compared to maize, where probably five loci (Smith et al. 1978), and tomato where three loci, are known to affect the level of endogenous ABA, in *Arabidopsis* only one and perhaps two loci have been identified so far among 14 independently induced mutants. A simple explanation might, apart from the limited scale of the present mutation-induction experiments, be the fact that our *aba* locus has a relatively high mutation frequency (cf. Koornneef et al. 1981a). Finally, it has to be realized that many ABA-deficient mutants may be lethal because they might as well be deficient in carotenoïds as has been found in maize (Robertson 1955; Smith et al. 1978). All these substances have mevalonic acid as a common precursor. It may be significant that a viviparous mutant of sunflower was also characterized by a reduced pigment content (Wallace and Habermann 1958). It is quite possible that this mutant and other viviparous mutants described in other plant species, e.g. barley (Gustafsson et al. 1969), prove to be deficient in or in sensitive to ABA.

In addition to studies with mutants as cited above, differences in ABA content have also be observed between varieties of cultivated species. Largué-Saavedra and Wain (1974, 1976) found that ABA content was higher in both wilted and non-wilted leaves of drought-resistant cultivars of maize and sorghum as compared to less resistant ones. A correlation between reduced ABA content and short dormancy and low sprouting resistance was found for two barley varieties (Goldbach and Michael 1976). Compared to the drastic effects observed in deficient mutants, the minor and more specific physiological differences between varieties may reflect rather a specific genetic regulation of ABA metabolism than an effect on biosynthesis as is probably the case in mutants. Likewise it is not clear whether relatively small differences in ABA content between different genotypes, as found by Lee and Looney (1977) and Yadava and Lockard (1977) between compact and normal apple types, are a primary effect of mutations in genes directly involved in the regulation of ABA biosynthesis.

The fact that the absence of seed dormancy of ABA-deficient mutants segregates as a single recessive gene in the progeny of heterozygous plants (Table 2), shows that dormancy in ripe seeds related to ABA metabolism is mainly determined by the genotype of the embryo and not by the genotype of the mother plant including the seed coat. The genetic control and the role of ABA in relation to seed dormancy will be described in detail in a later paper.

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CHAPTER 3

INDUCTION OF DORMANCY DURING SEED DEVELOPMENT BY ENDOGENOUS ABSCISIC ACID: STUDIES ON ABSCISIC ACID DEFICIENT GENOTYPES OF *ARABIDOPSIS THALIANA* (L.) HEYNH.

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SUMMARY

Mutant lines of *Arabidopsis thaliana* (L.) Heynh. which are characterized by symptoms of withering and the absence of seed dormancy showed much lower levels of endogenous ABA in developing seeds and fruits (siliquae) than the wild-type.

Reciprocal crosses of wild-type and ABA-deficient mutants showed a dual origin of ABA in developing seeds. The genotype of the mother plant regulated a sharp rise in ABA content half-way seed development (maternal ABA). The genotype of the embryo and endosperm was responsible for a second ABA fraction (embryonic ABA), which reached much lower levels, but persisted for some time after the maximum in maternal ABA. The onset of dormancy showed a good correlation with the presence of the embryonic ABA fraction and not with the maternal ABA. Dormancy developed in absence and presence of maternal ABA in the seeds. In this respect maternal ABA resembled exogenously applied ABA which did not induce dormancy in ABA-deficient seeds. However, maternal and applied ABA both stimulated the formation of a mucilage layer around the testa, which could be observed during imbibition of the mature seeds.

ABA-deficient seeds germinated in the mature state in the siliquae on the plant, but only when the atmosphere surrounding the plant was kept at high relative humidity. In younger stages germination in siliquae occurred after isolation from the plants and incubation on wet filter paper. Therefore, it seems that limited access to water is the primary trigger for developmental arrest in these seeds.

KEYWORDS:

Abscisic acid - Abscisic acid deficient mutants - *Arabidopsis thaliana* - Dormancy - Seed development.

ABBREVIATIONS

ABA = abscisic acid.

INTRODUCTION

The developmental program which proceeds in plants from zygote formation via seed development and germination to seedling growth is in nearly all species interrupted by a period of developmental arrest, characterized by dehydration and termination of growth and resulting in either quiescent or dormant seeds. Quiescent seeds only require rehydration for germination, whereas in dormant seeds the range of suitable conditions for germination is further restricted.

It has been suggested that endogenous ABA plays a major role in both the arrest of development and the induction of dormancy (Walbot 1978; Walton 1980; Wareing 1978). Indeed, it has been shown in embryo cultures of several species that addition of ABA is a prerequisite for normal embryogeny. In these cultures ABA inhibited precocious germination but enhanced the synthesis of certain mRNA and protein fractions, the accumulation of reserve food and the activity of certain enzymes (Crouch and Sussex 1981; Choinski and Trelease 1978; Dure et al. 1981).

Evidence for a comparable dual role of endogenous ABA during seed development *in vivo* is mainly restricted to the occurrence of the maximum rise in ABA content and the most active phase of fresh and dry weight increase and cell enlargement (Hsu 1979; Quebedeaux et al. 1976; King 1976; Goldbach and Michael 1976) and/or the inability for precocious germination of isolated embryos (Quebedeaux et al. 1976; King et al. 1979; Van Onckelen et al. 1980). Application of ABA to developing grains of wheat promoted cessation of grain growth and drying of the seeds (King 1976). In barley a similar treatment stimulated both the transport of ^{14}C-glucose from leaves to grains and the dry weight of mature grains (Tietz et al. 1981). A role of ABA in developmental arrest is also suggested by its last position in the sequence of rising and falling hormone levels during seed development (Eeuwens and Schwabe 1975).

Convincing evidence for a role of endogenous ABA in the induction of seed dormancy is still missing.

The use of mutants with an ABA deficiency adds an important tool to the investigation of the possible role of ABA in seed physiology. A first indication of their value is found in a study with seeds of viviparous corn mutants which either contained less ABA or were less sensitive to applied ABA than the wild-type seeds (McDaniel et al. 1977; Smith et al. 1978; Robichaud et al. 1980).

In the present experiments the role of ABA in the onset of dormancy is studied using ABA-deficient mutants of *Arabidopsis thaliana*. The induction and isolation of these monogenic mutant lines and their genetic and physiological characterization has been described in a previous report (Koornneef et al. 1982). The lines are characterized by symptoms of withering, increased transpiration and a lowered ABA content in ripe seeds and leaves. Therefore, the phenotype was called ABA-type and the recessive mutant allele *aba*. Dormancy of seeds of the ABA-type is strongly reduced, as judged from a reduced requirement for light and cold treatment. The sensitivity of the ABA-type seeds to applied ABA is only slightly less than that of the wild-type. Reciprocal crosses between wild-type and ABA-type are used to study the origin of ABA in the seeds and the location of the dormancy mechanisms.

MATERIAL AND METHODS

Seed material. The origin of the wild-type and ABA-type seed stocks used in the present experiments and the conditions of culture during seed formation were described in our previous report (Koornneef et al. 1982).

Seed development and performance of crosses. Flowers were tagged at the moment of anthesis or artificial pollination. For the latter treatment a dissection microscope was used. Reciprocal crosses were performed with male sterile (*ms*) mother plants. The *ms*-allele with perfect expression had been crossed into the present lines for the purpose of large scale crossing. It implies that the present material had the cytoplasma of the female wild-type *ms*-donor. The lines were maintained by crossing *ms ms* x *Ms ms*, instead of by selfing.

Germination tests. To collect unripe seeds the siliquae were cut open with scalpels using a dissection microscope. The immature seeds were carefully collected by means of needles and directly sown in plastic petri dishes(Ø 8.5 cm) on filter paper (Ederol no. 261) saturated with 2 ml of sterile distilled water. To avoid rapid evaporation, each dish was wrapped in a small polythene bag. Conditions during the tests were a temperature of 25 ^{o}C and continuous light (Philips TL 57) at an intensity of 8 W.m^{-2}. Germination was scored 7 and 14 days after the start of incubation. After 7 days one extra ml of water was added to compensate evaporation.

Germination tests were performed in duplo. Mature seeds were stored after harvest in dry conditions at room temperature. Germination tests were performed as described above.

ABA determinations. Weighed badges of siliquae containing seeds were stored in liquid nitrogen within half an hour after harvest until the start of extraction procedures. High relative humidity prevented dehydration in the period before storage.

Extraction procedures and quantitative determination by gas chromatography using an electron capture detector were performed according to techniques

described by Knegt et al. (1981) with a few additions as in Koornneef et al. (1982). Determinations occurred in duplo.

Measurement of mucilage layer. In order to colour the mucilage layer seeds were incubated in a solution of Ruthenium Red in water (Witzum et al. 1969). The thickness of the mucilage layer was measured under the microscope.

RESULTS

ABA levels and precocious germination in wild- and ABA-type. In a first experiment ABA content, fruit growth and the capacity for precocious germination of isolated seeds were compared during seed development after self pollination of the genotypes *Aba/Aba* (wild type) and aba^3/aba^3 (ABA-type, line G4) (Fig.1). The very tiny proportions of seeds and siliquae of *A. thaliana* strongly restricted the workable experimental approaches. A dry mature seed weighs about 20 µg and measures 0.4 x 0.2 mm. Siliquae contain a mean number of 60 seeds and have an average weight of 6 mg. One ABA determination asked for approximately 800 siliquae. It turned out to be infeasable to separate the seeds of the siliquae for all ABA determinations at the different phases of development. Therefore, ABA was generally determined in the siliquae containing the seeds, except for one experiment where ABA was determined separately in seeds and siliquae at an age of 10 days after pollination. Seeds contained 94% of total ABA in siliquae plus seeds.

In seeds of wild-type the ABA level reached a maximum half-way development (Fig. 1A). During its decline the curve showed a shoulder between 14 and 16 days after pollination. The siliquae and seeds of ABA-type contained a much lower level of ABA throughout development. It should be realized that the mutation of the aba^3 allele represents a rather mild inactivation of the

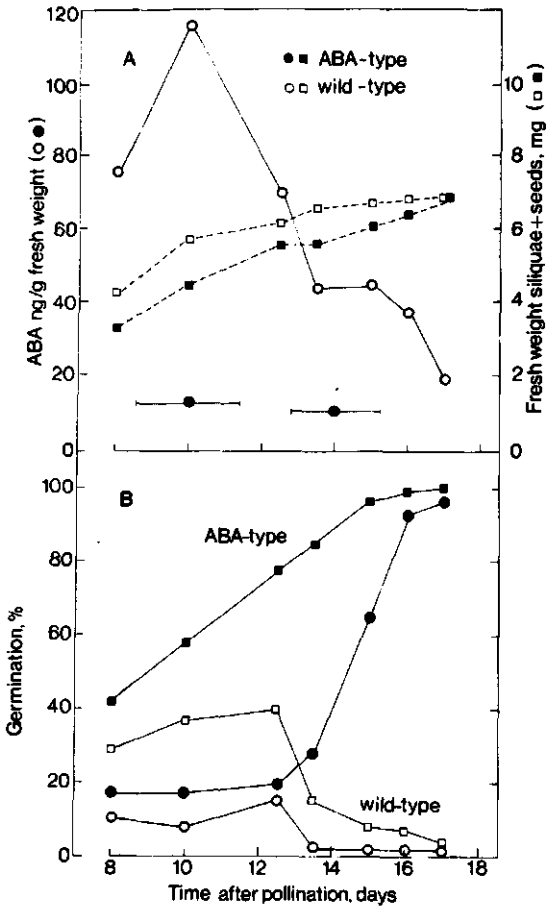

Fig. 1. Change with time from pollination in (A) ABA content and fresh weight of siliquae containing seeds, and in (B) germination of isolated seeds. Both for wild-type (open symbols) and ABA-type (line G4; *aba*^{3}) (closed symbols). Horizontal lines in A indicate sample width for ABA extraction in ABA-type. Germination in B was scored after 7 days (circles) and 14 days (squares).

aba gen. The ABA levels in mature seeds of two other ABA-type mutant lines (A26 and A73) were found to be reduced below the level of detection (Table 1). The G4 line was chosen for the present experiments because the withering of the siliquae was less severe than in the other two lines and therefore enabled a workable number of siliquae and seeds to develop into ripeness (Koornneef et al. 1982). Nevertheless, the development of siliquae in the G4 line was still reduced to such an extent that ABA determinations could not be performed on the same narrow age ranges as with the wild-type. Therefore, siliquae and seeds differing in age for several days were combined for one determination,

and consequently the dates in Fig. 1A for ABA content in ABA-type represent the mean values of the indicated periods of development.

The difference in genotype also strongly influenced the capacity for precocious germination of the developing seeds (Fig. 1B). Seeds of ABA-type gradually developed full germination capacity, whereas in the wild-type seeds dormancy developed to full extent after a temporary period of germinability in a fraction of the seeds. In most young seeds of both genotypes precocious germination took more than 1 week. Germination rate increased to the end of maturation in the ABA-type seeds, however.

These results indicate that the induction of dormancy during seed development in *A. thaliana* is correlated with the presence of ABA in the seeds. It is noteworthy that the pattern of fruit growth was hardly influenced by the mutation in the *aba* gene (Fig. 1A). Also the weight of mature seeds of the different genotypes did not differ strongly (Table 1). The mutation clearly influenced, however, the thickness of the mucilage layer upon imbibition of mature seeds in water.

Table 1. Some characteristics of mature seeds of different lines of ABA-type compared with the wild-type

	Aba/Aba (wild-type)	aba^3/aba^3 (G4)	aba^1/aba^1 (A26)	aba^4/aba^4 (A73)
Seed weight, mg/100 seed	1.9	1.8	1.7	2.1
Thickness mucilage layer, μm	67±0.1	52±0.1	27±0.1	25±0.2
ABA content, pg/seed	0.54	0.14	<0.02	<0.02
Germination in light, %	0	100	100	100

Site of induction of dormancy and origin of ABA, germination studies. The previous experiment raised questions about the regulatory site of the dormancy induction and about the origin of endogenous ABA in seeds. Genetic experimentation provides excellent tools to answer these questions. In the F_1 seeds of ABA-type x wild-type crosses the genotype of the maternal and embryonic tissues will be different. If these crosses are made reciprocally the development of seeds with the *Aba/aba* genotype in embryo can be studied on both a wild-type and an ABA-type mother plant. We compared the development of such seeds with F_1 seeds of *aba/aba* x *aba/aba* and *Aba/aba* x *aba/aba* crosses. In the latter case the embryos will have genotypes *Aba/aba* and *aba/aba* in equal proportions.

Precocious germination was tested during development of these different F_1 seeds (Fig. 2). Dormancy again was absent when both parents were ABA-type, but it fully developed in the heterozygous F_1 seeds, irrespective of the genotype of the mother plant. The capacity for precocious germination in F_1 seeds of the *Aba/aba* x *aba/aba* crosses followed a course which happened to match perfectly with the curve constructed from the addition of halved values of the curves for *Aba/Aba* x *aba/aba* crosses. Therefore, it is concluded that the development of dormancy with regard to the *aba* gene is regulated by the genotype of the embryo and is not a maternal effect.

The genotype of the mother plant was not completely without an effect in the present experiments, however. Firstly, it is shown in Fig. 2 that the timing of dormancy induction was slightly influenced. On an ABA-type mother plant the induction started two days later than on a wild-type plant. The retardation of induction was part of an overall increase in length of the period of seed development in ABA-type seeds with 2 to 4 days (data not shown). The retardation is most obviously related to the weak condition of ABA-deficient mother plants (Koornneef et al. 1982). Secondly, germination tests with ma-

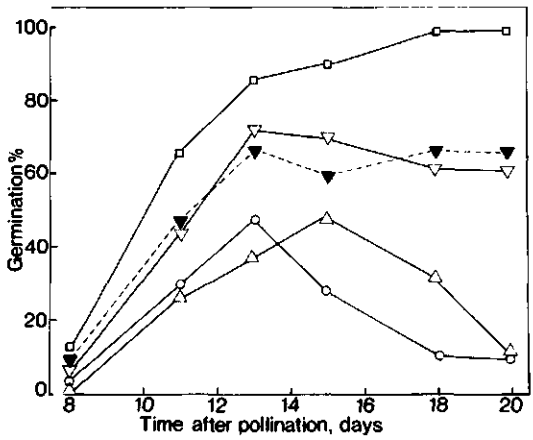

Fig. 2. Germination of isolated developing F_1 seeds from different crosses. Germination was counted after 14 days.

	♀	♂	F_1
□	*aba/aba* x	*aba/aba*	→ *aba/aba*
▽	*Aba/aba* x	*aba/aba*	→ *Aba/aba* ($\frac{1}{2}$) + *aba/aba* ($\frac{1}{2}$)
o	*Aba/Aba* x	*aba/aba*	→ *Aba/aba*
△	*aba/aba* x	*Aba/Aba*	→ *Aba/aba*

The curve indicated with ▼ is the calculated average of curves □ and o.

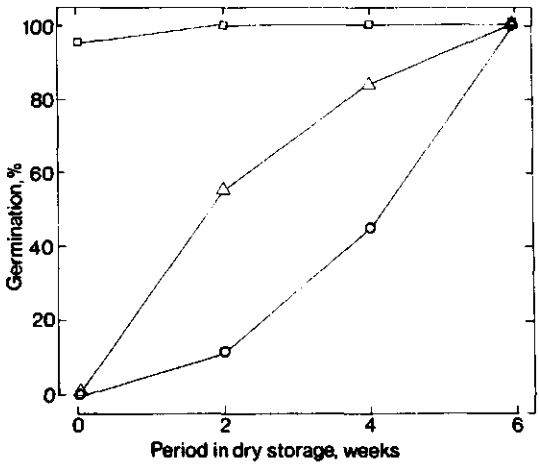

Fig. 3. Change with time of dry storage in germination of mature seeds generated from the crosses *aba/aba* x *aba/aba* (□), *aba/aba* x *Aba/aba* (△) and *Aba/Aba* x *aba/aba* (o). Germination was counted after 7 days.

ture seeds showed that heterozygous F_1 seeds generated on an ABA-type plant required a shorter after-ripening period at dry storage to increase germination in the light than did seeds from a wild-type plant (Fig. 3).

ABA determination. It was attempted to affirm these conclusions with ABA determinations in developing siliquae containing the F_1 seeds of the different crosses. Sampling had to be restricted for practical reasons to two stages of development. Extractions were performed 10 and 16 days after pollination, representing the peak and the shoulder in the ABA curve (Fig. 1A), respectively. The data on precocious germination obtained in this experiment showed a reasonable agreement with comparable tests in the previous experiments (Table 2; Figs. 1B, 2).

The ABA content in siliquae and F_1 seeds of the *aba/aba* x*aba/aba* crosses was very low at both sampling dates, as could be expected from the data in Fig. 1A. The results obtained with the progeny from the *Aba/Aba* x *aba/aba* crosses agreed with data obtained with self pollinated wild-type material in the first experiment: high ABA levels at 10 days after pollination and a lower level at 16 days. The difference in the value at 10 days between the two experiments can not be explained yet. We presume that season dependent differences in cultivation conditions are involved. If the dominant *Aba* allele was only present in the embryo (*aba/aba* x *Aba/Aba*) the ABA level at both sampling dates stayed at a level of about 40 ng/g. This value was characteristic for the shoulder in the ABA curve in the first experiment (Fig. 1A). This level was also present in F_1 seeds from the *Aba/aba* x *aba/aba* crosses at 16 days after pollination. After 10 days the ABA level in these seeds from heterozygous mothers was roughly half of that in seeds descending from homozygous mothers. In judging this result it should be realized that the steepness of the ABA

Table 2. ABA content and germination of immature (10 and 16 days) and mature (26 days) F_1 seeds of different crosses and some morphological characteristics of mature seeds

	Days after pollination	♀ : o : F_1 :	*Aba/Aba* : *aba/aba* : *Aba/aba*	*aba/aba* : *Aba/Aba* : *Aba/aba*	*Aba/aba* : *aba/aba* : *Aba/aba* (½) *aba/aba* (½)	*aba/aba* : *aba/aba* : *aba/aba*
ABA content, ng/g[a]	10		598	35	259	8
	16		59	36	38	1
Germination, %	10		39	44	42	64
	16		29	49	55	99
	26		0	0	55	95
Seed weight, mg/100 seed	26		2.8	3.0	2.6	2.6
Thickness mucilage layer, m	26		77	40	-	-

[a]Per g fresh weight of siliquae containing seeds

curve may cause big differences in ABA content at small differences in seeds ages or rate of development.

It is concluded that endogenous ABA in developing fruits and seeds of *A.thaliana* has two different origins. The first fraction is regulated by the genome of the mother plant (maternal ABA). It is responsible for the peak in ABA content half-way seed development. The second fraction is regulated by the genome of the embryo (embryonic ABA). This fraction is present during maturation, but also at earlier stages of development. The exact course of embryonic ABA has to be determined yet.

Dormancy was only induced in seeds of *A.thaliana* if the genome of embryo contained the dominant *Aba* allele and, thus, the embryonic ABA fraction. Maternal ABA was not related to dormancy induction. Dormancy developed in spite of its absence (*aba/aba* x *Aba/Aba*) (Table 2). If, on the contrary, maternal ABA was present the pattern of dormancy induction still perfectly correlated with the genotypes of embryo and endosperm (Fig. 2, Table 2).

The weight of the different F_1 seeds did not differ significantly, whereas a maternal effect was observed on the thickness of the mucilage layer (Table 2).

Effects of ABA sprays. Dormancy was not induced if the ABA level in the seeds was raised artificially. A spray with 100 µm ABA solution 10 days after pollination raised the ABA level in seeds and siliquae of ABA-type plants from the very low level shown in Fig. 1 and Table 2, to a value of 220 ng/g fresh weight (data not shown). The amount was determined 3 days after the spray. Sprays with 100 µm ABA solutions did not induce dormancy in ABA-type seeds, however, even if they were repeated at regular intervals (Fig. 4). On the contrary, the seeds developed the capacity for precocious germination at an earlier moment.

The sprays stimulated the development of the mucilage layer in both genotypes (Table 3).

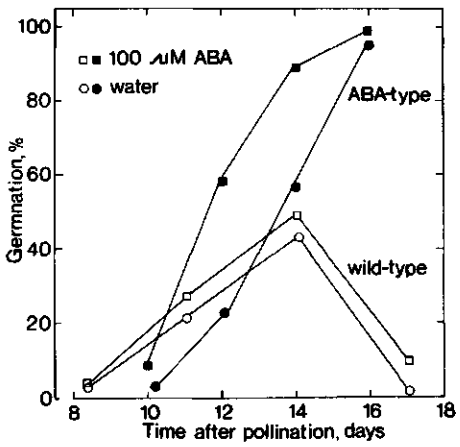

Fig. 4. Change with time after pollination in germination of isolated seeds from wild-type (open symbols)or ABA-type (closed symbols). During seed development plants were sprayed at intervals of 4 days with distilled water (circles) or 100 µm ABA (squares). Germination was counted after 14 days.

Table 3. Thickness of mucilage layer (in µm) of mature seeds of wild-type and ABA-type. During seed development plants were sprayed with intervals of 4 days with distilled water or 100 µm ABA.

Spray	Wild-type	ABA-type
100 µm ABA	85±0.2	97±0.1
water	77±0.1	60±0.2

Precocious germination in vivo. In contrast to the precocious germination obtained with isolated immature seeds (Fig. 1, 2, 4) we never observed any germinated seeds in the fruits of any of the genotypes during cultivation of the plants in the greenhouse. Seeds of the ABA-type only germinated in the fruits when during seed development the plants were enclosed in plastic bags to maintain a high relative humidity in the atmosphere surrounding the plants (data

not shown). Germination did occur at first at about 28 days after pollination, when the siliquae were very mature and had coloured yellow. If we consider that in the precocious germination tests a seed which was isolated after 14 days took more than one week to germinate (Fig. 1B), it seems possible that the late moment of visible germination in the siliquae is caused by the slow rate of the germination process.

Wild-type seeds did not germinate in the siliquae on the plant under humid conditions. Germination was still prevented when wild-type siliquae were taken from the plants at different stages of development and were incubated on wet filter paper in petri dishes (Fig. 5). After isolation of the seeds from the siliquae the same germination pattern occurred as was shown before (Figs.1,2,4). On the contrary, ABA-type seeds germinated better when they were left in the siliquae (Fig. 5).

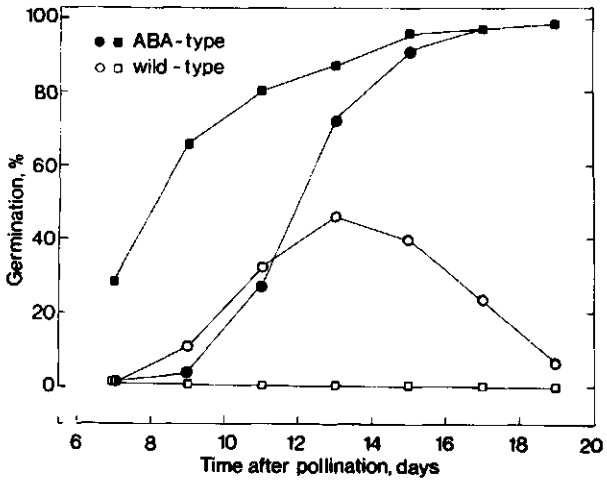

Fig. 5. Change with time after pollination in germination of seeds in isolated siliquae (squares) or of isolated seeds (circles) of wild-type (open symbols) or ABA-type (closed symbols). Germination was counted after 14 days.

DISCUSSION

Dormancy induction by embryonic ABA. Increased transpiration and symptoms of withering and within have been described so far as the main characteristics of ABA-deficient mutants in tomato (Imber and Tal 1970), potato (Quarrie 1982) and *A.thaliana* (Koornneef et al. 1982). The present results clearly show that in *A.thaliana* absence of dormancy induction in developing seeds is another of the pleiotropic effects of the same recessive mutation. A similar situation seems to exist in potato (Quarrie 1982) and tomato (Koornneef unpublished). The probability that ABA and dormancy induction are not causally related is very low since genetic analysis has shown beyond doubt that a single gene is involved in the mutation (Koornneef et al. 1982). The failure of ABA sprays to induce dormancy in ABA-type seeds, in spite of its penetration, seems at first sight an argument against such a causal relationship (Fig. 4). The argument weakens considerably, however, when it is realized that applied ABA resembled maternal ABA in this respect. This ABA fraction is also present in the seeds but fails to induce dormancy (Fig. 1). Dormancy in *A.thaliana* seeds is only induced if the genome of the embryo contains a dominant *Aba* allele (Fig. 2) and therefore the embryonic ABA fraction is present (Table 2). Embryonic inheritance of dormancy was also described for lettuce and tobacco (Globerson et al. 1974; Honing 1930).

The localization of the different ABA fractions could not be studied in detail due to the tiny proportions of the seeds. Maternal ABA might be restricted to the testa as the only maternal tissue in the seeds. It can not be excluded, however, that the maternal ABA fraction also penetrates into the endosperm and embryo, but then it should be located in another cell compartment than the embryonic ABA since it has a different physiological effect.

Thus, it is concluded that dormancy induction is located in the embryo.

In order to induce dormancy ABA has to be synthesized close to its site of action or has to be localized in a specific subcellular way, which can not be reached after transport from the maternal tissues.

The mechanism of action of embryonal ABA is unknown. The several specific biochemical changes which are enhanced by ABA in developing seeds (Choinski and Trelease 1978; Crouch and Sussex 1981; Dure et al. 1981) indicate that the induction of dormancy does not need to be restricted to the inhibition of growth.

The present experiments indicate that after the onset of dormancy endogenous ABA was not required for its maintenance. During maturation ABA levels were reduced to very low levels (Fig. 1A) which seems to be insufficient to inhibit germination. During incubation in a ^{14}C labelled ABA solution at the minimal inhibitory concentration of 10 µm, about 7 pg ABA entered per seed (Brinkhorst-van der Swan unpublished results). Based on the presence of approximately 95% of all ABA in the seeds it can be calculated that such an ABA content was only present in the seeds at the time of the peak value in the ABA curve (Fig. 1A).

Functions of maternal ABA. The present results show that the role of the maternal genotype and of maternal ABA on dormancy induction in *A.thaliana* is restricted to small, modifying effects on the general pattern determined by the genotype of the embryo, *i.e.* by embryonic ABA. In general, maternal ABA, and in a similar way applied ABA, tended to shorten the period of seed development and dormancy induction (Figs. 2, 5). This is most obvious due to the absence of withering phenomena which inhibit plant and seed development.

In other species the maternal genotype is sometimes engaged in dormancy induction. In tobacco crosses the female parent had a slightly greater in-

fluence on dormancy induction than the pollen parent (Honing 1930; Kasperbauer 1968). In tomato the reduction of germination due to the presence of the lateral suppressor gen (*ls*) was also shown to be an effect of the maternal genotype rather than of the embryo (Taylor 1979).

In *A.thaliana* maternal and applied ABA both stimulated the development of a mucilage layer around the seeds (Tables 1, 2, 3). Mucilage layers have been described as barriers to germination in certain species (Witzum et al. 1969). In *A.thaliana* the embryonic origin of dormancy makes such a function unlikely.

Plasma inheritance was not involved in the maternal effect. To construct male sterility segregating ABA-type lines, a *ms*-donor line which was otherwise wild-type was taken as female parent. So all subsequent progeny contained wild-type cytoplasma.

Inhibition of precocious germination in the siliquae. Addition of ABA to cultures of isolated embryos of several species favoured the conclusion that ABA is a prerequisite for the arrest of development during maturation of seeds. It has been hypothesized that during embryo development, endogenous ABA on the one side, helps to stimulate the uptake of solutes and the development of a large negative water potential, which favours rapid hydration, but on the other side, prevents such hydration by the inhibition of cell expansion (Walbot 1978).

In the ABA type dehydration and termination of growth also occurred, however, in the absence of ABA. Restricted access to water seems to be the most important limiting factor for the prevention of precocious germination *in vivo*, thus triggering developmental arrest. Such water deficiency might develop naturally when the open xylem connection between seeds and mother plant becomes obstructed.

The germination experiments with seeds inside the isolated siliquae had a confusing result (Fig. 5). Whereas the siliquae inhibited germination in wild-type, they stimulated in ABA-type. It is assumed that isolation of seeds from the siliquae has a negative effect on the germination capacity of the seeds, due to wounding of tissues, increased leakage of solutes or other reasons. Therefore, ABA-type seeds germinate better in the intact seed. In wild-type this advantage turns into its opposite, because in the intact fruits endogenous ABA may leack to a much smaller extent from the seeds. Moreover the siliquae slightly add to the total ABA content.

Origin of the ABA fractions. A dual origin of ABA in seeds, regulated either by the embryonic or by the maternal genotype, has to our knowledge not been demonstrated clearly in seeds of other species. It might be implicated in the results obtained in developing bean seeds, where two distinct peaks in the ABA content occur during development (Hsu 1979). The first one was found in both testa and embryo, whereas the second one appeared in the embryo only. If such a dual origin of ABA also exists in other species, it will certainly have escaped attention because the peaks in both fractions most often will coincide. Moreover, the small amount of embryonal ABA might have been hidden under the large amount of maternal ABA.

It is to be expected that the ABA fraction regulated by the genotype of the embryo is also formed in those tissues. Endosperm and embryo of wheat incorporated ^{14}C-mevalonic acid into ABA (Milborrow and Robinson 1973). Wheat grains from detached ears grown in culture accumulated ABA to the same extent as in the intact plant (King 1979). Therefore, maternal ABA in *A.thaliana* might also originate from the siliquae and testa itself. Transport of ABA from other parts of the mother plant also has to be considered, however. Particularly after a

condition of water stress ABA transport to the seeds has been demonstrated from leaves (Goldbach and Goldbach 1977; Dewdney and McWha 1979).

If maternal ABA is transported to the fruits and seeds it is obvious that the trigger for this transport must be located in the receiving organs. At one moment an *Arabidopsis* plant contains siliquae of many different ages. Nevertheless, at one time ABA rises only in a few of these developing siliquae. The mechanism of such a timing device is unknown. Other hormonal factors might be involved in the regulation of the sink activity of the seeds and fruits determining the point of time that the organs become active sinks for phloem-transported ABA.

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CHAPTER 4

Genetic Control of Light-inhibited Hypocotyl Elongation in *Arabidopsis thaliana* (L.) HEYNH.

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With 3 figures

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Summary

Reduced sensitivity to the inhibitory action of white light on hypocotyl elongation was observed in 41 mutants induced in *Arabidopsis thaliana* at 5 different loci. Compared to wild type, these mutants show a locus-specific altered inhibition spectrum when grown in light of restricted spectral regions. Spectrophotometrically detectable phytochrome in dark-grown tissue of those mutants (loci *hy-1* and *hy-2*) in which the inhibitory effect of far red light is almost or completely absent, is either greatly reduced or below the level of detectability. The spectra of the different mutants and their recombinants provide evidence for the presence of more than one photoreceptor pigment for the High Irradiance Reaction and demonstrate the genetic control of light-induced inhibition of hypocotyl growth.

Key words: Activity spectra, Arabidopsis, High Irradiance Reaction, hypocotyl elongation, physiological mutants, phytochrome.

Introduction

Light regulates many morphogenetic processes in plants. Among the pigments mediating these reactions, phytochrome is the most intensively studied. Its main absorption bands are in the red and far red. The nature of photoreceptor pigments responsible for specific blue and violet light effects is still a matter of dispute. In addition to a possible involvement of several distinct pigments, another complicating factor in the study of photomorphogenetic processes is the increasing amount of evidence for multiple phytochrome reactions (e. g. JOSE and VINCE-PRUE, 1977 b; SMALL et al., 1979; SPRUIT et al., 1979).

Abbreviations: UV: near ultraviolet light; B: blue light; G: green light; R: red light; FR: far red light; HIR: High Irradiance Reaction; P_r: phytochrome, red-absorbing form; P_{fr}: phytochrome, far red-absorbing form.

Several effects of light in plants, such as floral induction, seed germination, phototropism, chlorophyll synthesis, and regulation of growth processes, are under genetic control. Many examples exist of genetic variation within species for daylength requirement in floral induction (reviews: SKRIPCHINSKY, 1971 and MURFET, 1977). In such cases, the genes involved may control either the synthesis of morphogenetic pigments or subsequent reactions in which the electronically excited or photochemically transformed pigment molecules normally enter. If a given photomorphogenetic process is controlled by more than one pigment, or if a photomorphogenetically active pigment has multiple points of attack within the cell or plant, we may expect that mutation of the genes affecting synthesis of these pigments may alter the morphogenetic behaviour of the mutants towards light. Obviously, mutants of this type can be very useful for photophysiological research. This approach, that has been so fruitful in studying metabolic pathways, has been neglected in photobiology so far. The search for mutants of *Phycomyces* with a deviating phototropic sensitivity (a blue light reaction) is a recent example of this approach (LIPSON et al., 1980).

The inhibition by light of hypocotyl elongation in dark grown seedlings is the classical example of the High Irradiance Reaction (HIR) (review: MANCINELLI and RABINO, 1978). Action spectra for this reaction have been determined (e. g. EVANS et al., 1965; HARTMANN, 1967) a. o. for lettuce and some varieties of *Petunia*. It is generally held that phytochrome is the pigment responsible for the HIR (SCHÄFER, 1976). The question whether the usually very considerable activity of the blue-violet region of the spectrum is also due to absorption by this pigment still forms a subject of controversy. There are also reports suggesting that in some plants red-absorbing pigments other than phytochrome may be involved (JOSE and VINCE-PRUE, 1977 b; VANDERHOEF et al., 1979). An induced mutant of *Arabidopsis* with a hypocotyl (in white light), more than twice as long as that of the wild type (= normal type) has been described by REDEI and HIRONO (1964) who proposed the gene symbol *hy*. This gene was mapped on chromosome 2 (REDEI, 1965) between the marker loci *py* and *er*. STUBBE (1966) described a mutant of *Antirrhinum majus, elongata,* which is very similar to the *hy* mutants of *Arabidopsis*. No attempts have been made so far to characterize these mutants physiologically. KRANZ (1977 b) and SCHEIDEMANN (1978) reported on the hypocotyl lengths in red, far red, and blue light of mutants of *Arabidopsis* which did not show elongated hypocotyls under white light.

The determination of complete action spectra from fluence-response curves for a large number of wavelengths is a laborious and time-consuming enterprise. As a first approach and as a method for screening our mutants for possible spectroscopically interesting types, we have determined activity spectra at constant quantum irradiances for the inhibition of hypocotyl elongation in a number of restricted wavelength regions. Phytochrome was determined spectrophotometrically in tissue of the mutants, grown in darkness.

Materials and Methods

Plant material

Arabidopsis thaliana (L.) HEYNH. (2n = 10) is a small, fast-growing self-fertilizing crucifer. Seed stocks used in the experiments derive from the pure line «Landsberg-*erecta*» (REDEI, 1962).

Culture medium, culture conditions and light sources

Seeds were sown in 9 cm petri dishes (25 or 30 per dish), equally spaced on perlite statured with a standard mineral solution. The composition of this solution was as described by OOSTINDIER-BRAAKSMA and FEENSTRA (1973). To break dormancy the petri dishes were first kept at 2–4 °C for 5 days after sowing. They were subsequently placed into a climate room at 24 °C under continuous illumination by fluorescent light (Philips TL 57) at roughly 8 W m^{-2}.

After 8 days the seedlings were transplanted into soil (7 cm pots) and cultivated in an air-conditioned greenhouse, where additional continuous light was given by frames of TL 57 tubes from October until April. This implies long-day conditions throughout the year.

For the growth tests under monochromatic light the seeds were also sown on filter paper moistened with distilled water in 4 cm high glass dishes. Under these conditions light inhibition was more pronounced than on the perlite medium, especially in continuous TL 57 light. However, the correlation between the results on perlite and on filter paper is high for each light colour.

The light sources were similar to those described earlier (JOUSTRA, 1970) with the addition of a separate cabinet for the near ultraviolet. Spectral energy distributions were measured spectroradiometrically, with a calibrated tungsten ribbon lamp as a reference. Equivalent spectrometer slit widths were 3 nm, throughout. The relative energy distributions are shown in fig. 1. Some additional data are:

Near ultraviolet (UV): fluorescent tubes type Philips TL 40/08 with a clear glass plate as a filter. Spectral half width (HW) 38 nm. Contributions from individual mercury lines to the total emitted energy: 365 nm 0.8 %; 404.7 + 407.7 nm 2.2 %; 435.8 nm 0.35 %.

Blue (B): Philips TL40/18 with 3 mm Plexiglas blue 0248 (Röhm u. Haas).

HW 75 nm. Mercury lines: 365 nm 0.07 %; 404.7 + 407.7 nm 3.8 %; 435.8 nm 17.7 %; 546.1 nm 0.34 %.

Green (G): Philips TL 40/17 with a sheet of yellow glass. HW 42 nm. Mercury lines 435.8 nm 0.09 %; 546.1 nm 4.9 %; 577 + 579 nm 1.02 %.

Red (R): Philips TL 40/103339 with 3 mm Plexiglas red 501. HW (envelope) ± 18 nm, main peak 13 nm.

Far red (FR). Bank of 60 watt 240 volt incandescent lamps operated at 220 volts with 10 cm running tap water and 3 mm Plexiglas red 501 plus 3 mm blue 627. The energy in this light regime was calculated by subtracting from the total energy the fraction transmitted by 3 mm RG 780 (Schott u. Gen.). The resulting effective energy distribution is the one shown in fig. 1 as a broken line. Since the maxima of the action spectra in the far red are not known but are probably at a wavelength, lower than the 750 nm maximum, the effective quantum fluency in the far red may have been 10–35 % lower than follows from the measurement. Light intensities at the level of the plants were measured with an Optometer 80X (United Detector Technology, Inc.). They were adjusted to give equal quantum irradiances of 3.06×10^{-6} E m^{-2} s^{-1} ± 5 % in each of the cabinets. The effective wavelengths assumed were: UV 359 nm, B 442 nm, G 527 nm, R 658 nm, FR 730 nm.

Measurements of hypocotyl elongation

After the standard cold treatment the seeds were given 15 minutes of red light (658 nm, 0.64 W m^{-2}) to induce germination. The seeds were kept in the dark for 24 hours and sub-

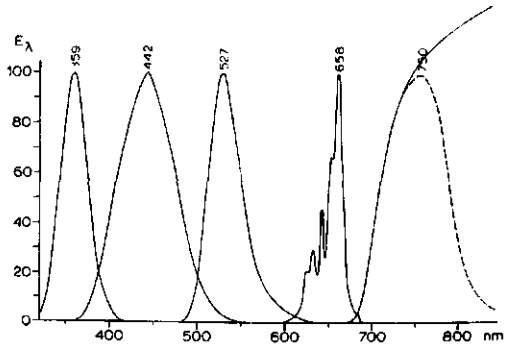

Fig. 1: Relative spectral energy distributions per nanometer of the light sources. Mercury lines not shown.

sequently placed in the cabinets with monochromatic light. The temperature was ± 22 °C. In wild type *Arabidopsis* as well as in all mutants tested, the period of exponential growth occurred between the 2nd and 4th day after the end of cold treatment. Although the final hypocotyl lengths were measured on the 7th day after this treatment, almost all growth occurred, therefore, in a 2-day period around the 3rd day after the end of the cold treatment. We have no indication that the action of the various wavelengths is different during the remainder of the 7-day period.

In the experiments described, the hypocotyl lengths of 10 plants per treatment per genotype were measured by putting the seedlings between glass plates, which were placed in the negative stage of a photographic enlarger. Prints were made with an enlargement of 5–6 times linear. From these prints the length was measured and the true length was calculated.

Phytochrome measurements

Phytochrome was determined spectrophotometrically as described earlier (SPRUIT, 1970). Since it would have been an extremely laborious task to collect a sufficient amount of material by dissecting apart various plant organs, the dark-grown plants were packed as such into the absorption cells (path lengths 2.5 mm). The bulk of the volume consisted of hypocotyl tissue. All measurements were made at 0 °C.

Isolation of Mutants

Arabidopsis seeds were either treated with ethylmethanesulfonate (EMS; 10 mM, 24 h, 24 °C) or irradiated with X-rays or fast-neutrons submersed in water (preimbibed) (for details of mutagenic treatment see KOORNNEEF and DELLAERT, 1980 and DELLAERT, 1980). The resulting plants (the M_1 generation) were individually harvested and the seeds were stored for at least 3 weeks at 30 °C. The M_2 lines grown from the individual seed lots were scored in the climate room for segregation of seedlings with elongated hypocotyls at day 8 after the end of cold treatment. Wild-type seedlings then have a hypocotyl length of 1.5–2.5 mm. Selected mutants were grown to maturity in the greenhouse and progeny tested (M_3 lines) to verify the phenotype and to test for recessivity. To obtain *hy* mutants in an otherwise undisturbed genetic background, line selection (within M_1 plant descents) was practized for a number of generations. In several cases the mutants were first backcrossed to the original parent line (wild type).

Genetic analysis

The different mutants were tested for allelism vs. non-allelism on the basis of non-complementation vs. complementation to normal hypocotyl type in their F_1's. In case of phenotypically less pronounced *hy* mutants and in case of incomplete dominance (mutants at the *hy-3* and *hy-4* locus; see later), an additional criterium was provided by the corresponding F_2's (non-segregation vs. segregation of normals). In addition several F_2's between mutants at different loci were grown for the selection of double-recessive recombinants. The location of the *hy* loci on the 5 *Arabidopsis* chromosomes (see Table 1) was by trisomic analysis (KOORNNEEF and VAN DER VEEN, 1978).

Table 1: The distribution of 41 independently induced *hy* mutants over the 5 loci involved, and the chromosomes on which these loci are located.

Mutagen	Locus:	*hy-1*	*hy-2*	*hy-3*	*hy-4*	*hy-5*	Total
	Chromosome:	2	3	2	?	5	
EMS		4	4	11	9	2	30
Fast neutrons		3	–	3	1	–	7
X-rays		–	–	3	1	–	4
		7	4	17	11	2	41

Results

Genetic characterization

All 41 independently induced mutants with an elongated hypocotyl isolated so far (under continuous TL 57 light and on perlite) were found to be alleles at one of five loci, numbered *hy-1* to *hy-5*. Their distribution over loci (EMS vs. ionizing radiations) gives no indication of mutagen specificity ($\chi^2_4 = 4.15$; $0.5 < P < 0.7$).

All mutants were monogenic recessives, almost completely recessive at loci *hy-1*, *hy-2* and *hy-5*, and incompletely recessive at loci *hy-3* and *hy-4* (Table 2).

Table 2: The hypocotyl length of parents and F_1 in a number of representative wild type × mutant crosses. The three genotypes were grown together in one petridish in three replications.

Mutant	Locus	Length in mm			Degree of dominance*) of wild type allele
		wild type	wild type × mutant	mutant	
21.84	*hy-1*	1.6 ± 0.1	1.9 ± 0.2	6.1 ± 0.2	0.9
TO76	*hy-2*	2.0 ± 0.1	2.3 ± 0.1	8.3 ± 0.3	0.9
Bo64	*hy-3*	1.7 ± 0.1	3.0 ± 0.1	7.8 ± 0.2	0.6
2.23N	*hy-4*	1.6 ± 0.1	3.8 ± 0.2	6.0 ± 0.2	0.0
Ci88	*hy-5*	1.5 ± 0.1	1.8 ± 0.1	5.6 ± 0.2	0.9

*) Degree of dominance calculated as: $\frac{F1\text{-}MP}{W\text{-}MP}$,

Where F_1 = the length of the F_1, MP = the mean length of wild type and mutant, W = the length of the wild type.

From linkage analysis, including the chromosome 2 markers *er* and *py*, the sequence *hy-3*, *er*, *hy-1*, *py* can be inferred with intervals of 15, 3, and 1 centimorgan, respectively. On the basis of comparable linkage relationships (REDEI, 1965) and a similar phenotypic description (REDEI and HIRONO, 1964) we tentatively equate our *hy-1* locus to REDEI's *hy* locus.

The 5 loci (with their sets of allelic mutants) can be roughly classified into two groups on the basis of hypocotyl length (3–5 times vs. < 3 times wild type) and the degree of pleiotropic effects on other characters (pronounced vs. less pronounced).

Pleiotropism is inferred from non-recombination between these effects with the elongated hypocotyl and from the fact that all mutants were locus specific in the phenotypic spectrum. For details see Table 3. It appeared that all less extreme mutants were allelic to the *hy-4* and *hy-5* locus, except one (mutant d412) that proved to be an allele at *hy-1*. The more extreme mutants were alleles at either *hy-1*, *hy-2* or *hy-3*.

Table 3: Pleiotropic effects of representative mutants at *hy* loci in one experiment in two replications. Per replication the measurements were based on 5 plants for hypocotyl length (white light, on perlite) and for 8–12 plants for other parameters.

Property	wild type	*hy-1*		*hy-2*	*hy-3*	*hy-4*	*hy-5*
		d412	others				
Number of mutants tested	1	1	4	4	5	6	2
hypocotyl length (in mm)[1])	2.1	4.1	6.9 – 8.2	7.2 – 9.8	6.4 – 8.3	4.1 – 6.4	5.2 – 6.1
colour (gr = green)	gr	gr	yellow-gr	light gr	light gr-gr	gr	gr
lenght of largest rosette leaf (in mm)[1, 2])	41	32	12 – 19	14 – 25	31 – 43	35 – 45	26 – 37
final plant length (in cm)[1])	27	25	23 – 26	26 – 28	32 – 40	26 – 33	25 – 28
apical dominance[1, 3])	3.5	2.6	1.5 – 2.0	0.3 – 2.0	1.5 – 2.0	2.7 – 3.0	3.4 – 3.7

[1]) Values indicate the range of the means of individual mutants.

[2]) Leaves of *hy-1*, *hy-2* and *hy-3* mutants were also narrower compared to wild type leaves especially at the leaf base.

[3]) Indicated by the number of grown-out side shoots in the rosette.

The wavelength dependence of hypocotyl inhibition

In wild type, all wavelength regions tested display inhibiting effects (Fig. 2), the most effective being far red, near UV and blue. It appeared that mutations in the *hy* genes may change the hypocotyl-inhibition spectrum very drastically. The spectra shown were found to be locus specific as they were qualitatively similar for all alleles at a particular locus tested (viz. four *hy-1*, three *hy-2*, five *hy-3*, four *hy-4* and two *hy-5* mutants).

The most remarkable effects are the almost complete absence of inhibition by far red in *hy-1* and *hy-2* mutants, and the reduced inhibition in *hy-5*. Compared with

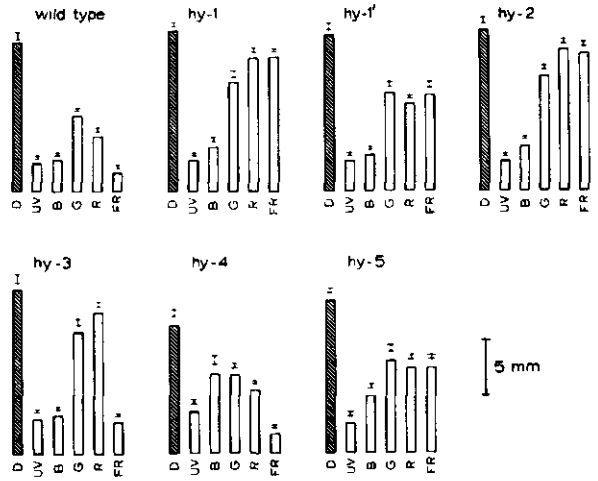

Fig. 2: Hypocotyl lengths of wild type Arabidopsis and a number of mutants in the various spectral regimes. Shaded bars: dark controls. Isolation numbers of the mutants used: *hy-1* = d400; *hy-1'* = d412; *hy-2* = d1127; *hy-3* = Bo64; *hy-4* = 2.23 N; *hy-5* = Ci88..

wild type, inhibition by red is nearly absent in *hy-1*, *hy-2* and *hy-3* mutants. Blue light is less effective in *hy-4* and to some extent also in *hy-5* mutants.

Double-recessive genotypes

F_2's were derived from all possible crosses between representative mutants at the five loci (except the cross *hy-2* × *hy-5*). In all F_2's involving *hy-4* or *hy-5* mutants, recombinants occurred with a more extreme hypocotyl phenotype than that of both mutant parents (Table 4). These phenotypes combine homozygous alleles of both genes (*hy-x hy-x, hy-y hy-y:* denoted in Fig. 3 as *hy-x hy-y*). In crosses between *hy-1, hy-2* and *hy-3* recombinant genotypes could only be identified by crossing F_2 plants with both parents. These were about as long as the parents (Table 4).

The inhibition spectra of some of these double-recessive genotypes with an «extreme» phenotype are presented in Fig. 3. They can be interpreted as

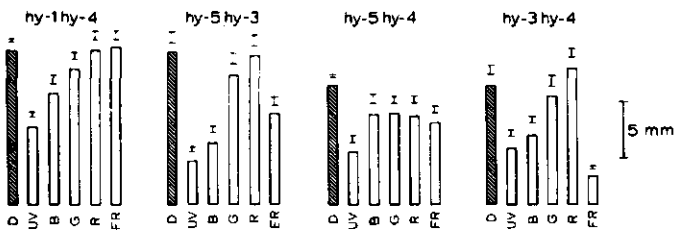

Fig. 3: Hypocotyl lengths of a number of recominants in the various spectral regimes.

Table 4: Hypocotyl length (in mm) of *hy* mutants and some of their recombinants when grown for seven days on perlite in continuous white light.

		Mutant	V197	2.23N	Ci88	e751
		Locus	*hy-3*	*hy-4*	*hy-5*	*hy-5*
		Hyp. length	8.4±0.1	5.8±0.4	5.7±0.3	5.3±0.2
Mutant	locus	Hyp. length				
21.84	*hy-1*	9.5±0.4				12.1±0.3
TO75	*hy-1*	9.2±0.2	8.0±0.2	17.1±0.3		
TO76	*hy-2*	9.0±0.2	9.1±0.2	16.2±0.2		
Bo64	*hy-3*	7.7±0.2		15.3±0.4	12.9±0.3	
V197	*hy-3*	8.4±0.1				
2.23N	*hy-4*	5.8±0.4			12.3±0.4	

demonstrating the effects of both parental mutants in a cumulative way. It is interesting to note that the genotype in which light inhibition is most strongly reduced (*hy-1 h-1, hy-4 hy-4*), still shows some inhibition in the UV and blue region although less so than in both parents.

Phytochrome content

The quantity of spectrophotometrically detectable phytochrome varied greatly between the various genotypes (Table 5). *Hy-1* and *hy-2* had no photometric phytochrome or only traces of it, with the exception of the d412 mutant, which had a reduced phytochrome content compared to wild type. The limit of detectability in these measurements was such that concentrations lower than 5–10 % of those in wild-type tissue could not be detected. We cannot, therefore, exclude the possibility that our «phytochrome-less» mutants still contain some pigment at concentrations below this level. Mutants at the other loci, *hy-3, hy-4* and *hy-5*, show about the same quantity as wild type, taking into account the varation between repeated

Table 5: Phytochrome content of dark-grown, one week old seedlings of a number of induced mutants of *Arabidopsis* and their length when grown under FR.

Mutant	Locus	Phytochrome content $\times \Delta\Delta A \times 10^3$	Length in FR in mm	
			exp. on perlite	exp. on filter paper
wild type	+	1.15	2.8	1.8
21.84	*hy-1*	0.00	11.4	n.t.
d412	*hy-1'*	0.26	n.t.	8.8
d1127	*hy-2*	0.00	n.t.	12.6
TO76	*hy-2*	0.05	10.6	n.t.
Bo64	*hy-3*	1.35	3.5	3.0
d504	*hy-3*	1.21	2.7	n.t.
2.23N	*hy-4*	0.97	3.1	1.9
Ci88	*hy-5*	1.03	7.3	7.7

measurements. Additional experiments with the same genotypes and another *hy-2* and *hy-5* mutant (not shown) confirmed the results of Table 5.

Discussion

Photomorphogenic effects as shown in Fig. 2, are not easily explained as resulting from the action of a single light receptor pigment. Attempts have been made to explain the HIR's, notably those of the inhibition of hypocotyl elongation in lettuce (HARTMANN, 1966; SCHÄFER, 1976) and in *Sinapis* (e. g. WILDERMANN et al., 1978) on the basis of phytochrome as the sole pigment involved. The model of SCHÄFER provides a plausible explanation for the far-red part of the HIR action spectrum for lettuce. Our results, presented above, demonstrating that there is no inhibiting action of red and far red on the *Arabidopsis* mutants that lack spectrophotometrically detectable phytochrome (*hy-1* and *hy-2*), add weight to the conclusion that this pigment mediates effects of the long-wavelength part of the visible spectrum.

In wild type *Arabidopsis*, like in lettuce, near-UV, blue, and far red are the most effective wavelength regions (see also KRANZ, 1977 b; SCHEIDEMANN, 1978). However, compared with lettuce, green and red have appreciable activity in wild type *Arabidopsis*. Whereas the effect of green might possibly be ascribed to the contribution of wavelengths < 500 nm (Fig. 1), green possibly acting as weak «blue», our red source is rather narrow-banded and contains particularly little radiation > 700 nm. No definite conclusions can be drawn until we will have determined detailed action spectra for the most important mutants. However, our results suggest that there may be considerable differences between the long-wavelength action spectra of lettuce, wild type *Arabidopsis* and its mutants, e. g. *hy-1*, *hy-2*, *hy-3* and *hy-5*.

We conclude, that the *hy-1* and *hy-2* genes regulate the synthesis of phytochrome, at least in the hypocotyl. Mutants of these genes provide the first known examples of phytochrome-deficient genotypes. Mutant *d412* (*hy-1'*), in which the *hy-1* gene apparently is still functioning partially, provides an example of a so-called «leaky» mutant.

KRANZ (1976, 1977 a) compared the absorption spectra of wild type *Arabidopsis* and several mutants mainly characterized by chlorophyll defects without hypocotyl elongation under white light (a. o. *ch* and *im* mutants). He concluded that some genotypes including wild type were abnormal in both biogenesis and/or phototransformations of phytochrome. However, apart from the chlorophyll content, other phytochrome-induced effects in the genotypes studied such as seed germination, hypocotyl inhibition (KRANZ, 1977 b) and daylength sensitivity (KRANZ, 1979), appear rather normal. The conclusion that wild type is the most abnormal genotype with respect to the phytochrome system (KRANZ, 1976) is surprising in so far as most mutations result in either loss or change of function. It is difficult to comment on the remarkable phototransformation spectra reported by KRANZ except to suggest that, in

view of the fact that this author has been working with chlorophyll mutants, reactions of the photosynthetic pigments may have interfered. Somewhat similar results published by JACQUES (1968) for oats may well have the same explanation. In view of the complicated pigment transformations following the inital phototransformation of protochlorophyllide in dark-grown plants, a more thorough analysis would be required before such findings could be accepted and ascribed to phytochrome. One possible approach to this problem would be to remove the cotyledons before phytochrome measurement. In view of these reported abnormal phytochrome types in *Arabidopsis* we have paid special attention to the occurrence of possible long-lived phytochrome intermediates in our material but nothing deviating from the normal was observed. We can say with some confidence that intermediates with half lives at 0 °C greatly in excess of those observed in other plants, were not formed. We also determined a difference spectrum for phototransformation of phytochrome *in vivo* in wild type *Arabidopsis*. The peaks at 664 and 732 nm were in the expected range.

Since the activities of UV and B, in contrast of those of FR, are hardly diminished in the *hy-1* and *hy-2* mutants, it seems to follow that the short-wavelength part of the HIR in these mutants is mainly due to non-phytochrome pigments. Since both P_r and P_{fr} have weak absorption maxima in the blue and near-UV region (e. g. GARDNER and BRIGGS, 1974) phytochrome should, according to the model of SCHÄFER (1976), contribute some HIR activity somewhere near the UV and violet asbestic points. A comparison between the wild type *Arabidopsis* on the one hand, and our *hy-1* and *hy-2* spectra on the other, suggests that this phytochrome activity is minimal, however. This leads to the conclusion that, at least for the inhibition of hypocotyl elongation in dark-grown *Arabidopsis*, high activity of the short-wavelength regions in the HIR is not related to phytochrome.

This conclusion gets additional support from our results with mutant *hy-4*. This mutant is strongly inhibited by FR, somewhat less by UV. Blue, on the contrary is the spectral region with about the weakest activity. This suggests two additional conclusions. First, that this mutant is normal with regards to phytochrome, but deficient in the specific «blue» light receptor. Second, it appears likely, that there are separate pigments for the UV and B effects as well. It should be recalled here that MEYER (1968) and GABA and BLACK (1979) previously obtained evidence, along entirely different lines, for different mechanisms operative in the blue and long-wavelength effects on hypocotyl elongation in *Cucumis*. Our results indicate that the long- and short-wavelength parts of the spectrum act independently. This appears to exclude the possibility of UV- and blue-absorbing pigments acting by transferring their excitation energy to phytochrome as there is strong action of these wavelength regions in those mutants that are weakly or not at all sensitive to red and far red. The latter situation is reminiscent of the action spectra for polarotropism in *Dryopteris* and *Sphaerocarpos* (STEINER, 1969) where in the former blue proved about 100 times more effective than red, whereas in the latter wavelengths above 550 nm

were entirely inactive notwithstanding a nearly complete identity of the action spectra for these two organisms in the blue region. If in all these cases an energy transfer mechanism were operative it would be hard to understand why blue is so infinitely more effective than red.

There are reports to the effect that such pretreatment of plants as deetiolation by white, red, or blue light can alter the subsequent sensitivity of the HIR to various wavelengths, notably red and far red (GRILL and VINCE, 1966; GRILL and VINCE, 1970; TURNER and VINCE, 1969; JOSE and VINCE, 1977 a). It is conceivable that the effect of such treatments consists of coupling or uncoupling the actions of one or another of several pigment systems. Our experiments do not address the question of a possible interaction between the various pigments during simultaneous irradiation with more than one wavelength.

A comparison of the spectra of Fig. 2 shows some more remarkable differences between the various mutants. Mutant *hy-3*, which has normal phytochrome content and is about as sensitive to far red as the wild type, deviates from the latter in its greatly reduced sensitivity to red. Although a definite conclusion must be postponed until we will have determined the intensity dependence of the HIR in these mutants and made detailed action spectra, we suspect that there may be interesting differences in the shapes of the action spectra in the red and the far red. In view of the findings of JOSE and VINCE-PRUE (1977 b) and VANDERHOEF et al. (1979) it appears possible that also in wild type *Arabidopsis* as well as in mutants *hy-4* and *hy-5*, a red-absorbing pigment other than phytochrome contributes markedly to the HIR.

Mutant *hy-5* is a separate case. Whereas its spectroscopic phytochrome content is fairly high, it is only moderately sensitive to far-red light. Apparently the reactivity to light in this genotype is blocked by some other factor than its capacity for phytochrome synthesis.

The extreme absence of inhibition in double-recessive genotypes shows that the photoreceptors behave independently from each other. The absence of such recombinants with «extreme» phenotype in crosses among *hy-1*, *hy-2*, and *hy-3*, indicates that these genes regulate biosynthetic pathways or regulating steps in physiological pathways that are not independent. It is significant that genotypes combining phytochrome-deficient mutants (*hy-1 hy-1*) with those of reduced sensitivity to blue (*hy-4 hy-4*), still show some inhibition by UV and blue light. This fortifies our conclusion that most of the remaining sensitivity to blue and UV in *hy-4* mutants is not due to absorption by phytochrome. The remaining effect in the recombinant might be caused by another system still functioning.

The general view that emerges from this study is that the light-induced hypocotyl inhibition may depend upon a complex system of photoreceptor pigments. It appears likely that the same may turn out to hold for other HIR effects also. Our results suggest that these systems are able to operate independently of one another. Since experiments of this type usually are of extended duration, during which developmental processes operate, the possibility has to be kept in mind that the

hypocotyl inhibition is the composite of several reactions that occur either simultaneously or in succession. The use of the genotypes described might be very useful to attribute specific effects to specific systems.

The determination of detailed action spectra for the different genotypes might elucidate some of the complexity of the overall action spectra constructed for wild type plants. These spectra should be made for other reactions that are known to be under light control in *Arabidopsis*, such as seed germination (SHROPSHIRE et al., 1961), photoperiodism (NAPP-ZINN, 1969), phototropism, etc.

Finally, it should not be left unmentioned that plants of the genotypes described above, will develop into phenotypically almost or completely normal plants when cultivated under appropriate conditions in white light. Since it is likely that phytochrome has one or more functions during several stages of normal plant development, the mutants *hy-1* and *hy-2* probably should not be designated «phytochromeless». So far, we have only observed that the hypocotyls of these types, if grown in the dark, do not contain detectable phytochrome. Other parts of the plants, formed in light, may do. This is at present under investigation. We know already that the seeds of these mutants also differ greatly in their phytochrome content.

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CHAPTER 5

EMS- and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana* (L.) Heynh.

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Summary

Average mutation frequencies per locus per dose of mutagen — EMS, X-rays and fast neutrons — were estimated from 15 different loci of *Arabidopsis thaliana*. These loci relate to clearly distinguishable phenotypic mutant groups, each affected by a limited number of loci. There were significant differences in mutation rate between individual loci. The proposition of a linear relationship between DNA content and mutation frequency per locus is rejected.

The probability of inducing mutations with respect to a specified character, i.e. obtaining mutants belonging to a specific phenotypic group, depends both on the number of loci involved and on the mutation frequencies at these loci. For making comparisons between mutagens or between biological species, mutation frequencies should be expressed per locus per cell (or per haploid genome) and per dose of mutagen.

In plants, mutation frequencies are usually presented on the basis of phenotypic groups, without further identification of the loci involved. With phenotypic groups such as *erectoïdes* and *eceriferum* mutants and especially chlorophyll mutants where a large number of loci are involved, such analyses are of course laborious.

To single out one or a few loci and generalizing their mutation frequency to the whole of the genome seems a doubtful procedure, because not only may different loci within the same phenotypic group vary considerably in mutation frequency (e.g. Lundqvist, 1978), but also different phenotypic groups might differ in the average mutation frequency of their loci. The issue of comparing mutation frequencies based

on limited-scale experiments and incorrect calculations has been critically examined by Schalet and Sankaranarayanan (1976) and by Schalet (1978), who reanalysed the claim of some authors (Abrahamson et al., 1973; Heddle and Athanasiou, 1975) that there exists a linear relationship between mutation frequency per locus and DNA content per haploid genome throughout the spectrum of biological species.

Our experiments with *Arabidopsis thaliana* enable us to identify the individual loci per mutant group as well as to estimate their mutation frequency per cell per mutagen dose unit. Our set of mutant groups fulfills 2 basic requirements: (1) unambiguous phenotypic classification even when scoring is done by different observers and/or in different environments, and (2) a limited number of loci per phenotypic group as judged from a comparatively large number of independently induced mutants per group.

For plant species there are only few data in the literature on mutation frequencies induced per locus. In Arabidopsis, data are available for thiamine auxotrophy (Feenstra, 1964; Redei and Li, 1969) and for chlorate resistance (Oostindiër-Braaksma and Feenstra, 1973; Braaksma and Feenstra, 1975). In the present report the *eceriferum* group (many loci, incompletely identified) has been added to compare this group of Arabidopsis with the intensively analysed *eceriferum* group of barley (Lundqvist and von Wettstein, 1962; Lundqvist, 1978; von Wettstein-Knowles, 1979).

For mutation induction, EMS, X-rays and fast neutrons were used in experiments performed in the same period and the same environment (greenhouses). From this material a comparison between the 2 irradiation sources on the basis of *overall* frequencies per phenotypic group in a more extended set of distinct groups has been published recently by one of us (Dellaert, 1981).

Material and Methods

Plant material

Seed stocks used in the experiments were of the pure line "*erecta*" from ecotype "Landsberg" (Redei, 1962). *Arabidopsis thaliana* ($2n = 10$) is a strict self-fertilizer under our greenhouse conditions.

Mutants

The mutants were induced by us, except those listed under "Mutants from other sources" in Table 1, and belong to mutant groups chosen on the basis of the stated prerequisites (unambiguous scoring, limited number of loci; see Introduction).

(a) *Gibberellin-sensitive mutants (ga).* For detailed description see Koornneef and van der Veen (1980). This group comprises mutants that require gibberellin (GA) both for germination and elongation growth (non-germinating GA dwarfs) and mutants that have this requirement only for elongation (germinating GA dwarfs).

(b) *Hypocotyl mutants (hy).* Hypocotyl elongation 3–6 times of the wild-type, when grown for a week under continuous TL light. Some of the loci involved

TABLE 1

RESULTS OF COMPLEMENTATION TESTS WITH ARABIDOPSIS MUTANTS

Mutant group/locus	Chromosome location	Number of mutants induced by			Mutants from other sources (mutagens unknown)	Total
		EMS	X-Rays	Fast neutrons		
Non-germinating dwarfs						
ga-1	4	19(+2)	1	4		26
ga-2	1	4	1	1		6
ga-3	5	5	–	–		5
		28(+2)	2	5		37
Germinating dwarfs						
ga-1	4	5	–	1		6
ga-2	1	2	–	–		2
ga-3	5	1(+1)	–	–		2
ga-4	1	7	1	–		8
ga-5	4	1	–	–		1
		16(+1)	1	1		19
Hypocotyl mutants						
hy-1	2	1(+2)	–	2(+1)	2[a]	8
hy-2	3	3(+1)	–	–	–	4
hy-3	2	9(+2)	2(+1)	3	–	17
		13(+5)	2(+1)	5(+1)	2	29
Glabra mutants						
gl-1	3	1	1	2	1[b]	5
gl-2	1	–	–	1	–	1
gl-3	5	2	1	1	–	4
ttg	5	3(+2)	–	1	1[c]	7
		6(+2)	2	5	2	17
Other mutants						
an	1	1	–	2	1[d]	4
ag	4	2(+1)	–	1	–	4
ap-1	1	3(+1)	–	2	–	6
		6(+2)	–	5	1	14

Mutants not from the M_2 lines used for the calculation of mutation frequencies are given between parentheses.

[a] *hy* described by Redei and Hirono (1964) and V317 from the AIS gene bank (Bürger, 1971).

[b] *gl* described by Redei and Hirono (1964).

[c] F31 from the AIS gene bank (Bürger, 1971).

[d] *an* from Redei (Lee-Chen and Steinitz-Sears, 1967).

regulate the presence of phytochrome in hypocotyls (Koornneef et al., 1980b) and seeds (Spruit et al., 1980). Less pronounced *hy* mutants were excluded from the present study.

(c) *Agamous (ag).* "Double flowering", resembling the *multipetala* mutant de-

scribed by Conrad (1971). Sterile (no pistils or stamens).

(d) *Angustifolia (an).* Narrow leaves and slightly crinkled siliquae (Lee-Chen and Steinitz-Sears, 1967; Demchenko et al., 1976).

(e) *Apetala (ap-1).* Fertile without petals or with rare rudimentary petals. The *ap-1* mutants represent a clearly distinguishable type within a large group of mutants with a deviating flower morphology (cf. McKelvie, 1962).

(f) *Glabra (gl, ttg).* Mutants without trichomes or with reduced trichomes on the leaves (Redei and Hirono, 1964; Lee-Chen and Steinitz-Sears, 1967; Bürger, 1971; Demchenko et al., 1976).

(g) *Eceriferum (cer).* Bright green siliquae and/or stems due to a deviating structure of the wax layer (Dellaert et al., 1979).

Except for 1 single *cer-1* mutant, none of the mutant types described in this report was found in non-mutagen-treated wild-type progenies grown in the course of years at our laboratory. This indicates a low spontaneous mutation frequency for the loci described.

Culture conditions

Seeds were sown (equally spaced) in 9-cm petri dishes on perlite with a standard mineral solution as described by Oostindiër-Braaksma and Feenstra (1973). After a cold treatment (4–6 days at 2–4°C) to break seed dormancy, the seeds were put to germinate in a climate room at about 24°C under continuous illumination by fluorescent light tubes (Philips TL 57, about 8 W/m^2), except for the mutagenic treatment. 8 days after the end of cold treatment the seedlings were transplanted into soil in an air-conditioned greenhouse.

Ethyl methanesulphonate (EMS)-treatment

Before the EMS treatment, seeds were kept on moist filter paper for 5 days at 4°C to break seed dormancy, then redried (in the dark, 24°C, 24 h). Subsequently the seeds were submerged in a freshly prepared unbuffered 10 mM EMS solution and left for 24 h in the dark at 24°C. After being rinsed off with tap water, the seeds were sown in petri dishes and put to germinate in the climate room as described above.

Radiation treatment

Dormancy breaking and redrying was as before EMS treatment. Before irradiation the seeds were submerged in water at 22°C for 3 h. Details on the X-ray machine, the source of fast neutrons and the use of the radioprotector dithiothreitol (DTT) as applied in some treatments, have been given by Dellaert (1980c). X-Ray doses were 140, 233, 327 and 420 Gy (10 Gy = 1 krad) for seeds submerged in tap water; and 280, 467, 653 and 840 Gy for seeds submerged in a 1.2% DTT solution. Fast neutron doses were 20, 33, 47 and 60 Gy for seeds submerged in tap water; and 40, 67, 93 and 120 Gy for seeds submerged in a 1.2% DTT solution. The temperature of the solutions was 22°C. The doses chosen were not so high as greatly to reduce M_1 fertility.

Corrections for recessive deficits

In M_3 lines the average segregation frequency of recessive mutants is usually less than 0.25 (Moh and Nilan, 1956; Doll, 1968; Jacobs, 1969; Delleart, 1980d). For our mutant groups, zygotic selection can be neglected (cf. Dellaert, 1980d). The most likely explanation for the deficit then is male gametic selection (certation). In M_2 lines an additional cause for a recessive deficit is M_1 chimerism.

The irradiation and EMS experiments were initially started by the first 2 authors (Dellaert and Koornneef, respectively) independently from each other. This involved a somewhat different procedure in harvesting M_1 plants. For irradiation, only one well-filled siliqua from the top region of the main inflorescence was taken. Within-flower chimerism can be neglected here (Ivanov, 1973; Dellaert, 1980d). However, with the EMS material, a number of siliquae from the top region were harvested. (M_1 plants with clearly reduced fertility were discarded.) Though chimerism is progressively lost in the direction of the top (Balkema, 1972; van der Veen, unpublished), it is not completely absent. So an M_2 line derives in part from selfed heterozygous siliquae (fraction α_B) and in part from selfed non-mutant siliquae (fraction 1-α_B).

The joint effect of chimerism and certation gives rise to a mixed M_2 population as follows:

	AA	Aa	aa
α_B [	$\frac{1}{2}-f$	$\frac{1}{2}$	f]
$+(1-\alpha_B)$ [	1	0	0]

where $\alpha_B < 1$ in the presence of chimerism and $f < 0.25$ in the presence of certation.

The following parameters can be extracted from the experimental data (cf. Dellaert, 1980d):

p_1, the average frequency of recessives in segregating M_2 lines.

p_2, the average frequency of heterozygotes among normals in segregating M_2 lines. For this purpose, large-scale progeny testing (M_3) is necessary.

p_3, the average frequency of recessives in segregating M_3 lines.

The estimates $\hat{p}_1$, $\hat{p}_2$ and $\hat{p}_3$ and their standard deviations were obtained by the method of Li and Mantel (1968), which corrects for small line sizes. For further details on the estimation procedure, see Dellaert (1980d).

Now, $p_1 = \alpha_B \cdot f$ and $p_2 = \alpha_B/2(1-p_1)$, so one can estimate $\hat{\alpha}_B = 2(1-\hat{p}_1)\hat{p}_2$ and $f = \hat{p}_1/\hat{\alpha}_B$. If the estimate $\hat{p}_2$ is not available, one can use the estimate $f = \hat{p}_3$ and $\hat{\alpha}_B = \hat{p}_1/\hat{p}_3$ as a correction for recessive deficit in M_2. A check on the reliability of using $\hat{p}_3$ for M_2 data will be presented later.

Mutation frequencies

For the estimation of mutation frequencies in self-fertilizing diploid plants a number of methods is available. (For a critical review see Yonezawa and Yamagata, 1975.) For our experiments, Gaul's M_2 plant method was used as this estimate of mutant frequency (m') is independent of M_2 progeny size and the degree of M_1 chimerism (Gaul, 1957; Frydenberg, 1963). m' is expressed as the total number of mutants among the total number of viable M_2 plants. The standard error s of m' is

calculated by a formula given by Snedecor (1966) (Cf. Dellaert, 1980b):

$$s_{m'} = \frac{\sqrt{N}}{\Sigma n_i} \sqrt{\frac{1}{(N-1)} \left\{ \Sigma a_i^2 - 2m' \Sigma a_i n_i + (m')^2 \Sigma n_i^2 \right\}}$$, where

N = number of M_2 lines scored,
n_i = number of viable plants in M_2-line i,
a_i = number of mutants in (segregating) M_2-line i,
m' = total number of mutants among the total number of viable M_2 plants.

The mutation frequency per locus per cell is $m = m'/f$. By dividing m by the dose of mutagen (D; in Gy's for radiation and mMh for EMS) one obtains the mutation rate per dose of mutagen per locus per diploid cell (m''). For correction to haploid genomes m'' should be divided by 2.

When only the number of segregating M_2 lines among the total number of scored M_2 lines is recorded one cannot apply Gaul's M_2 plant method, but one has to resort to the method of Li and Redei (1969). This method has the disadvantage that it leads to an underestimation of mutation frequencies when the M_2 lines are relatively small, especially when the degree of M_1 chimerism is high. Moreover, the estimate of $\hat{\alpha}_B$ which must be used is mostly rather inaccurate (Yonezawa and Yamagata, 1975). Li and Redei (1969) introduced the parameter "genetically effective cell number" (GECN $= 1/\alpha_B$) of the germ line. Their formula for mutation frequency per cell (m) reads $m = M/(S \times \text{GECN})$, where M is the number of M_2 lines segregating recessives and S the total number of M_2 lines tested. Note that $m = M\alpha_B/S$ equals $m = p_1 M/fS$ (see previous section) and that $p_1 M/S = m'$, so that $m = m'/f$ as before (Gaul's method).

Results

Genetic analysis of the mutants

Allelism versus non-allelism was assessed on the basis of non-complementation versus complementation within mutant groups. Complementation tests were not done simultaneously (in a diallele) but sequentially, by crossing mutants still to be tested with a gradually built-up locus representative tester set.

All mutants proved to be monogenic recessive to wild type.

Table 1 presents the results of complementation analyses along with location of the loci on the chromosomes ($2n = 10$) by means of trisomic analysis (Koornneef and van der Veen, 1978). To all loci listed in Table 1 distinct positions on the linkage map could be assigned (Koornneef et al., 1980a; Koornneef, in preparation), which confirms the results of the complementation analysis. Within mutant groups no close linkage was found.

A non-random distribution of mutants among the loci was found for the *ga* loci 1, 2 and 3 ($\chi_2^2 = 25.6$; $P < 0.01$) and for the 3 *hy* loci ($\chi_2^2 = 9.1$; $P < 0.05$), the frequency being highest for *ga-1* and *hy-3*, respectively, both among EMS- and radiation-induced mutants. At most loci, mutants were found with both EMS and irradiation. The number of mutants per locus was too small for mutagen specificity per locus to be analysed.

Locus-specific phenotypic expression within the groups of Table 1 was found for germinating dwarfs at *ga-4* and *ga-5*, these being clearly distinct from the dwarfs at *ga-1*, *ga-2* and *ga-3* (Koornneef and van der Veen, 1980). Locus-specific phenotypic expression was also conspicuous in the *glabra* group:

gl-1 is trichomeless or has a reduced number of normal trichomes; *gl-2* has rudimentary trichomes on the first leaf pair, and a reduced number of normal trichomes at higher leaves; *gl-3* has a reduced number of trichomes, which are unbranched or single branched; *ttg* is trichomeless. The *ttg* mutants also have transparent seed coat (seeds yellow, reflecting the embryo colour in ripe seeds). Conversely, transparent seed coat mutants were found with normal trichomes (not in Table 1), which were not allelic to *ttg*.

The *eceriferum* group, consisting of over 150 mutants induced by both EMS and radiation, was subdivided into 7 sub-groups by Dellaert et al. (1979) on the basis of differences in the pattern of wax deposition on siliquae and stem, visual impression of waxlessness and (locus-) specific pleiotropic effects. On the basis of incomplete dialleles within sub-groups including 38 of the mutants, a minimum of 14 loci was estimated. The complementation tests were extended and some sub-groups were pooled. Assuming no allelism between the 2 remaining sub-groups the complementation tests among 57 of the mutants revealed mutations at minimally 20 and maximally 25 different loci. The highest frequencies were found at locus *cer-3* (chrom. 5; 16 mutants) and at locus *cer-1* (chrom. 1; 8 mutants).

Mutants were isolated at a number of loci that have a less extreme phenotype than others, namely germinating dwarfs versus non-germinating dwarfs for *ga-1*, *ga-2* and *ga-3*, and a reduced number of trichomes versus absence of trichomes for *gl-1*. These mutants probably represent "leaky mutants", which implies a partial functioning of the gene product. 2 such mutants (one *ga-1* and *gl-1* mutant) were induced by fast neutrons. Some fast-neutron-induced non-germinating dwarfs were included in an intragenic recombination analysis of the *ga-1* locus. These behaved like overlapping intragenic deletions, except one which behaved like a "point mutation" (Koornneef, 1979).

Segregation frequencies (EMS-experiments)

The segregation frequencies $\hat{p}_1$ and $\hat{p}_3$ for different mutant groups are given in Table 2. No significant differences between mutant groups were found for $\hat{p}_1$ or for $\hat{p}_3$. Though $\hat{p}_3$ for the small group of germinating dwarfs (at *ga-1, 2, 3*) is not significantly lower than 0.25, one may say that there is also an average recessive deficit in M_3. The average EMS value $\hat{p}_3 = 0.219 \pm 0.005$ compares well with the average X-ray values 0.211 ± 0.010 (X-rays) and 0.208 ± 0.010 (X-rays + 1.2% DTT) and the average fast-neutron values 0.203 ± 0.010 (FN) and 0.221 ± 0.011 (FN + 1.2% DTT) found by Dellaert (1980d) for a larger and more diverse set of mutant groups. The value is also close to the 0.198 given by Jacobs (1969) for EMS-induced chlorophyll mutants in Arabidopsis.

To check whether $\hat{p}_3$ values obtained from EMS-induced mutants, like those obtained with radiation-induced mutants (Dellaert, 1980d), can also be used as an estimate for the correction factor f (see Material and Methods) a sample of 35

TABLE 2

M_2 AND M_3 SEGREGATION FREQUENCIES OF EMS-INDUCED MUTANTS

Loci	M_2 segregation frequency ($\hat{p}_1$)	Number of mutants tested	"M_3" segregation frequency ($\hat{p}_3$)[a]	Number of mutants tested
ga-1, ga-2, ga-3 (NG)	0.137 ± 0.013	28	0.211 ± 0.007	29[b]
ga-1, ga-2, ga-3 (G)	0.073 ± 0.030	8	0.239 ± 0.013	9[b]
ga-4, ga-5	0.111 ± 0.032	8		
hy-1, hy-2, hy-3	0.111 ± 0.032	13		
gl-1, gl-2, gl-3, ttg	0.103 ± 0.049	6		
an, ag, ap-1	0.125 ± 0.039	6		
eceriferum	0.148 ± 0.010	97	0.221 ± 0.010	35
Averages and totals	0.142 ± 0.007	166	0.219 ± 0.005	73

NG, non-germinating GA dwarf; G, germinating dwarf.

[a] For *ga-1, 2* and *3* loci, $\hat{p}_3$ was obtained from F_2's following the cross mutant × wild-type (Koornneef and van der Veen, 1980) which is taken to be equivalent to M_3 segregation frequency.

[b] Including some mutants from previous EMS experiments and a mutant first found in M_3.

EMS-induced *eceriferum* mutants was used, M_3 progenies being grown from 6 non-mutant plants per M_2 line. The corresponding $\hat{p}_1$ value is 0.156 ± 0.013. Further, $\hat{p}_3 = 0.221 \pm 0.010$ (Table 2) and $\hat{p}_2 = 0.430 \pm 0.038$. This leads to $\alpha_B = 2(1 - 0.156) \times 0.430 = 0.726$ (a non-negligible degree of chimerism between siliquae) and $f = 0.156/0.726 = 0.215$, which is close to $\hat{p}_3 = 0.221$. It follows from the *eceriferum* data that $\hat{p}_3$ can be safely used as an estimate for f.

Analysis and discussion

Mutation frequencies

Table 3 gives, for each mutant group, the 'calculation' of the average EMS-induced mutant frequency per locus. The weighted mean for the 15 identified loci is 0.20×10^{-3}. Taking the maximal estimate of 25 for the number of *eceriferum* loci as deduced from complementation tests with 57 of these mutants, one obtains for the *eceriferum* group an average mutant frequency per locus as $5.11 \times 10^{-3}/25 = 0.20 \times 10^{-3}$, which happens to be the same as the weighted average for the other loci. Dividing mutant frequencies by $\hat{p}_3$ (= 0.219 for EMS) one obtains mutation frequencies per locus per cell (Gaul's method). These can be converted to mutation frequencies per locus per dose of mutagen (in mMh), in our case by dividing by 10 (mM) × 24 (h). This is realistic, because in earlier unpublished experiments (van der Veen) performed under strictly comparable conditions (i.e. redried seeds after cold treatment; 24°C), the mutagenic effect (embryonic lethality, chlorophyll mutant frequency) was a linear function of time, since (a) at fixed dose (mM) × duration (h) it happened to be approximately constant, except for very short duration (Fig. 1), and (b) under the same conditions these genetic effects were linear with dose in the

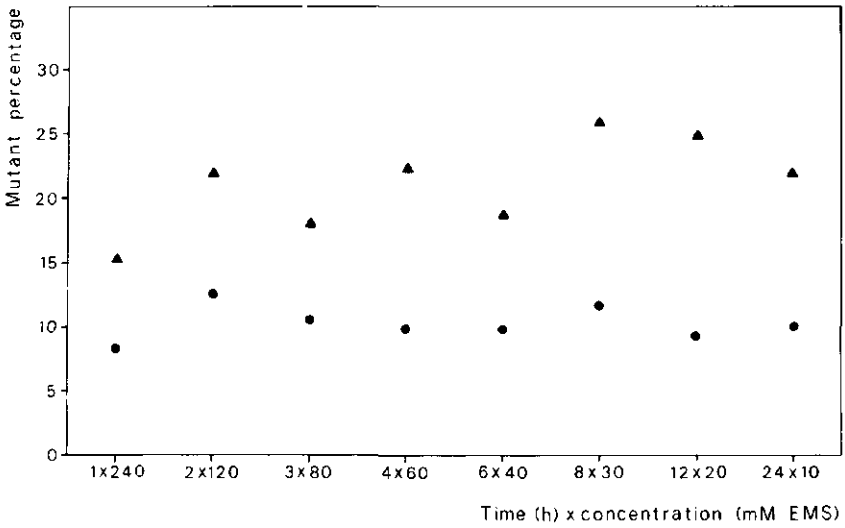

Fig. 1. Mutant percentage as a function of time×concentration. ▲, embryonic lethals; ●, chlorophyll mutants scored by Müller's embryo test (Müller, 1963). Each point represents about 2500 scored embryos.

range of 3.0–17.5 mM (24°C, 24 h) (van der Veen, 1968).

Tables 4 (X-rays) and 5 (fast neutrons) give the average mutant frequencies of the same mutant groups for experiments in which different doses (cf. Material and Methods) were used with and without the radio-protector DTT (Dellaert, 1980c, 1981). The doses applied with DTT were corrected for the protective effect of DTT as follows. Dellaert (1980c; Table 1) gives the mutant frequencies for viable mutants for the dose range applied both for X-ray and fast-neutron treatment with and without DTT. In all 4 cases the dose–response curve can be represented fairly well by a straight line through the origin ($y = ax$). For X-rays we find $y = a_0 x$ and $y = a_1 x$ for without and with DTT respectively. The ratio $a_0/a_1 = 0.00110/0.00074 = 1.49$ is called the dose-reduction factor (DRF) of DTT for X-rays. For fast neutrons the DRF is $a_0/a_1 = 0.00669/0.00543 = 1.23$. By correcting with (i.e. dividing by) the DRF, the "DTT doses" can be converted into corresponding "H_2O doses". When calculating the mean over doses, we weighted the values by the number of M_2 plants per dose.

With all 3 mutagens, the group of *ga-1*, *ga-2* and *ga-3*, shows the highest average frequency per locus (Tables 3, 4 and 5), which is specifically due to *ga-1* (Table 1). Also *hy-3* consistently had a high frequency (Table 1). This might be an intrinsic property of the genes, e.g. related to the length of transcription unit. It can be concluded that this locus specificity not only generates significant differences in mutation frequencies per locus within mutant groups, but also between groups. (Cf. the *ga*-group and *gl*-group; Tables 3, 4 and 5.)

For the loci tested, no data are available from the literature. However, data on 3 other well-defined phenotypic groups (Table 6) can be used for comparison. As in these cases only the fraction of M_2 lines segregating recessives (M/S) is given, i.e.

TABLE 3

EMS-INDUCED MUTANT AND MUTATION FREQUENCIES FOR SEVERAL MUTANT GROUPS

Mutant group (loci)	Number of M_2 lines	Total number of M_2 plants	Number of mutants in M_2	Number of independently induced mutants	Average mutant frequency ($\times 10^{-3}$)		Mutation frequency per locus per diploid cell per dose (mMh) $\times 10^{-6}$ [a,b]
					per group (m')	per locus	
ga-1, ga-2, ga-3 (NG)	4485	114933	113	28	0.98 ± 0.25	0.42 ± 0.09	8.0 ± 1.8
ga-1, ga-2, ga-3 (G)	3213	45815	13	8	0.28 ± 0.11		
ga-4, ga-5 (G)	3213	45815	21	8	0.46 ± 0.21	0.23 ± 0.10	4.4 ± 1.9
hy-1, hy-2, hy-3	3979	92485	62	13	0.67 ± 0.22	0.22 ± 0.07	4.2 ± 1.4
gl-1, gl-2, gl-3, ttg	3213	45815	9	6	0.20 ± 0.08	0.05 ± 0.02	0.9 ± 0.4
an, ag, ap-1	3213	45815	15	6	0.33 ± 0.19	0.11 ± 0.06	2.1 ± 1.2
eceriferum	3213	45815	235	97 [c]	5.11 ± 0.63		
					Weighted average	0.20 ± 0.03	3.7 ± 0.5

[a] EMS treatment 10 mM × 24 h (24°C); f via $\hat{p}_3 = 0.219$ (Table 2).

[b] Individual loci with high mutation frequencies are *ga-1* (16.0×10^{-6}), *ga-4* (10.9×10^{-6}), *hy-3* (8.8×10^{-6}), *cer-1* (12.5×10^{-6}) and *cer-3* (37.6×10^{-6}).

[c] 31 of these tested for allelism thus far; *cer-3* was found 12 times; *cer-1* 4 times.

TABLE 4

X-RAY-INDUCED MUTANTS AND MUTATION FREQUENCIES FOR SEVERAL MUTANT GROUPS

Mutant group (loci)	Number of M_2 lines	Total number of M_2 plants	Number of mutants in M_2	Number of independently induced mutants	Average mutant frequency ($\times 10^{-3}$)		Mutation frequency per locus per diploid cell per dose (Gy) $\times 10^{-6}$ [a]
					per group (m')	per locus	
ga-1, ga-2, ga-3 (NG)	2457	18963	5	2	0.26 ± 0.06	0.09 ± 0.06	1.5 ± 0.1
ga-4, ga-5 (G)	2457	18963	1	1	0.05 ± 0.05	0.03 ± 0.03	0.5 ± 0.5
hy-1, hy-2, hy-3	2457	18963	2	2	0.11 ± 0.08	0.04 ± 0.03	0.6 ± 0.4
gl-1, gl-2, gl-3, ttg	2457	18963	4	2	0.21 ± 0.15	0.05 ± 0.04	0.9 ± 0.6
an, ag, ap-1	2457	18963	–	–	0.0	0.0	0.0
eceriferum	2457	18963	16	8	0.84 ± 0.33		
					Weighted average	0.04 ± 0.02	0.7 ± 0.3

[a] Average dose was 273.13 Gy, weighted on the basis of the number of M_2 plants per individual dose (Dellaert, 1980c) and taking for the experiments in which 1.2% DTT was applied a Dose Reduction Factor of 1.49 (calculated from Dellaert, 1980c); $f = 0.215$ (Dellaert, 1980d).

TABLE 5

FAST-NEUTRON-INDUCED MUTANT AND MUTATION FREQUENCIES FOR SEVERAL MUTANT GROUPS

Mutant group (loci)	Number of M_2 lines	Total number of M_2 plants	Number of mutants in M_2	Number of independently induced mutants	Average mutant frequency ($\times 10^{-3}$)		Mutation frequency per locus per diploid cell per dose (Gy) $\times 10^{-6}$ [a]
					per group (m')	per locus	
ga-1, ga-2, ga-3 (NG)	2623	20194	22	5	1.14 ± 0.53	0.38 ± 0.18	38.9 ± 18.1
ga-1, ga-2, ga-3 (G)	2623	20194	1	1			
ga-4, ga-5 (G)	2623	20194	–	–	0.0	0.0	0.0
hy-1, hy-2, hy-3	2623	20194	7	5	0.45 ± 0.22	0.15 ± 0.07	15.2 ± 7.3
gl-1, gl-2, gl-3, ttg	2623	20194	12	5	0.59 ± 0.29	0.15 ± 0.07	15.2 ± 7.4
an, ag, ap-1	2623	20194	10	5	0.50 ± 0.25	0.17 ± 0.08	16.9 ± 8.6
eceriferum	2623	20194	66	27	3.27 ± 0.77		
					Weighted average	0.18 ± 0.05	18.3 ± 4.7

[a] Average dose was 45.39 Gy, weighted on the basis of the number of M_2 plants per individual dose (Dellaert, 1980c) taking for the experiments in which 1.2% DTT was applied a Dose Reduction Factor of 1.23 (calculated from Dellaert, 1980c); $f = 0.215$ (Dellaert, 1980d).

TABLE 6

MUTATION FREQUENCIES PER LOCUS PER CELL PER DOSE[a] OF MUTAGEN $\times 10^{-6}$ CALCULATED BY 2 METHODS FOR DIFFERENT MUTANT GROUPS

Mutant group	Number of loci	Mutagen	Number of independent mutants	Gaul's method[c]	Li and Redei's method
Groups of Table 1	15	EMS	69	3.7	3.2
		X-rays	7	0.7	0.7
Thiamine deficiency[b]	4	EMS	51		0.6
		X-rays	11		0.2
Thiamine deficiency[c]	4	EMS	6		0.9
Nitrate reductase deficiency[d]	6	EMS	8		0.2
chl-1[d] (chlorate resistance)	1	EMS	37		6.8

[a] mMh for EMS and Gy irradiation.
[b] Redei and Li (1969).
[c] Feenstra (1964); assuming GECN = 2.
[d] Braaksma and Feenstra (1975); Braaksma, personal communication; assuming GECN = 2.
[e] Values from Tables 3 and 4

no number of M_2-mutant plants nor M_2-line sizes, one has to resort to the method of Li and Redei (1969). Though for our 15 loci a good correspondence is found with Gaul's method (Table 6), small M_2-line sizes lead to an under-estimate as a result of proband escape. The mutation frequencies in Table 6 were obtained by using a GECN $= 1/\alpha_B = f/\hat{p}_1 = \hat{p}_3/\hat{p}_1 = 21.9/14.2$ (Table 2) $= 1.54$ for our experiments and GECN = 2 for the literature references (Li and Redei, 1969). For the groups thiamine and nitrate reductase deficiency, the frequencies were relatively low (below 1.0×10^{-6}), but for the *chl-1* locus a high frequency (6.8×10^{-6}) was found, though not as high as for our *cer-3*, *ga-1* and *cer-1* loci (footnote b in Table 3).

It can be argued that, owing to the still relatively small size of the experiments, the mutation frequencies per locus must be regarded as maximal estimates in Arabidopsis, because mutants at loci with low and very low frequencies may have escaped detection. So the number of loci per mutant group may be underestimated.

A comparison of *eceriferum* mutants in Arabidopsis with those in barley is of interest. As said earlier, complementation tests with 57 independently induced mutants indicated that these mutants represented mutations at 20–25 different loci. Lundqvist and von Wettstein (1962) found comparable values for barley, namely 23 loci for 67 mutants. In a later publication the number of loci had increased to 65 when 1123 mutants had been tested (von Wettstein-Knowles, 1979).

On the basis of proportionality, one may infer the number of *cer* loci in Arabidopsis, which might ultimately also be found to be about 65. With 65 loci, and referring to a haploid genome, one obtains a mutation frequency per locus per haploid genome of $5.11/(65 \times 2 \times 0.22 \times 240) = 0.74 \times 10^{-6}$ per mMh. Schalet (1978) derived, from the barley results of Lundqvist and von Wettstein (1962), the value of 6.3×10^{-6} per mM which is $6.3 \times 10^{-6}/24 = 0.26 \times 10^{-6}$ per mMh. A

second correction can be made for the treatment temperature (20°C). As indicated by Konzak et al. (1963), a rise from 20 to 24°C would give in barley a correction factor of 1.5. One then obtains 0.39×10^{-6} per mMh for *eceriferum* loci in barley, which compares reasonably well with our values for Arabidopsis (0.74×10^{-6}), especially as the barley values may still be underestimates (Schalet, 1978).

When comparing the mutation frequencies per *cer* locus for radiation of barley and Arabidopsis, one again finds a comparable order of magnitude. (X-rays: barley, 0.20×10^{-6}/Gy; Arabidopsis, 0.11×10^{-6}/Gy. Fast neutrons: barley, 4.56×10^{-6}/Gy; Arabidopsis, 2.58×10^{-6}/Gy.) The barley values were calculated from the results of Lundqvist and von Wettstein (1962) in the way described by Schalet and Sankaranarayanan (1976). For both species, the values are per haploid genome and assuming 65 loci.

The 2 species are known to differ by a factor of 25 in DNA content (Bennet, 1972), Arabidopsis being one of the plant species with the lowest DNA content per haploid genome. Therefore, the Arabidopsis-barley comparison is clearly at variance with a linear relation between DNA content and induced mutation frequency as suggested by Abrahamson et al. (1973) and by Heddle and Athanasiou (1975) on the basis of a literature survey. This claim had already been opposed on good grounds by Schalet and Sankaranarayanan (1976) and Schalet (1978).

Comparison between EMS and radiation

A second generalization made by Heddle and Athanasiou (1975) is that of a constant "equivalent" between 1 M EMS and 1 rad X-rays for all organisms. This could highly simplify the estimation of genetic risks, but as Schalet (1978) pointed out, this equivalent may differ by a factor of 10 for the same genetic parameter depending on the phase of development in which the mutagen is applied, and the effect of EMS is greatly affected by temperature and duration of treatment. Secondly, the existence of pronounced mutagen specificity is not consistent with this concept. Some rather extreme examples of mutagen specificity are described for the *eceriferum* mutants in barley (Lundqvist, 1978), where mutants at particular loci were isolated exclusively after radiation (e.g. *cer-i* mutants) and mutants at *cer-j* almost exclusively when chemical mutagens were used.

Mutagen specificity has been reported for Arabidopsis by Mesken and van der Veen (1968), who found that, compared with X-rays, EMS induced 4 times as many embryonic chlorophyll mutants at a given level of M_1 sterility (percentage of non-fertilized ovules on M_1 plants among all ovules). Hussein (1968) found the same ratio for flowering-time mutants. Mutagen specificity for phenotypic groups has been reported elsewhere in Arabidopsis (Röbbelen, 1962; McKelvie, 1963; Jacobs, 1969; Dellaert, 1981) and in other plant species. (For review see Dellaert, 1980a.)

This implies that the spectra of genetic damage caused by the mutagens compared are different and that mutational events leading to the particular types of genetic damage are different.

As has been argued by Dellaert (1980c), a possible explanation is that, with irradiation, the ratio "strand breaks"/"base damage" decreases in going from M_1 sterility via embryonic lethality to M_2 viable mutants. Since EMS is known to induce

relatively few "strand breaks" as compared with irradiation, the lower ratio EMS/X-rays for M_1 sterility than for chlorophyll mutants can be explained on this basis.

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CHAPTER 6

THE TRISOMICS OF *ARABIDOPSIS THALIANA* (L.) HEYNH. AND THE LOCATION OF LINKAGE GROUPS

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Submitted for publication

SUMMARY

The 5 primary trisomics and 4 out of the 10 possible telotrisomics were isolated in *Arabidopsis thaliana* (L.) Heynh. (2n = 10) var. Landsberg *"erecta"*. Identification was on the basis of meiotic chromosome counts and the characteristic segregation ratio's of specific linkage group markers with the corresponding trisomics. By means of trisomic analysis and additional linkage data, Rédei's 6 linkage groups and McKelvie's 4 linkage groups could be assigned to the 5 different chromosomes. These results are summarized in Table 4.

Female transmission of the different trisomics ranged from 16% to 30% and male transmission from 0 % to 32%. The higher male transmission rates were found among the telotrisomics.

The *er/er* genotype is found to have a pronounced adverse effect on female fertility of the trisomics.

INTRODUCTION

The selffertilizing cruciferous plant *Arabidopsis thaliana* (L.) Heynh. (2n = 10) has been widely used for genetical and physiological studies, favourable properties for experimentation being its small size, its very short generation interval and its high reproductive rate. Yet, its gene map is rather fragmentary so far, and the assignment of linkage groups to chromosomes is subject of contradictory conclusions.

Rédei (1965) presented six linkage groups (1 to 6) - each with one or two representative markers -, which by means of trisomic analysis were assigned to the five chromosomes as follows: 1, 2, 3, 4 + 6 and 5 (Lee-Chen & Steinitz-Sears, 1967; Sears & Lee-Chen,1970).

Independently, McKelvie (1965) had found four linkage groups (1 to 4), of which group 1 contained 15 markers, the other groups 2 to 4 markers each. By trisomic analysis Lee-Chen & Bürger (1967) found McKelvie's groups 2 and 3 to be on the same chromosomes as Rédei's groups 3 and 5,respectively.

Fischerová (1975) inferred Rédei's group 5 and 6 to be on the same chromosome, for the representative markers *lu* (group 5) and *ve-2* (group 6) were both linked to her chlorophyll markers M 4-6-18 (*alb-2*) and *ch-42*, which linkage data were in part erroneous (see later).

Feenstra (1978) showed by linkage analysis with intercalated markers that Rédei's group 1 and 4 should be assigned to the same chromosome.

So far trisomic sets of *Arabidopsis thaliana* have been developed at Columbia, Missouri (Steinitz-Sears, 1963; Lee-Chen & Steinitz-Sears, 1967; Sears & Lee-Chen, 1970), at Göttingen (Röbbelen & Kribben,1966) and later by the present authors at Wageningen (Koornneef & van der

Veen, 1978). The latter set is in the background of Landsberg *"erecta"* and also includes a number of telotrisomics. In the present paper the 5 primary trisomics and 4 telotrisomics are described with respect to morphology, male and female transmission, fertility and association with representative markers.

In the meantime localization studies with the help of these trisomics served as a starting point of an extensive linkage mapping program (Koornneef et al., 1980; Koornneef et al., submitted). Some preliminary results of the trisomic analysis were published by Koornneef & van der Veen (1978); Koornneef & den Besten (1979) and Koornneef & van der Beek (1979). Also segregation data for both telotrisomics of chromosome 1 were published in relation to centromere localization (Koornneef, submitted).

MATERIAL AND METHODS

Upon colchicine treatment of the diploid pure line Landsberg *"erecta"* a homogenous and very stable tetraploid line was obtained (Balkema, 1971; van der Veen & Blankestijn-de Vries, 1973). The triploid (from 4n x 2n) was pollinated by the diploid, and in the progeny a considerable number of suspected aneuploids were both selfed and pollinated by the diploid. In several of the progeny pairs, only one typical deviant type (on the basis of overall-morphology) was observed to segregate along with the normal diploids. By continued comparisons in subsequent generations, 6 morphologically distinct presumed trisomics were isolated. Later on, two of these (to be called Tr1A and Tr1B; see later) were found to segregate in low frequency the same very weak deviant type (to be called Tr1; see later), which type had already been observed in the 3n x 2n progeny but could not be maintained then

due to its high sterility. Finally,two other presumed trisomics,viz. Tr3 and Tr5 were found to segregate a very rare deviant type, each with intermediate habitus between its trisomic parent and the diploid. These types will be called Tr3A and Tr5A.

All 9 types to be designated as trisomics in the following can be distinguished on the basis of their typical morphology, the most conspicuous aspects of which are listed in Table 1.

Table 1. Characteristic morphological traits of *Arabidopsis* trisomics compared with the diploid line of origin (Landsberg *"erecta"*).

Tr1	Small rosette, which soon becomes necrotic, fragile plant with irregular flowers, highly sterile.
Tr1A	Dark green leaves, slightly lozenge shaped, semi-dwarf, irregular petals.
Tr1B	Small rosette leaves, which especially in winter soon become necrotic, slender plant type.
Tr2	Rosette leaves more round and often slightly bent, flowers a few days later.
Tr3	Narrow and greyish green rosette leaves, irregular petals, highly reduced fertility.
Tr3A	Like Tr3, but less extreme and much more fertile.
Tr4	Slightly smaller flatter rosette, flower buds thickened which makes the top of the influorescence seem flatter.
Tr5	Narrower leaves, semi-dwarf, stigmata remain pronounced on siliquae after flowering, rather sterile.
Tr5A	Like Tr5, but less extreme, leaf margins more serrated and fertility much better than Tr5.

To study marker association, the usual procedure was followed: in the F_1 from trisomic (*AAA*) x diploid (*aa*), the trisomic progeny (*AAa*) was selected and selfed. In F_2, marker segregation was scored in trisomics and diploids separately, or jointly when the contrast trisomic versus diploid can not be scored (e.g. with lethal chlorophyll markers, and with some seedling markers). For primary trisomics, association gives a 1:0 segregation among trisomics and an 8:1 segregation for diploids in F_2. With 25% trisomics a total segregation of 11:1 is expected. For telotrisomics the critical F_2 ratio's are 1:0 among trisomics, and among diploids 3:1 to approximately 7:1 depending on the locus involved being close to or far from the centromere (Koornneef, submitted).

The origin and description of the markers used is given in Table 2. All are monogenic recessives.

Recombination fractions between markers were obtained from F_2 data (when necessary combined with F_3 progeny testing) by means of the appropriate maximum likelihood procedures.

Meiotic chromosomes were counted according to Sree-Ramulu & Sybenga (1980): It should be noted that in general the very small chromosomes do not allow individual distinction.

Pollen was stained with Alexander's stain (Alexander,1969).Female fertility was expressed as percentage of fertlized ovules among all ovules as determined with Müller's embryo test (Müller,1963).

RESULTS AND DISCUSSION

Identification of trisomics

The presumed trisomics were selected on the basis of their typical habitus (see MATERIAL AND METHODS). Their segregation into trisomics

Table 2. Genetic markers used for the location of linkage groups

Gene Symbol	Source (obtained via)	Linkage group accordance to: Rédei (1965)	McKelvie (1965)	Name/description
alb-2	Relichová[1]			Albina (white embryos and seedlings; lethal)
an	Rédei (via Feenstra)[2]	1		Angustifolia (narrow leaves)
ap-1	Present authors[3]		1	Apetala (petals absent or rudimentary)
cer-2	Present authors[4]	6		Eceriferum (bright green stem and siliquae,wa
ch-1	Rédei (via Feenstra)[2]	4		Chlorina (green yellow)
ch-6	Relichová[1]			Chlorina (yellow green)
ch-42	Relichová[1]			Chlorina (yellow green)
dis-2	Feenstra[5]			Distorted trichomes
gl-1	Rédei (via Feenstra)[2]	3		Glabrous (trichomes on leaves absent)
le	McKelvie[3]		4	Lepida (dwarf with round dark leaves)
lu	Rédei (via Relichová)	5		Lutescent (yellow green)
py	Feenstra	2		2,5-dimethyl, 4-amino pyrimidine requiring
tt-3	Present authors			Transparent testa, anthocyaninless
tz	Feenstra	5		5-β-hydroxyethyl-4-methyl-thiazole requiring

[1]Fischervoa '1975); Relichová (1976); *alb-2* and *ch-6* are denoted as M 4-6-18 and M33 respectively by these authors.

[2]Described e.g. in Lee-Chen & Steinitz-Sears (1967).

[3]Our *ap-1* is allelic to McKelvie's *ap* (McKelvie, 1965) and was obtained via Röbbelen via Gotoh, who also provided *le*.

[4]Our *cer-2* is allelic to Rédei's *vc-2* (obtained via Relichová).

[5]Described in Feenstra (1978).

and normals upon selfing and pollination with diploids and the segregation ratio's of associated markers (see later) provides strong evidence for the trisomic nature of the types selected. Confirmation came from meiotic observations. In metaphase I 4 bivalents and a trivalent or 5 bivalents and an univalent were found, while in metaphase II and anaphase II groups of 5 and 6 chromosomes were observed in sister cells. The small size of *Arabidopsis* chromosomes does not permit individual distinction of the chromosomes at the meiotic stages studied and only rarely can telocentric chromosomes be distinguished from the normal metacentric chromosomes (Koornneef,submitted). So far, no satisfactory meiotic preparations could be made for the types Tr1 and Tr5A.

Marker association

Table 3 gives the segregation in F_2's derived from crosses of trisomics x diploid marker lines. It is seen that for each trisomic type association with at least one specific marker has been found and conversely that each of the specific markers (except *alb-2*) is associated with at least one trisomic type. Markers associated with two trisomic types are *dis-2* (Tr1 and Tr1A), *ch-1* (Tr1 and Tr1B), *ch-6* (Tr3 and Tr3A), *tt-3* and *tz* (Tr5 and Tr5A). Other markers were carried only by the first of these pairs of trisomics. This can be explained only by assuming that the trisomic types Tr1A, Tr1B, Tr3A and Tr5A have only part of the corresponding chromosome (as indicated by the notation) additional to the diploid complement. In these cases the extra chromosome most probably is a telocentric chromosome. Such telocentrics are known to arise from centric split of univalents, which frequently occur in aneuploids. Evidence for the telotrisomic nature of Tr1A and Tr1B is obtained from the close-

Table 3. Segregation ratio's (dominant:recessive) in F_2's from trisomic x diploid markers

Marker	Linkage group	Tr1	Tr1A	Tr1B	Tr2	Tr3	Tr3A	Tr4	Tr5	Tr5A
an	1	-	232:7	80:34	21:7	15:2	-	23:10	184:60	-
			697:105	215:69	86:36	58:12		87:14		
dis-2	1+4	71:0	219:0	210:64	-	-	-	-	-	-
		214:23	472:102	404:108						
ch-1	4	71:0	80:22	392:0	85:29	97:17	-	66:21	-	-
		216:21	160:46	576:191						
py	2	-	106:35	97:39	113:9	42:13	-	102:35	194:50	-
						160:58				
gl-1	3	-	114:25	92:42	110:30	55:0	80:33	78:23	177:65	-
						191:27	98:40			
ch-6	3	-	-	-	-	71:0	37:0	-	-	-
						280:45	46:6			
cer-2	6	-	25:10	30:17	22:7	14:4	-	122:1	14:4	-
			59:23	52:13	82:21	93:28		150:24	52:30	
ch-42	6[2]	-	364:125	289-97	275:96	-	-	691:58	-	-
lu	5	-	214:58	113:27	112:29	115:29	-	118:34	182:26	39:13
										97:20
tz	5	-	149:46	117:47	87:39	93:32	-	90:39	272:18	171:29
tt-3	5[2]	-	-	-	-	-	-	-	29:0	63:0
									76:9	97:23
alb-2	5[2]	-	364:125	286:97	275:96	-	-	546:183	-	

- Not tested

underlined $\chi^2(3:1) > 6.6 \rightarrow P < 0.01$

1) Ratio's are given for the trisomic (topline) and diploid progeny (bottom) separately or for the combined trisomic + diploid progeny (one ratio per trisomic/marker combination)

2) As inferred from the present experiments and linkage analysis (Koornneef & Feenstra, submitted)

ly linked (4.3 cM; Koornneef et al., submitted) markers *tt-1* (transparent testa ; not in Table 3) and *ch-1*, which are specifically associated with respectively Tr1A and Tr1B (Koornneef, submitted). These markers show the typical telotrisomic ratio's (1:0 among trisomics and 3:1 for disomic offspring) for loci close to the centromere (see *ch-1* on Tr1B in Table 3). Similarly, *tt-3* (Tr5A) shows this segregation pattern. Also *dis-2* (Tr1A) is not far from the centromere, although the ratio among disomics significantly deviates from 3:1. This, however, may at least be partly due to a more general recessive deficit for *dis-2* (see *dis-2* with Tr1B). Such a deficit is by no means uncommon among induced mutants.

The specific relation between the telotrisomics and their corresponding primary trisomics is confirmed by the occurrence (in low frequency) of these trisomics in the progeny of the telotrisomics, both upon selfing and crossing.

Sears & Lee-Chen (1970) found their telotrisomic Nc to be associated with *tz* and not with *lu*. Therefore Nc can probably be equated with our Tr5A (Table 3).

Location of linkage groups

It can be concluded that Rédei's 6 linkage groups correspond to the 5 *Arabidopsis* chromosomes, group 1 (marker *an*) and 4 (marker *ch-1*) being on the same chromosome, though on different arms. By themselves *an* and *ch-1* are found to segregate independently, i.e. are far apart, but on the basis of linkage analyses with several intercalated markers (Feenstra, 1978; Koornneef et al., submitted), they were found to belong to a joint linkage group. This is in accordance with the trisomic analysis. Rédei's groups 2, 3 and 5 are associated with our Tr2, Tr3 and Tr5 respectively. Finally, his linkage group 6 is also on a separate

chromosome (Tr4).

Sears & Lee-Chen (1970) associated *an* with their "Fragilis" trisomic, whereas they found *ch-1* related to "Concave",the trisomic carrying linkage group 6 (marker *ve-2 = cer-2*) on its extra chromosome. The latter conclusion now turns out to be erroneous. It should be added that the authors (l.c.)state that the F_2 segregation ratio's of *ch-1* with "Concave" gave variable results. Moreover no direct tests of *ch-1* with "Fragilis" have been reported.

On the basis of our own trisomic analysis, the Missouri trisomic analysis, and the morphological resemblance inferred from the descriptions, our 5 primary trisomics can now be unambiguously equated, to the 5 Missouri primary trisomics (see Table 4).

Fischerová (1975) concluded that Rêdei's linkage groups 5(marker *lu*) and 6 (marker *cer-2*) were on the same chromosome as she found *lu* and *cer-2* both to be linked to the chlorophyll markers *alb-2* (M 4-6-18) and *ch-42*. However, only linkage of *lu* with *alb-2* and of *cer-2* with *ch-42* could be confirmed (Koornneef & den Besten,1979), which is in accordance with our trisomic analysis (Table 3).

Finally McKelvie's (1965) 4 linkage groups can be unambiguously located. Lee-Chen & Bürger (1967) already equated by trisomic analysis McKelvie's groups 2 and 3 with Rêdei's groups 3 and 5 respectively. The present authors found McKelvie's markers *ap-1* (his group 1) and *le* (his group 4) to be significantly linked ($r = 0.29 \pm 0.05$), and both to be linked to Rêdei's group 4 marker *ch-1*, viz. $r = 0.34 \pm 0.01$ for *ap-1* with *ch-1*, and $r = 0.07 \pm 0.03$ for *le* with *ch-1*.

Table 4. The chromosomes, trisomics and linkage groups of *Arabidopsis* with representative markers

Proposed chromosome code	Corresponding trisomic: Wageningen	Missouri	Corresponding linkage group: Rédei	McKelvie	Proposed representative markers*
1	Tr1	F(ragilis)	1 + 4	1 + 4	*an*, *ch-1*, *ap-1*
2	Tr2	R(ound)	2		*py*, *er*
3	Tr3	Y(ellow)	3	2	*hy-2*, *gl-1*, *cer-7*
4	Tr4	C(oncave)	6		*cer-2*, *ap-2*
5	Tr5	N(arrow)	5	3	*ttg*, *yi*

* Chosen because of their location on different parts of the chromosome (Koornneef & Feenstra, submitted) and their easy recognition in many different backgrounds, seedling markers underlined *hy-2;* long hypocotyl, *ttg;* hairless and yellow seeds, *yi;* yellow influorescence.

The transmission of trisomics

Data on female (2n+1 x 2n), male (2n x 2n+1) and combined (selfing) transmission were pooled from a number of experiments (Table 5). As found before in *Arabidopsis* (Sears & Lee-Chen,1970) and many other plant species (Khush, 1973),female transmission is well below 50%. One explanation for reduced transmission was given by Röbbelen & Kribben (1966) who found trisomics to be more frequent among the smaller seeds and the slow germinators. Probably trisomics are also more frequent among aborted embryo's. In this connection it is interesting to note that only with Tr3 and Tr5 which both have highly reduced fertility (Table 6), female transmission is significantly higher than combined transmission. Here all seeds obtained from crossing usually will be used, whilst the non-crossed siliquae jointly still procedure more (selfed) seeds than used for sowing. So in the latter group selection may unconsciously be against less well developed seeds, i.e. against trisomics. A second cause of reduced female transmission probably is univalent loss, especially with telotrisomics where the telocentric chromosome often is unpaired.

An important cause of reduced male transmission is certation: a slower pollen tube growth for unbalanced haploid genotypes. It is significant that the highest male transmission is found with Tr3A and Tr5A where , judging from their overall phenotypes, genomic unbalance is not very pronounced.

As expected, tetrasomics were only observed in progenies from selfing of trisomics with relatively high male transmission, i.e. in Tr3A, Tr4 and Tr5A. They were probably also observed in the progeny of Tr1A. All these tetrasomics were highly sterile and show the typical characteristics of the corresponding trisomics in a much more pronounced way.

Table 5. Transmission percentage of trisomics

Trisomic	♀ Transmission 2n+1 x 2n	♂ Transmission 2n x 2n+1	♀ + ♂ Transmission (selfing)
Tr1	22.4 ± 4.5	0.0	25.0 ± 2.4
Tr1A	28.2 ± 1.1	6.3 ± 1.3	26.5 ± 0.8
Tr1B	27.0 ± 1.0	6.9 ± 1.3	31.1 ± 0.8
Tr2	15.7 ± 0.8	3.9 ± 1.1	22.5 ± 0.7
Tr3	21.3 ± 1.4	2.3 ± 0.6	15.5 ± 0.8
Tr3A	26.3 ± 2.3	32.0 ± 3.8	43.2 ± 1.5
Tr4	28.9 ± 1.1	15.5 ± 2.3	36.7 ± 0.8
Tr5	30.1 ± 2.0	0.0	21.7 ± 1.1
Tr5A	18.3 ± 2.4	27.7 ± 2.7	32.5 ± 1.3

The effect of the genetic background on the fertility of trisomics

The trisomics described were isolated in the genetic background of the *erecta* (*er/er*) mutant obtained by Rédei (1962) in a line selected from the *ecotype* Landsberg. The differences in morphology between trisomics possessing different *Er* alleles as observed in populations segregating for trisomics and this gene was sometimes large and unexpected. This was especially the case with Tr4, where with *er/er* the thickened flower buds are very pronounced, compared to *Er/.*. Although the *er* mutation does not markedly affect fertility of the diploids, it clearly reduces ovule fertility of the more sterile trisomics (Table 6).This makes trisomics like Tr1 and Tr3 difficult to handle in *er/er* background.

Table 6. Pollen stainability and ovule fertility of *Arabidopsis* trisomics as influenced by the *erecta* gene

Trisomic	Pollen stainability		Ovule fertility	
	Er/.	*er/er*	*Er/.*	*er/er*
diploid	99.4 ± 0.2	98.7 ± 0.3	97.2 ± 0.5	95.1 ± 0.7
Tr1	74.4 ± 2.2	79.2 ± 2.3	23.8 ± 2.6	3.8 ± 1.8
Tr1A	92.4 ± 1.2	91.4 ± 1.3	16.9 ± 2.1	13.2 ± 2.2
Tr2	91.4 ± 1.3	93.4 ± 1.1	51.7 ± 2.9	42.7 ± 3.0
Tr3	95.9 ± 1.1	94.1 ± 0.9	43.9 ± 2.9	20.9 ± 2.6
Tr3A	97.3 ± 0.7	94.9 ± 1.0	79.3 ± 2.2	63.4 ± 2.9
Tr4	95.8 ± 0.9	97.9 ± 0.6	92.8 ± 1.4	79.0 ± 2.3
Tr5	86.4 ± 1.5	77.7 ± 1.9	81.3 ± 2.3	43.6 ± 3.3
Tr5A	94.5 ± 1.0	94.4 ± 1.0	83.1 ± 2.0	72.9 ± 2.7

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CHAPTER 7

THE LINKAGE MAP OF *ARABIDOPSIS THALIANA* (L.) HEYNH.

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ABSTRACT: For *Arabidopsis thaliana* (L.) Heynh. (2n = 10), 76 loci have now been assigned to 5 linkage groups, corresponding to the 5 chromosomes. From a large number of estimated recombination percentages internally consistent linkage maps were constructed, ranging in genetic length from 51 cM (chromosome 2) to 123 cM (chromosome 1). Map lengths and centromere positions agree well with cytological observations of previous authors.

INTRODUCTION

Although the selffertilizing cruciferous plant *Arabidopsis thaliana* (L.) Heynh. (2n = 10) has been widely used in genetical and physiological studies[37], its gene map is rather fragmentary so far. Most of the information on linkage published till 1977 has been reviewed by Kranz and Scheidemann[24]. In the early studies, linkage analysis by Rédei[33,38] led to 6 linkage groups[35], which by trisomic analyses[26,44] could be assigned to the 5 chromosomes. Independently McKelvie[29] had published 4 linkage groups. So far only for the chromosomes 1[9],2[34,38], 4[20] and McKelvie's linkage group 1[29], a limited number of loci could be arranged into linear maps. With regard to the location of linkage groups, the sometimes conflicting conclusions in literature could be resolved on the basis of trisomic analysis by Koornneef and van der Veen[19].

The present paper compiles all linkage data published in literature and adds a considerable amount of new information. This leads to the construction of internally consistent linkage maps for all 5 chromosomes, comprising in total 76 loci.

Previously, centromeres have been localized on the basis of double reduction in primary trisomics[44] (chrom. 2) and by means of telotrisomics (chrom. 1 and 5)[15,44]. Here we give additional data from telotrisomic analyses for chromosome 3 and 5.

MATERIAL AND METHODS

Mutants

The mutants used in this study are listed and described in Table 1. Almost all have been induced in the genetic background of the *erecta* mutant of the ecotype "Landsberg"[33]. All markers were incorporated into our experiments, except *Gf* and *sul* (data from literature only). Where Table 1 lists two sources, the mutants have proved allelic, for the thiamine auxotrophic *th-1* isolated by Feenstra[7], however, allelism to similar mutants described by Li and Rédei[27] is assumed on the basis of identical nutritional requirements and comparable linkage positions. Gene symbols refer to the main characteristic of the mutant allele. Distinct numbers indicate different loci as shown by complementation tests. Mutants at loci with a similar symbol but distinct numbers do not necessarily resemble each other in all characteristics (see e.g. *cer-1*, *cer-2* and *tt-1*, *tt-3*).

The conditions of culture were as published previously for the laboratories at Groningen[32] and Wageningen[18,21,23]. The specific requirements (e.g. gibberellin, thiamine) are described in the references of Table 1.

Estimation of recombination fractions and map distances

Most crosses involved three or more markers and two given markers often segregated in different crosses. In the latter case recombination percentages were estimated from pooled F_2 frequencies separately for coupling and repulsion phase. Because of this the size of the populations varied considerably (from a few hundred upto a few thousand plants) depending on the marker combination.

Table I. Genetic markers of *Arabidopsis thaliana*

Symbol	Mutant code	Name	Phenotype	Source/Reference	
aba		Abscisic acid deficient	Symptoms of ABA deficiency and little or no endogenous ABA	W	23
ag		Agamous	Double flowering	W	4
alb-1		Albina	White embryo's, resp. white seedlings, lethal	W	46
alb-1^{v}	V157		Light green-yellow, viable, allelic to *alb-1*	Go	42
alb-2	M 4-6-18	Albina	Like *alb-1*	B	10,40
an		Angustifolia	Narrow leaves and slightly crinkled siliquae	C	26
ap-1		Apetala	No petals or rudimentary petals	Ca,W	21, 28
ap-2		Apetala	Reduced petals, large sepals	W	20
as		Asymmetric leaves	Asymmetric and lobed leaves	C	34
bp		Brevipedicellus	Very short pedicels, siliquae bend downwards	W	
cer-1	F_4	Eceriferum	Bright green siliquae and stem due to a deviating wax layer, semi-sterile	W	6
cer-2	G_5=*vc-2*	Eceriferum	Like *cer-1*, but with normal fertility	C,W	6
cer-3	F_5	Eceriferum	Like *cer-1*	W	6
cer-4	G_7	Eceriferum	Like *cer-2*	W	6
cer-5	D_3	Eceriferum	Like *cer-2*	W	6
cer-6	G_2	Eceriferum	Like *cer-1*	W	6
cer-7	G_3	Eceriferum	Like *cer-2*	W	6
cer-8		Eceriferum	Like *cer-1*, but slightly less bright green	W	
ch-1		Chlorina	Green yellow	C	11
ch-5		Chlorina	Green yellow	W	
ch-6	M33	Chlorina	Paler green embryo's and green-yellow plants	B	10,40
ch-42		Chlorina	Paler green embryo's and pale yellow-green seedlings, lethal	B	10,40
chl-1	B-1	Chlorate resistance	Chlorate resistance due to a reduced uptake of chlorate	G	32
chl-2	B2-1	Chlorate resistance	Chlorate resistance due to a decreased nitrate reductase activity	G	3, 32
chl-3	B29	Chlorate resistance	Like *chl-2*	G	3
clv-1		Clavata	Club like pods	W	
clv-2		Clavata	Like *clv-1*, but less pronounced	W	
cnx	B73	Cofactor of NR and XDH	Involved in the molybdenum-containing cofactor of nitrate-reductase and xanthine dehydrogenase	G	3
cp-1		Compacta	Compact semi-dwarf	W	
cp-2		Compacta	Compact semi-dwarf	W	
cp-3		Compacta	Compact semi-dwarf with round dark green leaves	W	20
dis-1		Distorted trichomes	Trichomes (hairs) on stem and leaves are short,bent and more or less club-like	G	9
dis-2		Distorted trichomes	Like *dis-1*	G	9
dw-1		Dwarf	Very short dwarf, heterozygote intermediate	W	
er		Erecta	Compact inflorescence, more blunt fruits	C	33
f_b		Late flowering	Flowers later than wild type and has proportionally more rosette leaves	W	
f_{ga}		Late flowering	Like f_b	W	12
f_e		Late flowering	Like f_b	W	
f_g		Late flowering	Like f_b with heterozygote intermediate	W	
f_t		Late flowering	Like f_b	W	
f_y		Late flowering	Like f_b	W	

Table I. continued

Symbol	Mutant code	Name	Phenotype	Source/Reference	
ga-1		Gibberellin requiring	Gibberellin responsive dwarfs (many alleles also require GA for germination)	W	18
ga-2		Gibberellin requiring	Like *ga-1*	W	18
ga-3		Gibberellin requiring	Like *ga-1*	W	18
ga-4		Gibberellin requiring	Gibberellin responsive dwarf	W	18
ga-5		Gibberellin requiring	Like *ga-4*	W	18
Cf		Female gametophyt factor	No female transmission, relatively fair male transmission	C	34
gl-1		Glabra	Trichomes absent on leaf surface and stems	C,W	21, 26
gl-2		Glabra	Trichomes rudimentary on first two leaves and reduced in number on higher leaves	W	21
gl-3		Glabra	Trichomes unbranched and reduced in number	W	21
hy-1		Long hypocotyl	Elongated hypocotyl, yellow green, altered light inhibition spectrum	C,W	22, 34,38
hy-2		Long hypocotyl	Like *hy-1* but more normal green	W	22
hy-3		Long hypocotyl	Like *hy-2*	W	22
hy-4		Long hypocotyl	Only slightly elongated hypocotyl also with altered light inhibition spectrum	W	22
hy-5		Long hypocotyl	Like *hy-4*	W	22
im	V52	Immutans	White, green variegation	C,Go	36, 41
le		Lepida	Dwarf with round leaves and small pods	Ca	28
lu		Lutescens	Yellow green	C	26
min		Miniature	Small plant with greyish green leaves	W	
ms-1		Male sterile	Male sterile because pollen delays and anthers fail to open	W	45
pi		Pistillata	Anthers and petals absent	W	
py		Pyrimidine requiring	Leaves except cotyledons white, lethal, 2,5-dimethyl-4-aminopyrimidine and thiamine restores to normal	C,W	27
rgn	B25	Regulating nitrate reductase	Decreased nitrate-reductase activity, chlorate-resistant	G	1
sul		Sulfurata	Bright yellow green	C	33, 36
Su/su		Suppressor *rgn*	Suppresses the effect of *rgn*	G	2
th-1		Thiamine requiring	Leaves except cotyledons white-yellow, lethal, thiamine restores	C,W	27
th-2	1018/6	Thiamine requiring	Variegated, thiamine restores to normal	Can	27, 37
th-3	V345	Thiamine requiring	Slightly pale green/green variegated, thiamine restores to normal	W	17
tt-1		Transparent testa	Yellow seeds, due to a transparent testa	W	14
tt-2		Transparent testa	Like *tt-1*	W	14
tt-3		Transparent testa	Like *tt-1* and with anthocyaninless leaves and stems	W	14
tt-4		Transparent testa	Like *tt-3*	W	14
tt-5	A11	Transparent testa	Like *tt-3* and brighter green	W	6, 14
tt-6		Transparent testa	Brownish yellow seeds and reduced anthocyanin content in leaves	W	
ttg		Transparent testa, glabra	Like *tt-3*, hairless and deviating seedcoatstructure	W	14
ta		Thiazole requiring	Leaves, except cotyledons, white, 4-methyl-5-thiazole ethanol and thiamine restores to normal	C,W	27,8
yi		Yellow inflorescence	Yellowish flower buds and yellow greyish sharper leaves	W	

Br Brno,Chzechoslowakia (Relichová)
Ca Cambridge, U.K. (McKelvie); via Gotôh
Can Canberra, Australia (Langridge); via Ledoux
C Columbia, Missouri, U.S.A. (Rédei); *hy-1*, *ae*, *lu* and *va-2 (=aep-2)* via Relichová
G Groningen, The Netherlands (Braaksma and Feenstra)
Go Göttingen, Federal Rep. of Germany (Röbbelen);via Kranz
W Wageningen, The Netherlands (Dellaert, Koornneef and van der Veen); *py*, *th-1* and *ta* induced by Feenstra

Where F_2 scoring was not unambiguous (e.g. for *hy-4, hy-5, cnx, rgn)* large scale F_3 progeny testing was done as a check. F_3 progeny testing was also applied for additional assessment of F_2 genotypes, when this was easy or desirable. For chlorophyll markers with embryonic expression (e.g. *alb-1, alb-2, ch-6, ch-42*), F_2 genotypes *A/A* vs. *A/a* can be identified by opening their siliquae just before seed ripening (Müller's embryo test[30]). E.g. when double recessive recombinants (*a/a, b/b*) are absent (in repulsion phase F_2's with close linkage), F_3 progeny testing of *a/a, B/.* and/or *A/., b/b* phenotypes becomes desirable. From a random sample of such phenotypes their ratio b (frequency segregating lines (*a/a, B/b*) among all lines (*a/a, B/.*) (n) tested) is used to estimate

$$r = \frac{b}{2-b} \quad \text{and} \quad s_r = \frac{2\sqrt{b(1-b)}}{(2-b)^2\sqrt{n}}$$

Recombination fractions were estimated by the appropriate maximum likelihood procedures, that were not based on the expected frequencies (e.g 3:1 for F_2's) but in all cases on the observed frequencies.

This procedure was applied assuming certation (mutant pollen attributes less to fertilization than non-mutant pollen) as the only cause of the regularly observed recessive deficits[5]. On the diploid level all mutants studied have a rather normal viability or are lethals under our conditions. Not correcting for disturbed segregation would lead to underestimation of r and s_r. The Product Ratio Method is more robust, but here also close linkage gives considerable bias when certation is involved. With 3 F_2 classes (no double recessives and no additional assessment of F_2 genotypes by progeny testing) the estimate of r becomes 0.0 with variance $1/n$ (n = total number of F_2 plants).

A method to estimate simultaneously the map distances from all available recombination percentages has been developed by Jensen and Jörgensen[13]. The estimates of recombination percentages are first corrected for double cross-overs by the Kosambi mapping function, converting them to map distances (D) in centimorgans (cM):

$D = 25\ ln\ (\frac{100+2r}{100-2r})$ with standard deviation:

$S_D = \frac{2500\ s_r}{2500-r^2}$, where s_r is the standard deviation of the estimated recombination percentage (r).

When large discrepancies occur between the available map distance (D) and the final estimate (E) obtained from all available data, very deviant distances should be omitted. This was done with the data with the highest value of $\chi^2 = \frac{(D-E)^2}{S_D^2}$. The map was then recalculated with all data, except the one omitted etc., until no χ^2 values > 6.6 ($P < 0.01$) remained.

To apply the map estimation procedure, the order of the loci should be known. This order was determined from the estimated recombination percentages and from the presence or absence of joint recombination in multiple heterozygotes.

Centromere localization with telotrisomics and the characteristics of *Arabidopsis* telotrisomics have been described elsewere[15,19].

RESULTS AND DISCUSSION

Tables II till VI give the complete sets of recombination percentages for the 5 chromosomes. The genes are arranged in the final map order (Fig. 1).

Table II. Estimates of recombination percentages between 23 markers of chromosome 1

Markers	Method	Recombination %	Markers	Method	Recombination %	Markers	Method	Recombination %
an/pgn	R3	0.6 ± 0.3	*chl-1/th-1*	RT	18.9 ± 1.5	*dis-2/ap-1*	C2	37.8 ± 3.4
an/cer-1	R2	0.0 ± 3.1	*chl-1/th-1*	R2	25.5 ± 6.4	*dis-2/ap-1*	R2	46.5 ± 4.0
an/cer-1	R3	0.8 ± 0.6	*chl-1/dis-2*	R2	34.4 ± 4.2	*chl-3/ch-1*	RT	6.2 ± 1.5
an/alb-1	R2,3	6.8 ± 1.4	*dis-1/ga-1*	C2	4.1 ± 0.6	*tt-1/ch-1*	R2	0.0 ± 6.1
an/alb-1^{p}	C2	11.6 ± 2.9	*dis-1/ga-1*	R2	8.3 ± 3.4	*tt-1/ch-1*	R2,3	5.9 ± 3.4
an/alb-1^{p}	R2	12.6 ± 0.6** 42	*dis-1/du-1*	R2,3	5.5 ± 1.7	*ch-1/le*	R2,3	7.2 ± 3.3
an/alb-1^{p}	R2	0.0 ± 5.1	*dis-1/th-1*	R2	15.8 ± 2.8	*ch-1/clv-2*	R2	25.7 ± 3.6
an/chl-1	RT	11.0 ± 1.6	*dis-1/f*$_{b}$	C2	14.9 ± 1.8	*ch-1/f*$_{t}$	R2	29.5 ± 4.7
an/chl-1	R2	13.2 ± 3.1	*dis-1/f*$_{b}$	R2	21.9 ± 5.2	*ch-1/ap-1*	C2	34.2 ± 1.0
an/dis-1	CT	12.5 ± 1.3**	*dis-1/dis-2*	RT	22.4 ± 3.7	*ch-1/ap-1*	R2	36.1 ± 3.4
an/dis-1	C2	18.9 ± 0.6	*dis-1/tt-1*	R2	33.5 ± 2.3	*ch-1/clv-1*	R2	40.7 ± 2.1
an/dis-1	RT	10.0 ± 2.2**	*dis-1/ch-1*	RT	38.9 ± 2.9	*ch-1/ga-2*	R2	44.6 ± 2.3
an/ga-4	C2	19.5 ± 1.4	*ga-4/th-1*	R2	10.7 ± 3.2	*ch-1/gl-2*	C2	45.2 ± 1.4
an/ga-4	R2	30.4 ± 4.6	*ga-4/f*$_{b}$	C2	10.9 ± 1.6	*ch-1/gl-2*	R2	46.3 ± 4.4
an/du-1	R2,3	19.3 ± 2.8	*ga-4/f*$_{b}$	R2	13.7 ± 5.5	*ch-1/f*$_{e}$	R2,3	38.7 ± 5.9
an/th-1		21.5 ± 4.0 39	*ga-4/dis-2*	C2	21.0 ± 1.5	*le/ap-1*	R2,3	28.8 ± 5.4
an/th-1	R2	27.4 ± 2.4	*ga-4/dis-2*	R2	26.2 ± 4.5	*le/gl-2*	R2,3	40.5 ± 5.5
an/f$_{b}$	C2	28.4 ± 2.6	*ga-4/tt-1*	C2	31.4 ± 1.9	*clv-2/ap-1*	R2	13.7 ± 3.4
an/f$_{b}$	R2	27.3 ± 5.0	*ga-4/tt-1*	R2	26.5 ± 2.3	*clv-2/gl-2*	R2	25.8 ± 4.4
an/dis-2	RT	31.8 ± 3.3	*ga-4/ch-1*	R2	29.3 ± 4.3	*f*$_{t}$*/ap-1*	R2	13.2 ± 5.1
an/dis-2	C2	44.7 ± 2.7	*ga-4/cer-6*	R2	42.5 ± 3.9	*f*$_{t}$*/gl-2*	R2	29.0 ± 4.5
an/tt-1	R2	38.2 ± 2.5	*th-1/f*$_{b}$	R2	0.0 ± 4.3	*cer-6/ap-1*	R2,3	2.3 ± 3.8
pgn/chl-1	R3	18.3 ± 2.9	*th-1/f*$_{b}$	R3	0.3 ± 0.3	*cer-6/clv-1*	R2,3	14.8 ± 5.6
cer-1/dis-1	C2	19.7 ± 2.8	*th-1/dis-2*	R2	0.0 ± 4.3**	*ap-1/clv-1*	C2	10.8 ± 0.7
cer-1/dis-1	R2	17.4 ± 3.2	*th-1/tt-1*	C2	20.6 ± 1.4	*ap-1/clv-1*	R2	11.0 ± 2.8
cer-1/dis-1	R3	10.7 ± 2.2**	*th-1/tt-1*	R2	19.9 ± 6.4	*ap-1/ga-2*	C2	18.3 ± 2.4
cer-1,ga-4	R2	27.8 ± 5.1	*f*$_{b}$*/tt-1*	R2	23.3 ± 4.2	*ap-1/ga-2*	R2	19.1 ± 2.6
alb-1/dis-1	R2,3	10.6 ± 1.6	*dis-2/chl-3*	RT	15.6 ± 2.3**	*ap-1/gl-2*	C2	18.7 ± 0.9
alb-1^{p}*/dis-1*	C2	10.7 ± 1.4	*dis-2/tt-1*	C2	7.9 ± 3.5	*ap-1/gl-2*	R2	17.3 ± 5.4
alb-1^{p}*/dis-1*	R2	0.0 ± 5.1	*dis-2/tt-1*	C2,3	7.2 ± 3.3	*ap-1/f*$_{e}$	R2,3	23.8 ± 6.0
alb-1^{p}*/dis-1*	R3	9.1 ± 4.1	*dis-2/ch-1*	CT	16.9 ± 2.4	*clv-1/ga-2*	C2	9.0 ± 0.9
alb-1^{p}*/ga-4*	R2	0.0 ± 5.4**	*dis-2/ch-1*	RT	16.5 ± 2.5	*clv-1/ga-2*	R2	10.0 ± 5.2
alb-1^{p}*/tt-1*	R2	48.1 ± 4.4	*dis-2/ch-1*	C2	12.1 ± 1.0	*clv-1/gl-2*	R2	6.2 ± 3.5
chl-1,dis-1	RT	2.7 ± 0.8	*dis-2/ch-1*	R2	12.8 ± 4.2	*ga-2/gl-2*	R2	0.0 ± 3.2
chl-1/dis-1	R2	0.0 ± 4.2	*dis-2/ch-1*	R2	24.4 ± 4.7	*gl-2/f*$_{e}$	R2,3	0.0 ± 6.1
			dis-2/clv-1	R2	29.0 ± 5.4			

CT, RT: Results of test crosses in coupling resp repulsion phase; C2, R2: Results of F_2's in coupling resp. repulsion phase; C2,3, R2,3: Results of F_2's combined with partial F_3 progeny testing or with recognizable heterozygotes; R3: F_3 line progeny testing of specific F_2 phenotypes.

**Data omitted from the final analysis

Table III. Estimates of recombination percentages between 10 markers of chromosome 2

Markers	Method*	Recombination %	Markers	Method*	Recombination %	Markers	Method*	Recombination %
gf/py	RT	20.7 ± 1.3 [34]	*cp-2/as*	C2	26.9 ± 2.0	*hy-1/py*	R2	5.0 ± 1.3 [34]
hy-3/cp-2	R2	0.0 ± 5.3	*cp-2/as*	R2	20.2 ± 9.0	*hy-1/as*	C2	17.9 ± 1.0
hy-3/er	C2	13.6 ± 1.4	*cp-2/cer-8*	R2	40.5 ± 4.5	*hy-1/as*	R2	0.0 ± 5.3**
hy-3/py	C2	18.8 ± 1.4	*er/hy-1*	C2	9.7 ± 0.9	*hy-1/as*	R2	14.5 ± 0.8 [34]
hy-3/as	R2	30.2 ± 5.0	*er/hy-1*	R2	0.0 ± 0.8 [34]	*hy-1/cer-8*	C2	31.4 ± 2.7
chl-2/er	CT	11.9 ± 2.4	*er/py*	C2	4.8 ± 1.0	*hy-1/cer-8*	R2	21.4 ± 8.2
chl-2/er	C2	10.4 ± 2.9	*er/py*	R2	7.0 ± 1.4 [34]	*py/as*	R2	11.9 ± 5.3
chl-2/py	RT	13.6 ± 3.3	*er/as*	C2	17.4 ± 2.2	*py/as*	R2	9.3 ± 1.2 [34]
cp-2/er	C2	8.7 ± 1.4	*er/as*	R2	25.4 ± 2.4**	*py/cer-8*	R2	27.5 ± 8.8
cp-2/hy-1	R2	0.0 ± 4.6	*er/as*	R2	16.7 ± 1.1 [34]	*as/sul*	R2	9.6 ± 1.5 [34]
cp-2/py	R2	13.6 ± 5.6	*er/sul*	R2	24.0 ± 1.4 [34]	*as/cer-8*	R2	14.8 ± 4.6

* and ** see Table II.

Table IV. Estimates of recombination percentages between 6 markers of chromosome 3

Markers	Method*	Recombination %	Markers	Method*	Recombination %	Markers	Method*	Recombination %
hy-2/gl-1	C2	33.1 ± 1.0	*hy-2/tt-6*	R2	48.1 ± 3.5	*gl-1/cer-7*	C2	37.6 ± 3.5
hy-2/tt-5	C2	46.0 ± 3.6	*gl-1/tt-5*	C2	32.3 ± 3.0	*gl-1/cer-7*	R2	28.4 ± 5.5
hy-2/tt-5	R2	53.2 ± 2.6	*gl-1/tt-5*	R2	39.4 ± 3.0	*gl-1/tt-6*	R2	38.0 ± 3.8
hy-2/ch-6	C2,3	52.1 ± 3.0	*gl-1/ch-6*	C2,3	43.0 ± 3.0	*tt-5/ch-6*	R2,3	2.9 ± 0.8
hy-2/ch-6	R2	43.0 ± 4.0	*gl-1/ch-6*	R2	28.3 ± 4.3	*tt-5/cer-7*	R2	12.8 ± 5.6
hy-2/cer-7	C2	48.6 ± 4.1						
hy-2/cer-7	R2	51.0 ± 4.6						

* See Table II

Table V. Estimates of recombination percentages between 14 markers of chromosome 4

Markers	Method*	Recombination %	Markers	Method*	Recombination %	Markers	Method*	Recombination %
ga-1/bp	C2	10.3 ± 2.2	*f_{ca}/th-3*	R2	0.0 ± 4.9	*ag/cer-2*	R3	15.8 ± 9.0
ga-1/f_{ca}	C2	30.3 ± 1.9**	*f_{ca}/th-3*	R3	6.7 ± 3.0	*ag/ga-5*	C2	7.7 ± 1.0
ga-1/f_{ca}	R2	28.1 ± 3.4	*f_{ca}/ag*	C2	9.2 ± 0.8	*ag/cer-4*	C2	22.0 ± 5.0
ga-1/th-3	R2	13.5 ± 9.2	*f_{ca}/ag*	C2,3	15.2 ± 3.3	*ag/cer-4*	R2	12.0 ± 5.5
ga-1/ag	R2	29.6 ± 2.3	*f_{ca}/ag*	R2	10.3 ± 2.4	*ag/ap-2*	R2	22.7 ± 3.6
ga-1/ch-42	R2,3	27.6 ± 3.4	*f_{ca}/cer-2*	R2	16.4 ± 3.8	*ag/ap-2*	R2,3	25.1 ± 4.6
ga-1/cer-2	R2	33.5 ± 3.7	*f_{ca}/cer-2*	R2,3	24.6 ± 4.5	*ag/cp-3*	C2	30.9 ± 1.4
ga-1/ga-5	R3	30.7 ± 4.3	*f_{ca}/ga-5*	R2	23.4 ± 3.6	*ag/cp-3*	C2,3	30.5 ± 5.2
ga-1/cer-4	R2	50.0 ± 4.3	*f_{ca}/cer-4*	C2	27.1 ± 4.1	*ch-42/cer-2*	R2,3	10.0 ± 1.9
ga-1/ap-2	C2	45.1 ± 2.1	*f_{ca}/cer-4*	R2	19.8 ± 3.8	*ch-42/ap-2*	R2,3	17.8 ± 2.7
ga-1/ap-2	R2	48.1 ± 4.7	*f_{ca}/ap-2*	C2	28.3 ± 1.8	*im/cer-2*	R3	3.5 ± 1.3
hy-4/f_{ca}	C2,3	17.9 ± 6.4	*f_{ca}/ap-2*	R2	35.2 ± 2.5	*im/ap-2*	R3	18.7 ± 3.4
hy-4/th-3	RT	21.9 ± 3.4	*f_{ca}/cp-3*	C2	35.7 ± 1.5	*cer-2/ga-5*	R2	0.0 ± 4.4
hy-4/th-3	R2,3	16.3 ± 5.4	*f_{ca}/cp-3*	R2	37.5 ± 3.4	*cer-2/ga-5*	R3	0.8 ± 0.8
hy-4/ag	C2,3	27.7 ± 4.7	*th-3/ag*	C2	6.2 ± 2.4	*cer-2/ap-2*	CT	18.5 ± 3.2
hy-4/cer-2	RT	38.3 ± 4.0	*th-3/ag*	R2	0.0 ± 6.0	*cer-2/ap-2*	C2	16.6 ± 0.6
hy-4/cer-2	R2,3	35.9 ± 5.3	*th-3/ag*	R3	5.3 ± 2.6	*cer-2/ap-2*	C2,3	20.2 ± 4.2
hy-4/ap-2	RT	52.7 ± 4.1	*th-3/cer-2*	CT	24.7 ± 3.6**	*cer-2/ap-2*	R2	0.0 ± 5.6**
hy-4/ap-2	R2,3	43.8 ± 5.8	*th-3/cer-2*	C2,3	18.0 ± 4.0	*cer-2/ap-2*	R3	27.8 ± 8.4
bp/cer-2	R2	24.1 ± 7.0	*th-3/cer-2*	R2	7.6 ± 6.0	*cer-2/cp-3*	R2	24.0 ± 4.9
bp/ap-2	C2	42.2 ± 5.3	*th-3/ap-2*	CT	40.4 ± 4.1	*ga-5/ap-2*	C2	7.1 ± 3.5
bp/ap-2	R2	35.9 ± 3.9	*th-3/ap-2*	C2,3	34.1 ± 5.3	*ga-5/ap-2*	R2	12.6 ± 4.5
cp-1/ag	C2	10.1 ± 1.1	*th-3/ap-2*	R2	25.7 ± 5.4	*ga-5/ap-2*	R3	13.0 ± 3.3
cp-1/ag	C2,3	16.3 ± 1.4	*ag/ch-42*	R2,3	2.8 ± 1.8	*ga-5/cer-4*	R2	0.0 ± 12.8
cp-1/cer-2	R2	23.4 ± 3.8	*ag/cer-2*	R2	9.0 ± 5.3	*cer-4/ap-2*	R2	0.0 ± 5.3
cp-1/ap-2	R2	37.3 ± 3.4	*ag/cer-2*	R2,3	15.9 ± 3.6	*cer-4/cp-3*	R2	14.5 ± 4.0
						ap-2/cp-3	R2	11.2 ± 4.9

* and ** see Table II

Table VI. Estimates of recombination percentages between 22 markers of chromosome 5

Markers	Method*	Recombination %	Markers	Method*	Recombination %	Markers	Method*	Recombination %
f_y/*tt-4*	R2	25.9 ± 4.8	f_g/*tt-3*	R2,3	31.6 ± 5.8	*ttg/ch-5*	R2	0.0 ± 5.1
f_y/*ms-1*	C2	25.7 ± 3.2	f_g/*tz*	R2	46.5 ± 3.5	*ttg/ch-5*	R3	14.3 ± 6.3
f_y/*ms-1*	R2	13.6 ± 5.1	f_g/*cer-3*	R2,3	51.6 ± 3.0	*ttg/tz*	R2	45.0 ± 2.1
f_y/*tt-2*	R2	34.9 ± 4.5	f_g/*yi*	C2	43.5 ± 4.0	*ttg/cer-3*	R2	42.9 ± 2.4
f_y/*tz*	C2	53.1 ± 1.7	f_g/*yi*	R2	59.7 ± 4.1	*ttg/yi*	C2	42.5 ± 1.7
f_y/*cer-3*	R2	45.0 ± 3.8	*alb-2/ms-1*	R2,3	6.9 ± 1.6	*ttg/yi*	R2	46.5 ± 3.8
f_y/*aba*	R2	48.2 ± 4.1	*alb-2/ttg*	R2,3	11.6 ± 2.1	*ttg/aba*	R2	46.4 ± 4.6
hy-5/ms-1	C2	14.3 ± 1.8	*alb-2/ga-3*	R2,3	17.1 ± 2.3	*ga-3/ch-5*	R2	0.0 ± 5.1
hy-5/ms-1	C2,3	8.7 ± 2.8	*pi/ttg*	C2	10.4 ± 2.1	*ga-3/ch-5*	R3	11.1 ± 5.5
hy-5/ms-1	R2	11.1 ± 5.1	*pi/ttg*	R2	0.0 ± 6.2	*ga-3/tt-2*	R2	0.0 ± 5.2
hy-5/ttg	C2	18.7 ± 3.2	*pi/ttg*	R2,3	9.6 ± 3.8	*ga-3/gl-3*	C2	17.8 ± 1.9
hy-5/ttg	R2	11.7 ± 5.1	*pi/ga-3*	C2	15.0 ± 2.7	*ga-3/gl-3*	R2	25.0 ± 5.0
hy-5/ga-3	C2	20.7 ± 3.4	*pi/ga-3*	R2,3	12.0 ± 3.7	*ga-3/tt-3*	R2	16.5 ± 7.1
hy-5/th-2	C2,3	30.4 ± 3.5	*cmx/ttg*	RT	6.1 ± 1.9	*ga-3/tz*	C2	29.9 ± 2.2
hy-5/th-2	R2	31.2 ± 4.9	*ms-1/ttg*	CT	6.0 ± 0.8	*ga-3/tz*	R2	31.4 ± 4.7
hy-5/tt-3	R2,3	37.3 ± 5.5	*ms-1/ttg*	C2	5.8 ± 0.4	*tt-2/gl-3*	R2	0.0 ± 5.2
hy-5/yi	R2	51.0 ± 3.8	*ms-1/ttg*	R2	5.7 ± 2.8	*tt-2/tz*	C2	29.5 ± 3.1
lu/tt-4	R2,3	1.5 ± 1.7	*ms-1/ttg*	R3	6.8 ± 2.4	*tt-2/tz*	R2	32.2 ± 4.7
lu/f_g	C2	5.1 ± 1.0	*ms-1/ga-3*	C2	9.7 ± 1.1	*th-2/tt-3*	R2,3	17.1 ± 4.0
lu/f_g	R2	3.6 ± 1.3	*ms-1/ga-3*	R2	12.9 ± 4.1	*th-2/cer-3*	R2	40.2 ± 5.6
lu/alb-2	C2,3	4.4 ± 1.1	*ms-1/ga-3*	R3	7.7 ± 3.4	*th-2/aba*	R2	44.8 ± 5.4
lu/alb-2	R2,3	2.6 ± 0.8	*ms-1/su*	R3	10.6 ± 2.1	*gl-3/tt-3*	R2	0.0 ± 6.5
lu/ms-1	C2	13.4 ± 1.5	*ms-1/ch-5*	R2	20.2 ± 8.3	*gl-3/tz*	C2	20.5 ± 3.6
lu/ms-1	R2	13.1 ± 3.0	*ms-1/tt-2*	C2	17.3 ± 3.5	*gl-3/tz*	R2,3	20.0 ± 5.2
lu/ttg	C2	18.1 ± 2.2	*ms-1/th-2*	C2,3	18.4 ± 3.4	*gl-3/cer-3*	R2,3	22.9 ± 5.4
lu/ttg	R2	16.0 ± 3.5	*ms-1/th-2*	R2	24.4 ± 5.0	*gl-3/aba*	R2	38.5 ± 5.9
lu/ga-3	R2,3	17.4 ± 2.2	*ms-1/gl-3*	R2	28.5 ± 6.4	*tt-3/aba*	R2	28.5 ± 5.0
lu/tt-3	R2	36.4 ± 5.7	*ms-1/tt-3*	C2	17.2 ± 4.3**	*tz/cer-3*	C2,3	4.5 ± 1.1
lu/tz	R2	49.3 ± 3.8	*ms-1/tt-3*	R2	31.3 ± 3.3	*tz/cer-3*	R2	0.0 ± 3.7
tt-4/alb-2	R2,3	3.1 ± 1.0	*ms-1/tz*	C2	46.1 ± 3.9	*tz/cer-3*	R3	1.6 ± 1.6
tt-4/ga-3	C2,3	18.8 ± 2.2	*ms-1/tz*	R2	42.8 ± 3.4	*tz/yi*	R2	0.0 ± 6.0
tt-4/ga-3	R2	17.2 ± 5.1	*ms-1/cer-3*	R2	45.3 ± 3.1	*tz/yi*	R3	7.7 ± 3.1
tt-4/tz	R2	56.1 ± 3.5	*ms-1/yi*	C2	47.8 ± 3.6	*tz/min*	C2	8.1 ± 1.8
f_g/*pi*	R2,3	11.5 ± 2.2	*ms-1/yi*	R2	47.6 ± 3.8	*tz/min*	R2	27.9 ± 6.0
f_g/*ms-1*	C2	8.6 ± 2.9	*ms-1/aba*	C2	49.3 ± 3.0	*tz/aba*	R2	19.3 ± 3.7
f_g/*ms-1*	C2,3	9.9 ± 0.7	*ttg/ga-3*	C2	3.0 ± 0.6	*cer-3/yi*	R2	12.9 ± 5.7
f_g/*ms-1*	R2	8.8 ± 3.7	*ttg/ga-3*	R2	0.0 ± 2.8	*cer-3/min*	R2,3	8.3 ± 2.6
f_g/*ttg*	C2	12.5 ± 1.5	*ttg/su*	R3	7.2 ± 1.2	*cer-3/aba*	C2	13.9 ± 1.6
f_g/*ttg*	C2,3	15.2 ± 0.8				*yi/aba*	R2	15.0 ± 5.9
						min/aba	R2,3	6.8 ± 3.0

* and ** see Table II.

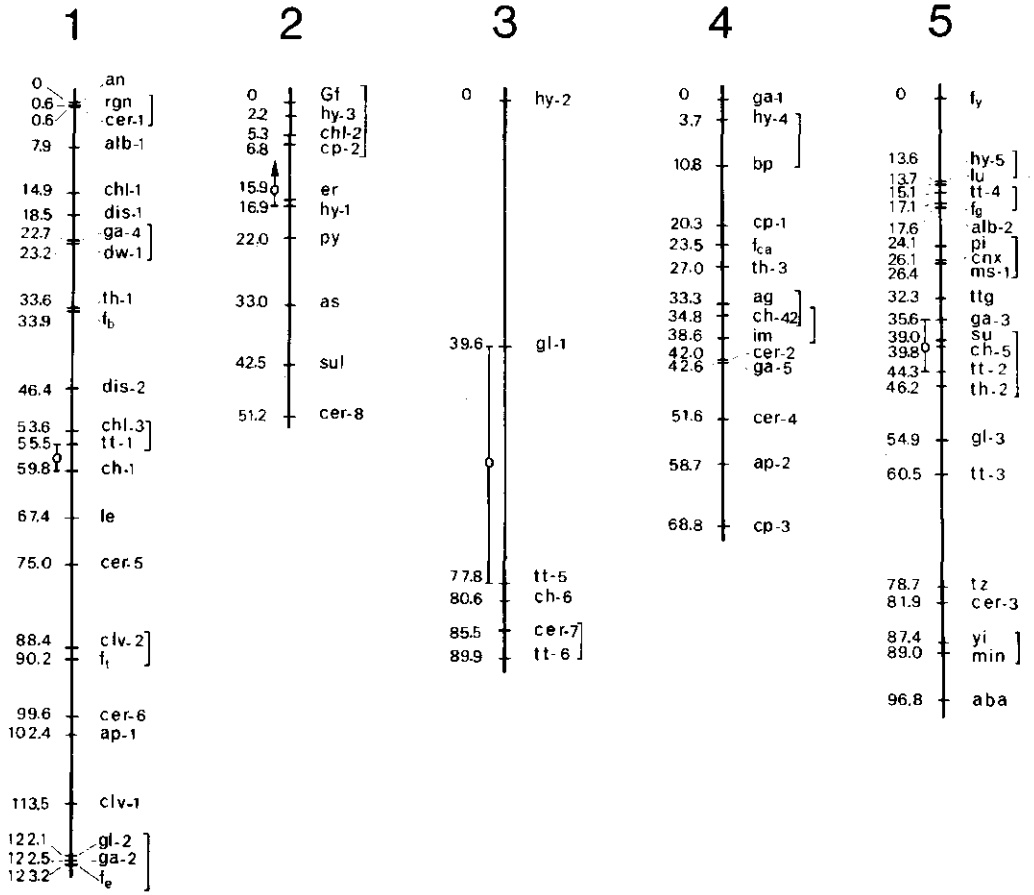

Fig. 1. Linkage map of *Arabidopsis thaliana*. Brackets indicate that the order within the bracketed group could not be conclusively established. Circles with range indicate position of the centromere (not known for chrom. 4).

Chromosome 1

It is seen that Rêdei's linkage groups 1 (*an*) and 4 (*ch-1*) as well as McKelvie's groups 1 (*ap-1*) and 4 (*le*) are located on chromosome 1. Although McKelvie's group 1 contains 15 markers comprising 145 map units, these data could not be combined with ours as only 1 marker (*ap-1*) was studied in relation with our markers. Moreover the accuracy of McKelvie's data is very limited as they were based on relative small populations of repulsion phase F_2's only. Hirono and Rêdei[11] found the gene order gi^2 - 24.8 - *ch-1* - 7.9 - *pa*, which has not be used in the present study for the following reasons. An X-ray treated heterozygote yielded a pale green sector (*ch-1/ch-1*) and from the progeny of this sector Hirono and Rêdei (l.c.) concluded that mitotic crossing-over had occurred between gi^2 and *ch-1* and also that the centromere was to the left of gi^2. This is clearly at variance with the short distance between *ch-1* and the centromere or rather between *ch-1* and *tt-1* as found by us. Telotrisomic analyses[15] had shown *ch-1* and *tt-1* to be on different arms.

Close linkage of *gl-2* and *ga-2* was further confirmed by absence of segregation for respectively *ga-2* and *gl-2* among the selfed progeny of 141 *gl-2/gl-2, Ga-2/.* and 147 *Gl-2/., ga-2/ga-2* plants from a repulsion phase F_2 of both markers. This gives an estimate of 0.0% with 95% confidence limits 0% and 0.5%.

Chromosome 2

Here Rêdei's data (ref. 34, Table 1) were incorporated after converting his coupling phase standard deviations into repulsion phase standard deviations by means of Stevens' tables[45]. Rêdei himself states that these data were from repulsion phase crosses.

The centromere location (Fig. 1) was based on the results of Sears and Lee-Chen[44].

Chromosome 3

Relatively few loci were mapped on this chromosome, which has two gene distances much larger than found on the other chromosomes (Fig. 1). Rédei and Hirono[38], however, state that 7 unspecified genes were found to be in this linkage group 3.

Telotrisomic analysis (Table VII) shows that *tt-5* is on Tr3A, whilst *gl-1* is not. This means that the centromere is located between these two loci.

Table VII. Segregation frequencies in F_2 populations derived from duplex plants (A^{telo} *A a*) obtained by crossing telotrisomics with recessive mutants

Cross	Trisomic progeny		$\chi^2_{3:1}$	Disomic progeny		$\chi^2_{3:1}$
	Dominant	Recessive		Dominant	Recessive	
Tr3A x *gl-1*	80	33	1.1	98	40	1.2
Tr3A x *tt-5*	68	0	22.7*	46	19	0.6
Tr5A x *ttg*	63	12	3.2	184	54	0.7
Tr5A x *tt-2*	56	0	18.7*	100	25	1.7
Tr5A x *th-2*	83	0	27.7*	72	28	0.5
Tr5A x *gl-3*	71	0	27.7*	96	32	0.0
Tr5A x *tt-3*	63	0	21.0*	97	23	2.2
Tr5A x *yi*	75	0	25.0*	212	26	25.1*

* $P < 0.01$

Tr5A x *ga-3* gave among total progeny 169:49 ($\chi^2_{3:1} = 0.7$; n.s.)

Chromosome 4

In a preliminary report[20], *cp-1* was only crossed with *ag* and f_{ca}. Later it turned out that *cp-1* could not be reliably scored in f_{cd}/f_{ca} background, so the recombination estimate ($f_{cd}/cp\text{-}1$) was omitted from the present calculations (Table V). As a result *cp-1* is now located on the other side of *ag*.

The close linkage of *cer-2* (= *vc-2*) with *im* found by Rédei[36] is confirmed. As discussed before[16,19] Fischerova's[10] conclusion that *ch-42* (chrom. 4) is linked with *lu* (chrom. 5) and *alb-2* (chrom. 5) must be rejected.

Chromosome 5

Telotrisomic analysis (Table VII) shows that *tt-2*, *th-2*, *gl-3*, *tt-3* and *yi* are on Tr5A, whilst *ttg* and *ga-3* are not. Sears and Lee-Chen[44] and Rédei et al.[39] found *th-2* and *tz* to be on their telotrisomic Nc (identical with Tr5A[19]), whilst *lu* was not. So their centromere localization agrees with our more precise position between *ga-3* and *tt-2*.

Genetic length

The total genetic length of the *Arabidopsis* genome now amounts to 430 cM. On the basis of individual chromosomes *Arabidopsis* matches well in map length with other well analysed plant species like maize[31] and tomato[43], although its chromosomes are much smaller at metaphase.

It is interesting to see that there is a good agreement of the linkage map with the cytological observations given by Sears and Lee-Chen[44] for the individual *Arabidopsis* chromosomes. The short chromosome 2 has the shortest map, the medium sized chromosomes 1, 3 and 5 have the longest maps. The longest (nucleolar) chromosome 4 has only a

medium map length, but here a large heterochromatic block probably comprising an entire arm is present, so when correcting for the heterochromatic region (presumed to be genetically empty) also chromosome 4 fits the picture. The heterochromatic arm may also render the search for its telotrisomics futile. Finally, chromosomes 1, 3 and 5 have both genetically and cytologically median or submedian centromeres.

Locus distribution

There is no indication that loci belonging to specific phenotypic groups are preferentially located on specific chromosomes (Table VIII).

Table VIII. Distribution of genes with specific phenotypes over the 5 *Arabidopsis* chromosomes

Genes affecting	Gene symbols	Number of loci on chromosome :					Total
		1	2	3	4	5	
chlorophyll	*alb, ch, im, lu, sul, yi*	2	1	1	2	4	10
epicuticular wax	*cer*	3	1	1	2	1	8
flowering time	*f*	3	-	-	1	2	6
flower morphology	*ap, ap, clv, pi*	3	-	-	2	1	6
gibberellin sensitivity	*ga*	2	-	-	2	1	5
hairs (trichomes)	*dis, gl, ttg*	3	-	1	-	2	6
hypocotyl length	*hy*	-	2	1	1	1	5
nitrate metabolism	*chl, cnx, rgn, su*	3	1	-	-	2	6
plant length	*cp, dw, er, ga, le, min*	4	2	-	4	2	12
testa colour	*tt, ttg*	1	-	2	-	4	7
thiamine metabolism	*py, th, tz*	1	1	-	1	2	5
		25	8	6	15	22	76

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CHAPTER 8

GENETIC FINE—STRUCTURE OF THE *GA—1* LOCUS IN THE HIGHER PLANT *ARABIDOPSIS THALIANA* (L.) HEYNH.

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SUMMARY

Non-germinating gibberellin (GA) responsive mutants are a powerful tool to study genetic fine structure in higher plants. Nine alleles (EMS- and fast neutron induced) of the *ga-1* locus of *Arabidopsis thaliana* were tested in a complete half-diallel. No wild type "recombinants" were found in the selfed progeny of 9 homoallelic combinations (in total 3×10^5 plants); in the progenies from the 36 selfed hetero allelics the wild type frequency ranged from zero to 6.6×10^{-4}. These frequencies allowed the construction of an internally consistent map for 5 different sites representing 8 alleles. The 9th allele covered 3 sites and thus behaved like an intragenic deletion. The estimate of the total genetic length of the *ga-1* locus was 0.07 cM. The order of the sites was also clearly reflected by the association with proximal outside markers. On the assumption that wild type gametes predominantly arise from reciprocal events, it was shown that a cross-over within the *ga-1* locus leads to positive interference in the adjacent region

The results are discussed with respect to the mutagen used, the frequencies found in other plant- and Drosophila genes, and the possible occurrence of gene conversion.

1. INTRODUCTION

Studies on the genetic fine structure of genes have given useful information about their physical structure, the nature of crossing-over and the nature of particular mutations.

Among eukaryotes, fungi with their high "resolving power" (high numbers of spores and the availability of "self-detection" systems) have proved ideal tools for this type of study by means of complementation, recombination and gene conversion analysis (for reviews see Catcheside, 1977, and Fincham *et al.*, 1979).

Studies in Drosophila led to qualitatively comparable results, which suggests a common molecular mechanism of meiotic recombination (Hilliker & Chovnick, 1981). Fine structure analyses in higher plants are rare due to the difficulties in handling the enormous numbers of plants required. For this reason the most extensive studies are with pollen-grain markers where very large numbers can be easily scored: waxy *(wx)* in maize (Nelson, 1958, 1962, 1968, 1975; Amano, 1968), *glx* in barley (Rosichan *et al.*, 1979; Nilan *et al.*, 1981) and *Adh1* in maize (Freeling, 1976, 1978). Seedling characters have been studied in maize *(gl-1)* by Salamini & Lorenzoni, 1970 and in barley (*cer-cqu* region) by Wettstein-Knowles and Søgaard, 1980. These genes control wax biosynthesis and deposition. Jörgensen & Jensen (1979) studied the mildew resistance gene *ml-o* in seedlings of barley.

For the analysis of intragenic recombination the recessive non-germinating gibberellin (GA) responsive mutants (gene symbol *ga*) isolated in *Arabidopsis thaliana* (Koornneef & van der Veen, 1980) and tomato (Koornneef *et al*, 1981) seem particularly suitable. For germination these mutants require gibberellin and without further addition of GA they develop into typical dwarfs, but with GA sprays at weekly intervals, they develop into the wild type phenotype or nearly so.

Depending on the allele, the environmental circumstances during seed development and the germination conditions, varying degrees of germination occur without GA ("leakiness" of some mutant alleles). However, subsequently such germinators invariably develop into dwarfs.

The suitability of the *ga*-system for intragenic analysis, among other things its high resolving power derives from the following aspects:

1. Recombinants can be identified with certainty as these are "self-detecting" as germinating seedlings, which are much more vigorous than the mostly rare spontaneous germinators of the *ga*-mutants. In cases of doubt, the dwarf versus non-dwarf contrast is a definite criterium for mutant versus wild type.
2. Several types of outside markers are available to study joint segregation.
3. As only few seedlings emerge, sowing can be done closely spaced in petri dishes and on artificial media (like filter paper, agar, perlite, etc.).

Special advantages of *Arabidopsis* for this type of research are:

1. It is self-fertilizing under greenhouse conditions.
2. 1 000 upto 5 000 of the small seeds go into a 9 cm petri dish.
3. Also mutants may produce as many as 5 000 seeds per plant.
4. The short generation interval (2 months for the early ecotypes used) and the small plant size allow the rapid production of large quantities of seeds in climate chambers.
5. A high number of independently induced non-germinating mutant alleles obtained with ethylmethanesulphonate (EMS), X-rays and fast neutrons (FN) are available at 3 different loci (Koornneef & van der Veen, 1980). All mutants are induced in the same genetic background (ecotype: Landsberg "*erecta*").

Two preliminary experiments, included in this report as the 1st and 2nd experiment, had indicated the substantial occurrence of wild type plants in the progeny of heteroallelic *ga-1* plants (Koornneef, 1979), such in contrast to experiments with *ga-3* mutants (Koornneef & Janssen, unpublished), which yielded only very few recombinants. Therefore in the present study 9 independently induced mutants at the *ga-1* locus were analysed in a complete half-diallel crossing-scheme. They included both EMS and FN induced mutants and one germinating *ga-1* dwarf (a clearly leaky allele); otherwise they were chosen at random.

2. MATERIAL AND METHODS

(i) *Mutant alleles*

The *ga-1* mutants used are listed in Table 1 with respect to mutagen and "spontaneous germination" (i.e. without addition of GA).

Table 1. *Ga-1* mutants used for fine structure analysis, mutagen used and germination % (without adding GA)

Mutant allele	Mutagen*	Germination %
NG4	EMS	0
NG5	EMS	1
A428	EMS	15
Bo27	EMS	1
d69	EMS	0
d352	EMS	59
6.59	FN (69 Gy)	0
29.9	FN (47 Gy)	0
31.89	FN (67 Gy)	0

* Ethylmethanesulphonate (EMS): 10 mM, 24 hrs, 24 ^{o}C, in the dark
Fast neutrons (FN): in Gy (Gray) dose as indicated. 1 Gy = 100 rad.

(ii) *Conditions of culture*

The seeds were sown in 9 cm petri dishes, either equally spaced (25 seeds/dish) or scattered (250-5 000 seeds/dish), on perlite saturated with a standard mineral solution, composed as described by Oostindiër-Braaksma & Feenstra (1973). To break seed dormancy the dishes were kept at 2-4 ^{o}C for 4-6 days. Germination was at approx. 24 ^{o}C under continuous illumination by fluorescent light tubes (Philips TL 57) at approx. 8 W.m^{-2}.8 days after incubation at 24 ^{o}C the seedlings were scored and when necessary transplanted into soil. To obtain F_1 seeds parental mutant lines were grown and crossed in an air-conditioned greenhouse.
For the emasculation technique see Feenstra (1965). To exclude as much as possible unwanted selfing, in the 3rd large experiment, parental lines were used, which carried an extra recessive marker. Available for this were lines 6.59 with *ms* (male sterility) and NG5 with f_{ca} (late flowering) and *ap-2* (apetala-without petals), and the recessivity of non-germination to the germination of d352. F_1 seeds were sown as described. After a week at 24 ^{o}C, checking for wild type contaminants and scoring of "spontaneous germination" was done, after which they received GA_{4+7} (mixture of gibberellin GA_4 and GA_7) up to a final concentration of 10 µM in the medium to induce complete germination. A week later the F_1 seedlings were transplanted into soil in an isolated climate chamber (standardized conditions; unwanted cross-fertilization excluded). Here temperature was 23 ^{o}C, relative humidity approx. 80% and continuous light was by TL 33 fluorescent tubes (12-17 W.m^{-2})supplemented with incandescent bulbs (4-5 W.m^{-2}). Two weeks after transplanting (dwarf phenotypes clearly visible) GA_{4+7} (100 µM) was sprayed at weekly intervals, in total 2 or 3 times. Harvested F_2 seeds were stored at room temperature for at least two months.

(iii) *Testing for wild type recombinants*

Seeds of selfed parental lines and F_2 populations derived from hetero allelic crosses were sown under the conditions described above. These conditions permit also 100% germination of wild type and of *ga-1* mutants (the latter only when 10 µM GA_{4+7} is included in the medium). The seeds were scattered into petri dishes at numbers ranging from approx. 250 (F_2's involving the "germinating allele" d352) up to approx. 5 000 (F_2's without "spontaneous germination"). Counting of the seeds was on the basis of seed weight, determined separately in each experiment for each parental line and each F_2 population. The weight of 1 000 seeds is mostly 20-25 mg. Wild type seedlings are easily recognized because, upon germination, they are much more vigorous than the *ga-1* mutants of which in case of some "leaky" alleles a certain proportion of the seeds will germinate (Fig. 1). All presumed wild type seedlings (including cases of doubt) were transplanted into soil to check their non-dwarf phenotype.

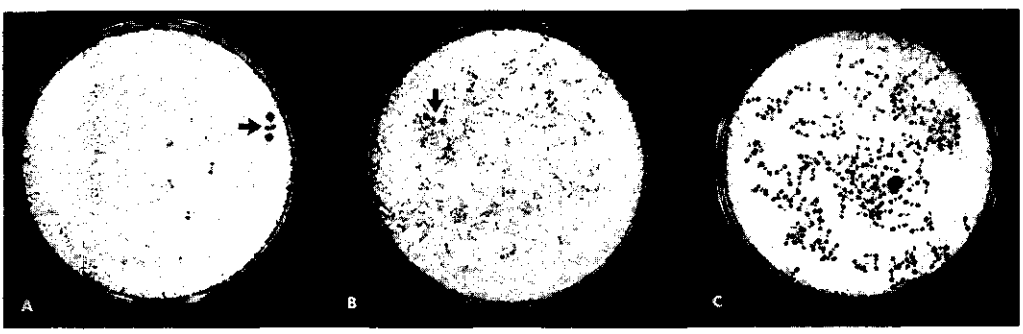

Fig. 1. Petri dishes with F_2 progenies of 6.59 x NG4 (A), 6.59 x NG5 (B) and d352 selfed. Arrows indicate wild type seedlings.

In experiment 3 all wild type recombinants were also progeny tested to check for the expected segregation of *ga-1* mutants: wild type contaminants are not expected to be heterozygous for the *ga-1* locus.

Since the intragenic recombinant gametes are rare and only one half of these are (dominant) wild types, the proportion of wild types found in F_2 populations is a direct estimate of the recombinant fraction (r). See: $\frac{r}{2}$ (maternal) + $\frac{r}{2}$ (paternal) - $\frac{r^2}{4} \simeq r$. For calculating the 95% confidence limits a Poisson distribution is assumed.

(iv) *The association of outside markers with intragenic recombinants*

To distinguish between cross-overs and gene conversions, closely linked outside markers are required at both sides of the locus studied. For *ga-1* the situation is not ideal, as this locus is at the end of the chromosome 4 map and the nearest markers are rather distant.

Line NG5 ($ga\text{-}1^1/ga\text{-}1^1$) was provided with the proximal outside markers f_{ea} (late flowering) and *ap-2* (apetala, reduced petals), *f* and *ap* for short. The map positions (in cM) are: *ga-1* - 27.2 - *f* - 30.0 - *ap* (Koornneef *et al.*, 1980). NG5 was then crossed with the other 8 lines carrying ga-1 alleles. This yielded F_1's $ga\text{-}1^1.f.ap/ga\text{-}1^x.F.Ap$.

In F_2 the wild type recombinants (with respect to the *ga-1* locus) arise from one recombinant gamete (*Ga-1*) and one non-recombinant gamete (*ga-1*). Both gametes can further carry *F.Ap*, *F.ap*, *f.Ap* or *f.ap*. So recombinants of 4 x 4 = 16 different genotypes (always combinations of *Ga-1* and *ga-1* gametes) may occur. Of these types only *Ga-1.f.ap/ga-1.f.ap* can be directly identified. To determine the other genotypes F_3 progeny testing is required. To distinguish the coupling and repulsion diheterozygotes 40 plants were raised per F_3 line, and when necessary another 40 plants.

With the homozygotes gamete assessment is straightforward: e.g. *F.ap/F.ap* necessarily has originated from one *Ga-1.F.ap* and one *ga-1. F.ap*. gamete. With the 4 monoheterozygotes and the 2 diheterozygotes, the linkage phase must be taken into account: e.g. *F.Ap/f.ap* arose from *ga-1.F.Ap* and *Ga-1.f.ap*, when the *ap-1* and *f* locus are in repulsion with *ga-1*. This can be applied to *F/f* heterozygotes but not to genotypes which are only heterozygous *Ap/ap*, since *ap* segregates almost independently from *ga-1*. In other words, complete gamete assessment cannot be done for *F.Ap/F.ap* and *f.Ap/f.ap*. To save work, none of the *f/f* phenotypes were progeny-tested which implies that *f.Ap/f.ap* also could not be distinguished from *f.Ap/f.Ap*. So in total 4 x 4 - 5 = 11 gamete combinations were completely assessed, the other 5 being assessed only for the loci *ga-1* and *f*. From these data (Table 5) the frequencies of the different types of recombinant *Ga-1* gametes with respect to *f* and *ap* are then estimated by a maximum likelihood procedure (in view of the incomplete assessment). The figures also allow an estimate of the recombination fraction between *f* and *ap* in gametes that did not originate from intragenic recombination at the *ga-1* locus. Estimates of "ordinary" recombination fractions were calculated by the method of maximum likelihood from F_2 segregation data derived from the cross *ga-1*^{1}*.f.ap/ga-1*^{1}*.f.ap* x wild type.

3. RESULTS

(i) *Intragenic complementation*

The germination percentages of both F_1's and F_2's of all 36 heteroallelic combinations did not exceed the percentages of the higher parent. So there is no indication of even partial intragenic complementation. The same holds for 23 other independently induced *ga-1* alleles as fas as mutually tested.

(ii) *Frequencies of wild type recombinants*

Table 2 presents the frequencies of wild type *ga-1* alleles found in F_2's of all 36 heteroallelic combinations of the 9 *ga-1* mutants. In Table 2 the results of the 2 preliminary experiments (Koornneef,1979) and of the large 3rd experiment have been pooled. Only the results from NG4 x 29.9 in experiment 2 were omitted as they were strikingly at variance with all other results, probably as a result of selfing admixture. In all other cases no significant differences were found between the different experiments.

Not a single recombinant was detected among the homoallelic combinations (3×10^5 seeds tested in total,indicating a spontaneous reversion frequency $< 10^{-5}$ ($P < 0.05$)). In the majority of the heteroallelic combinations the frequencies of wild type recombinants range from 1×10^{-5} upto 6.6×10^{-4}. In analogy to similar results obtained by previous authors in other organisms, it is concluded that these elevated frequencies reflect intragenic recombination which may include gene conversion.

Table 3 gives the results upon pooling alleles that did not show recombination among each other. These are a) NG4 and d69 and b) A428, d352 and 6.59. The results with 31.89 are not included as they clearly stand apart (see below). It appears possible to construct an internally consistent map (Fig. 2) from the data of Table 3. The distances in Fig.2 were calculated from the direct estimate of a particular segment and the estimates of its bordering segments, e.g. with an allele order $A\overset{1}{\text{---}}B\overset{2}{\text{---}}C\overset{3}{\text{---}}D$, $r_1 = (r^{AB} + r^{AC} - r^{BC})/2$ and $r_2 = (r^{BC} + r^{AC} - r^{AB} + r^{BD} - r^{CD})/3$. When using this procedure the sum of the intervals becomes $7.1 \times 10^{-4} = 0.071$ cM. From the results obtained allele 31.89 can be interpretated as an intragenic deletion which covers half of the present *ga-1* map (Fig. 2). The data give no indication for the occurrence of map expansion.

Table 2. Frequencies of wild type recombinants (x 10^{-4}) in selfed progenies of hetero allelic crosses of *ga-1* mutants, the 95% confidence intervals (between parentheses) and the approx. number of F_2 seeds tested

Allele	NG5	NG4	d69	A428	d352	6.59	Bo27	31.89
29.9	1.6(0.8-2.8)	4.9(3.2-7.1)	6.6(4.5-9.5)	5.6(4.6-6.8)	5.6(3.7-8.0)	5.1(3.8-6.7)	6.1(4.9-7.5)	3.4(2.0-4.5)
	75300	55300	45300	178400	50300	100300	156600	87300
	NG5	4.9(3.6-6.3)	4.1(3.0-5.4)	5.0(3.5-5.8)	4.5(3.0-6.5)	5.2(3.9-6.9)	5.5(4.1-7.4)	3.3(3.2-4.7)
		111000	125300	149100	62300	97300	83000	85300
		NG4	0.0(0.0-0.5)	1.9(1.2-2.8)	0.6(0.1-1.7)	0.5(0.1-1.7)	2.5(1.6-3.7)	0.0(0.0-0.3)
			65300	130900	50300	43300	99800	92900
			d69	2.0(1.2-3.2)	1.6(0.7-3.1)	0.5(0.1-1.3)	1.2(0.6-2.2)	0.0(0.0-0.4)
				85300	50300	80300	85300	80300
				A428	0.0(0.0-0.9)	0.0(0.0-0.2)	0.6(0.2-1.1)	0.0(0.0-0.2)
					34300	127100	126600	162300
					d352	0.0(0.0-0.6)	0.6(0.1-1.7)	0.0(0.0-0.6)
						46600	50300	50300
						6.59	0.1(0.0-0.6)	0.0(0.0-0.3)
							89300	109900
							Bo27	0.0(0.0-0.3)
								101800

Table 3. Summarized recombinational matrix (frequencies x 10^{-4}, between parentheses 95% confidence intervals) compiled by adding together the figures of alleles that do not show recombinants among each other (31.89 not included)

Allele	NG5	NG4 d69	A428 d352 6.59	Bo27
29.9	1.6(0.8-2.8)	5.7(4.3-7.3)	5.4(4.7-6.3)	6.1(4.9-7.5)
NG5		4.4(3.6-5.4)	4.8(4.0-5.2)	5.5(4.1-7.4)
NG4, d69			1.3(1.0-1.7)	1.9(1.3-2.6)
A428, d352, 6.59				0.4(0.2-0.7)

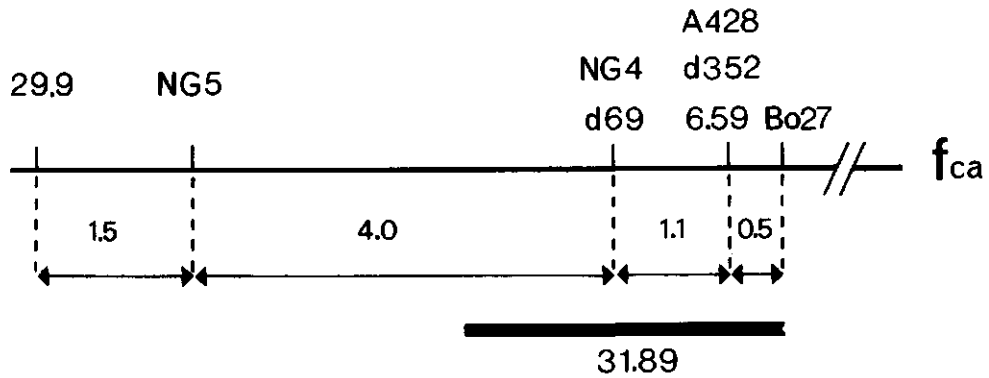

Fig.2. Genetic fine structure map of the *ga-1* locus. Distances: cM x 10^{-2}.

(iii) *Distribution of outside markers*

As *ga-1* is located close to the end of chromosome 4 no distal markers are yet available. The linkage relations of *ga-1* with the two proximal markers *f* and *ap* have been derived from the F_2 *(ga-1*^{1}*.f.ap/ga-1*^{1}*.f.ap)* x wild type as shown in Table 4. The estimates of recombination fractions agree with previously published results (Koornneef *et al.*, 1980).

The frequencies of *Ga-1* recombinants with respect to both *f* and *ap*, isolated from F_2's of NG5 x other *ga-1* mutants are presented in Table 5. No significant differences were found for the distribution of the different gametes between the different F_2's. Heterogeneity $\chi^2_{84} = 82.6$ ($0.4 < P < 0.6$). Therefore the crosses were pooled (see totals in Table 5) to estimate the marker distribution over the *Ga-1* recombinants (Table 6). However, the data obtained with 29.9 were not included as this allele is located at the other side of NG5 (see Fig. 3). From the predominance of the recessive *f* allele with the *Ga-1* recombinant and on the basis of the intragenic map, the order of the alleles with respect to *f* and *ap* is to be most likely 29.9 - NG5 - other *ga-1* alleles - *f* - *ap*.

Assuming that only reciprocal cross-overs give rise to *Ga-1* alleles (i.e. no conversions), it appears that a cross-over within the *ga-1* locus leads to a significant decrease of cross-overs in the adjacent *ga-1* to *f* region (positive interference), but does not affect recombination between *f* and *ap* (see Table 6; 2nd and 4th column). In addition the recombination fraction between *f* and *ap* which can be estimated from the non-recombinant gametes ($r = 0.201 \pm 0.032$) is in good agreement with that from the recombinant gametes ($r = 0.215 \pm 0.017$ in Table 6).

Conversely, the position of 29.9, distal to NG5, is expected to lead to a proportional excess of *F* as outside marker. However, the number of plants (12) is too small to confirm this.

Table 4. Linkage analysis of the F_2 from *(ga-1^1/ga-1^1, f/f, ap/ap)* x wild type

Plant phenotype	F_2 frequencies	Gamete genotypes	Estimated frequencies
Ga-1/. F/. Ap/.	516	*ga-1.f.ap*	0.481 ± 0.020
ga-1/ga-1 F/. Ap/.	91	*Ga-1.f.Ap*	
Ga1/. f/f Ap/.	61		
Ga-1/. F/. ap/ap	99	*ga-1.F.Ap*	0.234 ± 0.017
ga-1/ga-1 f/f Ap/.	63	*Ga-1.f.ap*	
ga-1/ga-1 F/. ap/ap	17	*ga-1.f.Ap*	0.215 ± 0.017
Ga-1/. f/f ap/ap	79	*Ga-1.F.ap*	
ga-1/ga-1 f/f ap/ap	50		
	986	*ga-1.F.ap*	0.070 ± 0.012
		Ga-1.f.Ap	

Estimates of recombination fractions

ga-1 - *f* : 0.304 ± 0.018

ga-1 - *ap*: 0.449 ± 0.022

f - *ap*: 0.284 ± 0.018

Table 5. Genotypes of F_2 *Ga-1* recombinant plants derived from crossing *ga-1*^{1}.*f.ap*/*ga-1*^{1}.*f.ap* with other *ga-1* alleles

Gamete genotype		Parent line (*ga-1* allele tested with NG5)								Total	Total
Recomb. *(Ga-1)*	Non-recomb. *(ga-1)*	29.9	NG4	d69	A428	d352	6.59	Bo27	31.89		29.9 excluded
F Ap	*F Ap*	2	1	2	1	3	0	2	2	13	11
F Ap	*f ap*	1	4	2	1	1	3	0	0	12	11
F Ap	*f Ap*	0	0	0	0	0	0	0	1	1	1
f Ap	*F Ap*	0	3	5	3	1	3	4	3	22	22
f Ap	*F ap*	0	2	0	0	2	1	1	1	7	7
F ap	*f ap*	0	2	2	1	0	0	2	0	7	7
F ap	*F ap*	0	1	0	0	0	0	0	0	1	1
F ap	*f Ap*	1	1	0	1	0	0	0	1	4	3
f ap	*F Ap*	0	16	13	4	7	11	14	8	73	73
f ap	*f ap*	2	7	11	10	4	8	9	2	53	51
f ap	*F ap*	1	3	2	3	3	2	2	2	18	17
F $\frac{Ap}{ap}$	*F* $\frac{ap}{Ap}$*	1	2	2	0	0	2	2	2	11	10
f ·	*f* ·**	4	3	10	3	4	7	6	6	43	39
		12	45	49	27	25	37	42	28	265	253

* single heterozygous *Ap/ap* could not be completely assessed (see text)

** Homozygous *f/f* were not tested (see text), so only *f/f*, *ap/ap* was completely assessed.

Table 6. Outside marker distribution of *Ga-1* recombinants from the pooled F_2's from crosses *ga-1*^{1}.*f.ap*/*ga-1*^{1}.*f.ap* *ga-1*^{x}.*F.Ap*/*ga-1*^{x}.*F.Ap* (The F_2 from NG5 x 29.9 was excluded (see text)

Marker association of *Ga-1* recombinants	Frequency of occurrence	Gamete originates after crossing-over between*	Frequencies expected on the basis of random crossing-over**
f ap	0.614 ± 0.037	no crossing-over	0.481 ± 0.020
F Ap	0.101 ± 0.020	*ga-1/f*	0.234 ± 0.017
f ap	0.212 ± 0.031	*f/ap*	0.215 ± 0.017
F Ap	0.073 ± 0.017	*ga-1/f* and *f/ap*	0.070 ± 0.012

* No gene conversion is assumed

** From Table 4

4. DISCUSSION

An internally consistent map could be constructed from the frequencies of wild types (intragenic recombinants) occurring in the selfed progenies of plants heteroallelic for mutations at the *ga-1* locus. The deviating results from crosses with allele 31.89 can be readily explained by the hypothesis that 31.89 is an intragenic deletion. It may be significant in this respect that 31.89 hardly shows any germination (Table 1), which indicates non-leakiness. It is in no way a general rule that intragenic recombinant frequencies are additive like we found for the *ga-1* locus (see e.g. Carlson, 1959; Fincham *et al.*, 1979). In higher plants this lack of additivity was conspicuous for the *Adh1* locus in maize (Freeling, 1976, 1978) and was also noted by Nelson (1968) for *wx* in maize, which made it impossible to construct a map on the basis of intragenic recombinant frequencies.

The frequencies of wild type recombinants among gametes found for the *ga-1* locus in *Arabidopsis* (upto $\frac{r}{2} = 3.3 \times 10^{-4}$) are comparable to those found for other well studied plant genes (over $\frac{r}{2} = 10 \times 10^{-4}$ for *wx* in maize (Nelson, 1968), upto 20×10^{-4} for *glx* in barley (Nilan *et al.*, 1981) upto 6.6×10^{-4} for *Adh1* (Freeling, 1978) and upto 5.8×10^{-4} for *gl-1* (Salamini & Lorenzoni, 1970)). The size of the plant genes including *ga-1* corresponds to the large Drosophila loci like *lz* (Green & Green, 1956), *r* (Carlson, 1971), *dp* (Grace, 1980). This suggests that plant genes in general are "large". However, it should not be overlooked that one of the reasons for studying these particular loci was the availability of a number of different alleles, which implies that preferentially loci were chosen with relatively high mutation frequencies.

In this connection it is interesting that the *ga-3* locus has a significantly lower mutation frequency than *ga-1* and also shows lower frequencies of intragenic recombinants (Koornneef & Janssen, unpublished). A relation between the genetic size of a gene and its induced mutation frequencies has also suggested by Chovnick *et al.* (1971) who compared the *ma-1* and *ry* locus of Drosophila.

Amano (1968) found for his 3 fast neutron induced *wx* mutants in maize no wild type pollen grains from the heteroallelic plants and moreover a reduced transmission of the affected chromosome, such in contrast to his 9 EMS-induced *wx* mutants, 7 of which were able to recombine with the same *wx* tester allele. This suggests that FN may preferentially induce gross chromosomal damage like large deletions. There is an apparent discrepancy with our results, where 2 FN-induced and all 6 EMS-induced alleles show recombination, whilst a third FN-induced allele shows only reduced recombination. This discrepancy may be explained by a difference in mutant selection procedure. Our mutants were selected in M_2 lines (the progenies from selfed mutagen treated M_1 plants), so that gross chromosomal aberrations have been sieved out due to their low transmissibility through the pollen (certation). In contrast, Amano's *wx* mutants were all identified upon pollination of M_1 plants with pollen from (non-treated) homozygous recessive testers. Therefore, the conclusion seems warranted that FN induces more gross genetic damage than EMS does, but that mutations that pass the certation sieve (i.e. those selected in M_2) are in general not of that type.

The isolation of leaky alleles induced by both EMS and FN at the *ga-1* locus (germinating dwarfs) (Koornneef & van der Veen,1980; Koornneef *et al.*, 1982a) also points to the recovery of FN-induced alleles with minor genetic damage.

As outside markers were not available at both sides of the *ga-1* locus (*f* and *ap* are both proximal), it was *a priori* impossible to exclude conversion (not followed by a reciprocal event) as a source of wild type gametes. Let *x* and *y* be *ga-1* mutants and *f* the outside marker in the order *x-y-f*. Then the heterozygote $\frac{x \quad f}{y \quad F}$ may produce *Ga-1.f* gametes by 1) reciprocal crossing-over between *x* and *y*, 2) conversion of *x*, and 3) conversion of *y* followerd by crossing-over between *ga-1* and *f*. It may produce *Ga-1.F* gametes by 1) conversion of , 2) conversion of *x* followed by crossing-over between *ga-1* and *f*. For the order *y-x-f* this relationship is interchanged. It follows that an excess of *f* alleles (or *F* alleles) among *Ga-1* recombinants only allows a conclusion about the order of *x* and *y* when reciprocal events are more frequent than conversion events. When the conversion events are in excess and when in addition conversion of the proximal site is far more frequent than at the distal site, the order inferred on the assumption of only reciprocal events may be erroneous.

On the other hand, no indication was obtained for the occurrence of map expansion. This phenomenon is explained by the relatively frequent occurrence of co-conversion of closely "linked" sites (Holliday, 1964). Its absence is an indication that recombinants with parental flanking markers (conversions) are relatively rare among the randomly sampled (wild type) recombinant gametes. Moreover the *ga-1* gene is relatively large and so are the intervals between the sites. With larger intervals these conversions are expected to be relatively infrequent as the recombination event has a greater probability of being detected as a cross over than as a pure conversion event (Chovnick *et al.*, 1971; Hilliker & Chovnick, 1981). However, with some large loci both in *Drosophila* (*r* locus, Carlson, 1971) and in maize (*wx*, Nelson, 1968, 1975; *gl-1*, Salamini & Lorenzoni, 1970) there seem to be exceptions to this rule.

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GENERAL DISCUSSION AND SUMMARY

This thesis describes the isolation and characterization in *Arabidopsis thaliana* (L.) Heynh. of induced mutants, deficient for gibberellins (GA's), abscisic acid (ABA) and photoreceptors.

These compounds are known to regulate various facets of plant growth and differentiation, so mutants lacking one of these substances are expected to be affected in several aspects of their physiology. It is shown in this thesis that the earliest expression of these mutants occurs during seed development and seed germination. Therefore these processes form an excellent phase to screen for these mutants.

Planthormone and photoreceptor mutants in relation to seed physiology.

In general three major periods may be distinguished in the history of a seed: 1) Seed development and maturation, 2) developmental arrest of the mature seed, characterized either by a dormant state in which seeds even do not germinate under favourable environmental conditions, or by a quiescent state in which seeds only require rehydration, and 3) germination, starting with water uptake and often requiring breaking of dormancy, which is triggered by specific environmental factors such as light and temperature. Planthormones may play a regulatory role in all three phases.

Non-germinating GA-responsive mutants as described in Chapter 1 have a strongly reduced gibberellin biosynthesis (Barendse, pers.comm.) which may lead to an increased level of dormancy and/or to the inability of the seeds to

break dormancy after imbibition of mature seeds. Clearly the presence of GA's, either by *de novo* GA synthesis, or by hydrolysis of bound forms, is not always a prerequisite for seed germination: genotypes that combine GA- and ABA deficiency like the revertants of non-germinating *ga-1* mutants described in Chapter 2 do readily germinate.

Apart from the absence of endogenous factors such as gibberellins, also the lack of receptors for environmental factors that normally break dormancy might prevent germination. An example are the *hy-1* and *hy-2* mutants (Chapter 4), which are characterized by an increased hypocotyl length in white light and the absence of detectable phytochrome in dark grown hypocotyls. It was shown by Spruit et al. (1980), that these mutants hardly show any germination and correspondingly, have strongly reduced levels of phytochrome in their seeds. Their reduced germination capacity is restored by (relatively high) concentrations of exogeneously applied GA_{4+7} (Koornneef et al., 1981). Consequently one might expect such phytochrome deficient mutants to occur among the GA responsive non-germination mutants in *Arabidopsis*, like van der Veen and Bosma actually found for a tomato mutant (see Koornneef et al., 1981). Remarkably this was not the case in *Arabidopsis*. The reason for this seems to be the absence of a light requirement in the *hy* mutants from the M_2 populations screened for non-germinating mutants of *Arabidopsis*. It happened that these M_2 seeds in all cases were harvested from M_1 plants grown in winter, in contrast to the seeds studied by Spruit et al. (1980) which were harvested in summer. We have observed during a number of years that seeds (including wild-type seeds), which developed in winter (natural daylight with additional continuous light by Philips TL 57) were less dormant than seeds from summer grown mother plants (long days, high light intensity, no additional light). Relevant environmental

factors in this respect may be light intensity, light quality (McCullough and Shropshire, 1970) and daylength (Karssen, 1970; Luiten, 1982). The effect of light quality (McCullough and Shropshire, 1970) indicates that phytochrome may be involved in the determination of the level of dormancy.

To select mutants with a reduced or absent seed dormancy, one may choose those conditions, where the wild-type is clearly dormant. However, the high and probably complex environmental variability of this character and the relatively rapid change in the level of dormancy during dry storage of the seeds makes this selection system less attractive.

Selection for revertants in the progeny of mutagen treated non-germinating *ga-1* mutants proved to be an effective procedure to isolate mutants with a reduced dormancy (Chapter 2). As the reverting effect (restored germination) was caused by a mutation at a different locus, the *ga-1* allele could be replaced by its wild-type allele by crossing the revertant with the wild-type parent followed by selection in F_2. These newly selected monogenic recessive mutants had a reduced level of ABA in the leaves and in both the developing and ripe seeds. Correspondingly the mutant allele was called *aba* (ABA-types are *aba/aba* plants).

The germination of seeds collected at different stages of their development on both ABA-and wild-type plants showed that dormancy developed during the last part of seed maturation in wild-type, but not in the *aba*-mutant. This shows that the function of ABA is dormancy induction. ABA determinations in unripe siliquae showed a peak level of ABA at about 10-12 days after anthesis, followed subsequently by a decrease, a short period at a constant level and a further decrease (Chapter 3). In addition to ABA-type mothers with ABA-type embryo's and wild-type mothers with wild-type embryo's, one can also obtain

by means of the appropriate reciprocal crosses ABA-type mothers with wild-type embryo's and wild-type mothers with (50%) ABA type embryo's. So the effects of maternal and embryonic genotype can be separated. It was found (Chapter 3) that the genotype of the mother plant regulated the sharp rise in ABA content halfway seed development (maternal ABA). The genotype of the embryo and endosperm was responsible for a second ABA fraction (embryonic ABA), which reached lower levels; but persisted for some time after the maximum in maternal ABA. The onset of dormancy showed a good correlation with the presence of the embryonic ABA fraction and not with the maternal ABA.

Another category of mutants which also may give some understanding of the role of ABA in seed germination are the ABA tolerant mutants recently isolated by us in *Arabidopsis*. Compared to wild-type these mutants require an upto 20 fold higher concentration of exogeneously applied ABA to inhibit seed germination. These mutants too are characterized by a reduced seed dormancy.

Other genetically determined factors than those mentioned above are certainly also involved in seed development and seed germination. Thus in *Arabidopsis* mutations leading to the absence of seed coat pigments (transparent testa) and simultaneously to the absence of a mucilage layer around the seed have a reduced dormancy (Koornneef, 1981). The latter seedcoat characters are determined purely by the maternal genotype.

Planthormone and photoreceptor mutants in relation to other physiological effects.

Non-germinating mutants at the loci *ga-1*, *ga-2* and *ga-3*, when made to germinate by adding gibberellin, initially develop into normal looking seedlings. Later on they become dark green bushy dwarfs with reduced petals and stamens.

Regular GA-spraying from the seedling stage onwards maintains the wild-type phenotype completely or nearly so (Chapter 1). The strong and quick response of the dwarfs to GA sprays (the elongation of the petals of older dwarfs becomes visible within two days) clearly demonstrates the essential role of gibberellin in elongation growth.

Recently the non-germinating *ga* alleles were shown to have a strongly reduced kaurene synthetase activity in young siliquae compared to wild type. These analyses were performed by Dr. G.W.M. Barendse (pers.comm.). This indicates that these genes control some early step(s) in GA biosynthesis.

Apart from mutants that do not germinate without GA,also more or less normally germinating GA responsive dwarfs were isolated. Half of these were found to be allelic to the non-germinating *ga-1*,*ga-2* and *ga-3* mutants. These mutant alleles behave like so called "leaky alleles",i.e. the alleles are only partly defective and produce sufficient GA for seed germination, but not enough to give normal elongation growth.

GA sensitive dwarfs were also found at two other loci (*ga-4*,*ga-5*)of which no non-germinating alleles have been isolated so far (Chapter 1).These mutants have normal or slightly reduced kaurene synthetase activity (Barendse, pers.comm.), which indicates that these genes regulate steps beyond kaurene, or affect GA metabolism in another way. It is also possible that in the mutants cell elongation factors are blocked for which the relatively high concentration of exogeneously applied GA may substitute. Locus *ga-4* seems to control interconversions between GA's, which is suggested by the insensitivity of *ga-4* dwarfs to GA_9, which gibberellin is effective with mutants at the other 4 loci.

Abscisic acid (ABA) deficient mutants are characterized not only by reduced seed dormancy but also by disturbed water relations (wiltiness, withering),

probably as a result of failure to close the stomata upon conditions of water stress (Chapter 2). This is characteristic for ABA deficient mutants in tomato (Tal and Nevo, 1973) and potato (Quarrie, 1982). ABA deficient mutants in maize are in addition to a reduced seed dormancy (viviparous mutants, gene symbol *vp*) characterized by the failure to synthesize carotenoids and they accumulate precursors of these pigments (Robichaud et al., 1980). As ABA deficient mutants in *Arabidopsis*, tomato and potato have normal pigments, it is suggested that in the latter species the ABA biosynthesis may be blocked in the last part of the pathway, whilst in the maize mutants it is blocked at an earlier stage, i.e. where ABA and carotenoids still have a common pathway.

Some of the photoreceptor mutants are affected in their germination behaviour as discussed above. However, the most conspicuous effect observed is the partial lack of light induced inhibition of hypocotyl elongation(Chapter 4). Mutants in *Arabidopsis*, and also in tomato and cucumber (Koornneef et al., 1981; Koornneef et al., unpublished), that have an elongated hypocotyl when grown in white light, were shown to have locus-specific alterations in the spectra of light inhibition when grown in light of restricted spectral regions. In these "colour blind" mutants at two loci (*hy-1* and *hy-2*) little or no spectrophotometrically detectable phytochrome was present in dark grown hypocotyls, nor was it in the seeds. In these mutants the inhibitory effect of red and far-red was almost absent. Mutants of other genes were characterized by the absence only of red inhibition (*hy-3*) or by a decreased sensitivity to the shorter wavelengths of the spectrum (*hy-4*, *hy-5*). *Hy-5* also showed a reduced inhibitory effect of far-red light. The differential sensitivity of the genotypes to specific spectral regions strongly suggests the involvement of more than one pigment in the inhibition by light of hypocotyl elongation and probably also

in other photomorphogenetic processes. Some authors ascribed this role solely to phytochrome (Schäfer, 1976).

Since under specific conditions phytochrome could nevertheless be detected in so called phytochrome deficient mutants (Koornneef and Spruit, unpublished) the genes *hy-1* and *hy-2* probably do not represent the structural genes of the phytochrome protein or the phytochrome chromophore, but instead may play a role in the regulation of phytochrome metabolism.

Further genetic aspects of plant hormone and light receptor mutants.

Mutation frequencies for the different groups of loci were estimated for ethylmethanesulphonate (EMS), fast neutrons and X-rays (Chapter 5). Average mutation frequencies calculated per diploid cell, per locus and per mM EMS during 1 hr at 24 ^{o}C, were for *ga-1*, *ga-2*, *ga-3* 8.0 $\pm$ 1.8 x 10^{-6}, for *hy-1*, *hy-2*, *hy-3* 4.2 $\pm$ 1.4 x 10^{-6} and for the *aba* locus about 27 x 10^{-6}. These mutation frequencies are relatively high compared to other loci studied by us and others. It is not excluded that in these categories loci escaped detection simply because of a low mutation frequency.

It is a good custum to locate newly induced mutations on the organisms gene map, especially when they are the basis of extensive research like our *ga*, *aba* and *hy* mutants. Unfortunately, the gene map of *Arabidopsis* was rather fragmentary, and contradictory or wrong conclusions about linkage relations could be found in literature. Since we had gradually built up the complete set of 5 primary trisomics supplemented with a number of telotrisomics (one chromosome arm extra) and also made a collection of mutations at many loci, induced in the course of various experiments at our department and supplemented with mutants described in literature, we had a good starting point to construct

a more representative gene map for *Arabidopsis*. The required scale of operations was only feasable thanks to the accurate assistance of many students who performed trisomic analysis and gene mapping as part of their university training program. Important further additional data were obtained from the department of Genetics of Groningen University and from literature.

The trisomic analysis aimed at assigning linkage groups (via representative markers) to the different chromosomes is described in Chapter 6. The gene maps in centimorgans for the five *Arabidopsis* chromosomes is presented in Chapter 7. On the basis of 76 loci mapped the genetic length of the *Arabidopsis* chromosomes now compares well with that of individual chromosomes in e.g. tomato and maize. This notwithstanding the small size of the *Arabidopsis* chromosomes.

Genes with a similar mutant phenotype (and probably comparable functions) seem to be distributed at random over the *Arabidopsis* genome.

Our set of mutants at the *ga-1* locus of *Arabidopsis* provides an excellent opportunity for fine structure analysis of the gene. The system has a very high resolving power,for the intragenic recombinants are found as the rare wild-type seedlings among thousands of non-germinating seeds per petri dish. The results show (Chapter 8) that 8 different alleles could be arranged into an internally consistent map on the basis of the frequencies of intragenic recombinants. One fast neutron induced allele behaved as an intragenic deletion. The order of the sites with respect to other genes on chromosome 4 could be established.

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SAMENVATTING

De groei en ontwikkeling van planten wordt mede gereguleerd door plantenhormonen zoals gibberellinen (GA), abscisine-zuur (ABA) en fotoreceptoren zoals bv. fytochroom. Mutanten, waarbij één van deze factoren in verminderde mate of niet aanwezig is of niet functioneert, kunnen een belangrijke bijdrage leveren tot de studie van de genetica van deze factoren en vormen ook een belangrijk hulpmiddel voor plantenfysiologisch onderzoek. De selectie en karakterisering van een aantal mutanten gestoord in de gibberelline (GA), abscisine-zuur (ABA) en fotoreceptor huishouding bij de crucifeer *Arabidopsis thaliana* (de zandraket) wordt in dit proefschrift beschreven. Deze plantensoort is als proefobject gekozen vanwege zijn geschikte eigenschappen voor genetisch onderzoek zoals de zeer korte generatieduur (± 2 maanden), het zelfbevruchtende karakter, de hoge zaadproduktie en de geringe afmetingen van de plant. Dit laatste kon soms problemen opleveren bij fysiologisch- en biochemisch onderzoek.

In hoofdstuk 1 wordt beschreven hoe mutanten zijn geisoleerd, die zonder het van buiten toevoegen van gibberelline niet kunnen kiemen. Worden deze genotypen in een gibberelline-oplossing tot kieming gebracht, dan ontwikkelen de aanvankelijk normaal lijkende zaailingen zich tot donkergroene dwergplanten. Door echter deze planten regelmatig met gibberelline te bespuiten kunnen ze uitgroeien tot planten, die bijna niet van het wildtype (het standaard ras Landsberg "erecta") zijn te onderscheiden. 37 van deze recessieve mutanten bleken allelen te zijn van 3 verschillende genen (*ga-1*, *ga-2* en *ga-3* genoemd). Deze genen zijn zeer waarschijnlijk verantwoordelijk voor de synthese van enzymen uit het begin van

de biosynthese weg van de gibberellinen. Behalve deze zogenaamde niet-kiemende GA dwergen werden ook 19 wel-kiemende voor gibberelline gevoelige dwergen geisoleerd. 10 hiervan bleken mutaties van dezelfde 3 *ga* genen die eerder genoemd zijn, alleen functioneert hier het gen blijkbaar nog juist voldoende om kieming in gang te zetten en te houden, maar niet voldoende voor een normale lengtegroei. De 9 andere dwergen zijn mutanten van 2 andere genen (*ga-4* en *ga-5*), die een andere functie lijken te hebben.

Na een mutatiebehandeling van niet-kiemende *ga-1* mutanten konden in de nakomelingschap van de behandelde planten (de z.g. M_2 generatie), wêl-kiemende dwergen (revertanten) geselekteerd worden, waarbij het herstel van de kieming een gevolg bleek van een mutatie in een gen verantwoordelijk voor de aanmaak van de remstof abscisine-zuur (hoofdstuk 2). Na kruising van de revertanten met het wildtype werden in de F_3 lijnen gevonden, die homozygoot recessief waren voor alleen dit *aba* gen. Van dit gen zijn tot nu toe 14 verschillende allelen geisoleerd. Deze genotypen worden gekenmerkt door de afwezigheid van kiemrust in de zaden en door verwelkings- en verdorringsverschijnselen van de plant. Dit laatste lijkt een gevolg te zijn van het permanent openstaan van de huidmondjes, waardoor de plant te veel verdampt. Beide verschijnselen blijken het gevolg van abscisine-zuurgebrek te zijn, waaruit de belangrijke rol van dit hormoon voor de regulatie van zowel de waterhuishouding als de kiemrust blijkt.

Dit laatste aspect is meer gedetailleerd onderzocht in hoofdstuk 3 waarbij gevonden werd dat het abscisine-zuur, dat door het embryo zelf aan het eind van de zaadontwikkeling geproduceerd wordt, kiemrust in zaden induceert, waarna het hormoongehalte afneemt. Halverwege de zaadontwikkeling blijkt er een hoog gehalte van dit hormoon voor te komen in zaden, dat echter van moederlijke oorsprong is. Dit laatste werd aangetoond met behulp van planten met zaden

waarbij het genotype van de moeder en het embryo verschilden (verkregen via reciproke kruisingen). De rol van dit moederlijke abscisine-zuur is minder duidelijk.

Bij *Arabidopsis* en ook bij andere plantensoorten zijn mutanten gevonden,die gekenmerkt worden door een extra lang hypocotyl. Bij *Arabidopsis* betrof het 41 mutanten van 5 verschillende genen. Wanneer deze mutanten opgekweekt werden onder licht van een beperkt golflengtegebied (een bepaalde kleur), bleek dat bepaalde kleuren, die bij het wildtype remmend werken, dit effect niet meer of in mindere mate vertonen bij mutanten van specifieke genen. De afwezigheid van remming door rood en ver-rood licht (de genen *hy-1* en *hy-2*) bleek samen te gaan met de afwezigheid van het pigment fytochroom, dat geheel of gedeeltelijk verantwoordelijk gesteld moet worden voor de remming door rood en ver-rood. Mutanten van bv. *hy-4* bleken veel minder gevoelig voor blauw licht dan het wildtype. Deze genspecifieke hypocotylremmingsspectra geven sterke aanwijzingen voor de betrokkenheid van verschillende min of meer onafhankelijk werkende pigmenten bij deze reactie. Naast fytochroom komen dus zeer waarschijnlijk nog andere fotoreceptoren voor.

De laatste 4 hoofdstukken geven een aantal genetische details van bovengenoemde en enige andere genen. Mutatiefrequenties voor ethylmethaansulfonaat (EMS) en 2 soorten straling (snelle-neutronen en röntgen) worden beschreven in hoofdstuk 5. Deze werden vergeleken met frequenties van andere genen en met frequenties van genen bij gerst. Verschillende genen kunnen een significant verschillende mutatiefrequentie hebben.

Het is een goed gebruik dat nieuwe mutanten, vooral als ze onderwerp zijn van diepgaand onderzoek, op de genenkaart van het organisme gelocaliseerd worden. De genenkaart van *Arabidopsis* was echter vrij fragmentarisch. Teneinde onze genen te localiseren op één van de 5 *Arabidopsis* chromosomen is gebruik

gemaakt van trisomen (genotypen met een extra compleet chromosoom; hoofdstuk 6). Dit is echter eerst vooral gedaan voor genen die karakteristiek zijn voor reeds eerder in de literatuur beschreven koppelingsgroepen. Dit vanwege tegenstrijdige gegevens in de literatuur die een goede genetische beschrijving van verschillende *Arabidopsis* chromosomen tot nu toe moeilijk maakten. Tevens werden een aantal telotrisomen (genotypen met een extra half chromosoom) gevonden, die gebruikt zijn om centromeren van 3 van de 5 chromosomen te localiseren

Door middel van koppelingsanalyses zijn een groot aantal van de relatieve (genetische) afstanden van een 76-tal genen bepaald (hoofdstuk 7). Op grond van deze gegevens kon een gedetailleerde genenkaart geconstrueerd worden. Bij planten zijn alleen de kaarten van mais, gerst, de erwt en de tomaat gedetailleerder.

Niet-kiemende gibberelline gevoelige mutanten van *Arabidopsis* blijken zeer geschikt om verschillende mutaties van een dergelijk gen in kaart te brengen. De hiervoor benodigde zeldzame recombinanten zijn namelijk eenvoudig te herkennen als wildtype kiemplanten tussen duizenden niet-kiemende zaden per petrischaal. Het bleek mogelijk voor het eerst bij een plantengen een intern kloppende kaart te construeren, waarbij ook de volgorde van de mutaties t.o.v. andere genen van chromosoom 4 vastgesteld kon worden. Eén mutant bleek zich te gedragen als een intragene deletie.

CURRICULUM VITAE

Maarten Koornneef werd geboren op 20 september 1950 te De Lier. Hij behaalde in 1965 het MULO diploma aan de Marnix MULO te Maassluis. In 1968 werd het HBS-B diploma behaald aan het Groen van Prinsterer Lyceum te Vlaardingen. In datzelfde jaar werd begonnen met de studie aan de Landbouwhogeschool. Met het behalen (met lof) van het ingenieursdiploma in september 1972 werd de studie in de richting van plantenveredeling afgesloten. De keuzevakken in de doctoraalstudie waren de Erfelijkheidsleer, Tuinbouwplantenteelt en Plantensystematiek en -Geografie. Van oktober 1974 tot september 1976 was hij als plantenveredelaar in dienst van het groentenzadenveredelingsbedrijf Vandenberg B.V. te Naaldwijk, waarna hij in dienst trad bij de Landbouwhogeschool als wetenschappelijk medewerker van de vakgroep Erfelijkheidsleer. In de sectie botanische genetica onder leiding van Prof. J.H. van der Veen werd meegewerkt aan het onderwijs van de vakgroep en werd genetisch-fysiologisch onderzoek verricht aan *Arabidopsis thaliana*. Dit onderzoek werd voor een belangrijk deel uitgevoerd in samenwerking met de vakgroepen Plantenfysiologie (Dr. C.M. Karssen) en Plantenfysiologisch Onderzoek (Dr. C.J.P. Spruit) en heeft geleid tot het schrijven van dit proefschrift.