Regulation of seasonal patterns in seed dormancy

Regulatie van seizoengebonden wisselingen in kiemrust van zaden

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Regulation of seasonal patterns in seed dormancy

Proefschrift

ter verkrijging van de graad van doctor

in de landbouw- en milieuwetenschappen

op gezag van de rector magnificus

dr. H.C. van der Plas

in het openbaar te verdedigen

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Stellingen

1. Gezien het reversibele karakter van kiemrust is het noodzakelijk bij het onderzoek naar de regulatie steeds zowel rustbreking als rustinductie te bestuderen.
   Dit proefschrift.

2. Licht- en/of temperatuur-gestuurde veranderingen in het gehalte aan gibberellinen en in de gevoeligheid voor gibberellinen bepalen of er wel of niet kieming kan plaatsvinden; ze zijn echter niet primair verantwoordelijk voor de regulatie van kiemrust.
   Dit proefschrift.

3. Wisselingen in kiemrust van geïmbibeerde zaden zijn niet gecorreleerd met veranderingen in metabolische activiteit; deze eigenschap bevordert de overleving in de bodem.
   Dit proefschrift.

4. Uitspraken over de activiteit en capaciteit van de diverse ademhalingsroutes dienen altijd voorafgegaan te worden door toetsing op eventuele neveneffecten van de gebruikte remstoffen.
   Y. Morohashi. 1986. Journal of Experimental Botany 37: 262-269.
   O. Leprince, A. Van der Werf, R. Deltour and H. Lambers. 1992. Physiologia Plantarum 85: 581-588.

5. Het remmen van de kieming door een lage concentratie benzohydroxyzuur is geen bewijs dat de alternatieve ademhaling nodig is voor de kieming, zoals beweerd door Gui, Wang en Huang, maar kan ook op een neveneffect van benzohydroxyzuur wijzen.
   M.X. Gui, X.M. Wang and W.Y. Huang. 1991. Physiologia Plantarum 81: 403-407.

6. Trewavas stelt ten onrechte dat het bepalen van de gevoeligheid van afzonderlijke cellen zinvol is voor het inzicht in de regulatie van ontwikkelingsprocessen in weefsels.
   A. Trewavas. 1991. Plant, Cell and Environment 14: 1-12.

7. Het gebruik van de term receptorreserve voor receptoren die op een bepaald moment niet met ligand bezet zijn, suggereert ten onrechte dat deze receptoren in die toestand geen functie hebben in de gevoeligheid van een cel of weefsel.
   M.D. Hollenberg. 1985. In: Neurotransmitter receptor binding (H.I. Yamamura, S.J. Erma and M.J. Kuhan, eds.), pp. 1-39.
   H.W.M. Hilhorst. 1990. Plant Physiology 94: 1090-1095.

8. De vooruitgang in het hormoononderzoek aan planten is lange tijd voornamelijk bepaald door het op de markt komen van nieuwe technieken en te weinig door de ontwikkeling van nieuwe theoretische concepten.

9. Om modellen die de opkomst van onkruiden in het veld voorspellen bruikbaar te maken voor een effectievere onkruidbestrijding, moet het microklimaat in de bouwvoor met voldoende nauwkeurigheid voorspeld kunnen worden.

10. Bij het incident met het tropisch quarantaine-organisme *Thrips palmi* op drie ficusbedrijven heeft het belang van de glastuinbouwsector geprevaleerd boven dat van de betrokken telers. Deze afweging is in de schadeloosstelling onvoldoende naar buiten gekomen.

11. Het door de overheid opleggen van diverse milieumaatregelen aan tuinbouwbedrijven bedreigt het voortbestaan van met name kleine bedrijven en bedrijven waarbij de bedrijfsopvolging niet gegarandeerd is.

12. Van Dijk bestrijdt het hechten van een milieuvignet aan milieu-vriendelijk geproduceerde bloemen met het argument dat bloemen de mens dichter bij de natuur brengen. Echter, de milieu-onvriendelijke productie van sommige sierteeltgewassen brengt de ontmoeting van de mens met de natuur op de lange termijn in gevaar.
G. van Dijk, NCR en LUW.

13. Wanneer het wachtgeld van Assistenten in Opleiding die na contractbeëindiging nog geen andere baan hebben, wordt verhaald op universiteiten, dreigt het gevaar dat promovendi de status van student krijgen. De belangstelling voor promovendi-plaatsen zal hierdoor afnemen.

14. 'Als je wat verder kijkt, zie je gewoon een .....apothekersassistente, tegelzetter, servicemonteur'. Het wegnemen van vooroordelen tegen buitenlanders is niet gebaad bij het eerst suggereren van vooroordelen.

15. Het feit dat de rol van een klunzig persoon op tv vaak gespeeld wordt door een nederlandstalige die een limburgs accent probeert te imiteren, getuigt niet alleen van weinig fantasie, maar degradeert het limburgs bovendien tot een zangerig nederlands met een zachte g.

Stellingen behorende bij het proefschrift: Regulation of seasonal patterns in seed dormancy.

M.P.M. Derkx
Wageningen, 18 juni 1993.

Abstract

Derkx, M.P.M. 1993. Regulation of seasonal patterns in seed dormancy. Thesis, Agricultural University, Wageningen, The Netherlands. English and Dutch summaries.

Buried seeds of many wild species pass annually through a pattern of induction and release of dormancy. These reversible changes in dormancy may be repeated for numbers of years when seeds are deprived from light and other germination-stimulating factors, and are a highly useful adaptation to the climatic conditions which prevail at the habitat.

This thesis studied the regulation of germination and reversible changes in dormancy in two related species *Arabidopsis thaliana* (L.) Heynh. and *Sisymbrium officinale* (L.) Scop. In *A. thaliana*, mutants that lack the capacity to synthesize GAs and/or are insensitive to GAs were compared with wild type.

Germination of both species is dependent on active phytochrome (Pfr). In seeds of *S. officinale* Pfr action absolutely requires the co-action of nitrate, seeds of *A. thaliana* only slightly respond to nitrate. It has been suggested that light stimulates GA biosynthesis since the need for light (and nitrate) can be replaced by gibberellins (GAs). Reversal of light-stimulated germination by tetcyclasis, an inhibitor of GA biosynthesis, supported this conclusion. Preliminary GA determinations showed that irradiation gave elevated levels of GA_1, GA_4 and GA_9 in *A. thaliana*. GA_4 was the most active GA. Light also increased sensitivity to GAs in both species.

Germination data fitted as logistic dose response curves showed that (soil) temperature is the most important factor in the mechanism by which seeds sense the time of the year. It regulates sensitivity to light (and to nitrate). Upon perception of light (and nitrate) some signal-transduction chain is initiated. In this chain GA biosynthesis is stimulated. Moreover, GA sensitivity is enhanced. Temperature can also directly increase GA sensitivity. Germination invariably depends on the level of bio-active GAs and the available GA-response system. When either factor is limiting, germination is prevented. Both factors do not necessarily accompany changes in dormancy and are therefore not primarily regulatory.

In *S. officinale* it was shown that changes in dormancy cannot be explained by changes in respiratory activity. During prolonged incubation levels of O_2 uptake and CO_2 release were not affected by dormancy-breaking and -inducing treatments. Germination clearly required an increased respiratory activity. The contribution of different respiratory pathways changed during prolonged incubation. A role of the alternative pathway in the regulation of dormancy of *S. officinale* seeds is unlikely.

Keywords: alternative pathway, *Arabidopsis thaliana* (L.) Heynh., CO_2 release, cytochrome pathway, dormancy, germination, gibberellin, hormone mutant, light, nitrate, O_2 uptake phytochrome, receptor, respiration, seasonal periodicity, seed, sensitivity, *Sisymbrium officinale* (L.) Scop., temperature, weed.

If you can look into the seeds of time,
And say which grain will grow, and which will not,
Speak then to me, who neither beg nor fear
Your favour nor your hate.

(Macbeth)

Aan mijn ouders
Voor John

Voorwoord

Aan de totstandkoming van dit proefschrift hebben vele mensen een steentje bijgedragen, die ik graag hierbij wil bedanken.

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Evert Vermeer voerde de gibberellinen-bepalingen in Hoofdstuk 3 uit en heeft zuivere gibberellinen ter beschikking gesteld.

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Arabidopsis thaliana zaad dat zowel gibberellinen-deficiënt alsook ongevoelig voor gibberellinen is, werd beschikbaar gesteld door Prof. Dr. Maarten Koornneef van de vakgroep Erfelijkheidsleer.

Technische ondersteuning kwam van Ruth van der Laan en Ben C.A. van der Swaluw.

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Hoewel het onderzoek zich afspeelt binnen de muren van een vakgroep, is ook steun van daarbuiten onontbeerlijk. Mijn ouders dank ik hartelijk voor de mogelijkheid die ze mij geboden hebben om te gaan studeren en voor hun belangstelling op alle fronten tijdens mijn studie- en promotie-tijd. John dank ik voor alle steun en afleiding in de afgelopen jaren.

Ria

Contents

List of abbreviations and symbols

ABA	abscisic acid
aba	abscisic acid-deficient mutant in *Arabidopsis thaliana*
abi1, *abi3*	abscisic acid-insensitive mutants in *Arabidopsis thaliana*
ATP	adenosine triphosphate
BHAM	benzohydroxamic acid
Fl_{50}	fluence required for half-maximum response
$g(i)_{alt}$	maximal contribution of the alternative pathway at a given concentration of BHAM
$g(i)_{cyt}$	maximal contribution of the cytochrome pathway at a given concentration of KCN
GA	gibberellin
$[GA]_{50}$	GA dose required for half-maximum response
ga1	gibberellin-deficient mutant in *Arabidopsis thaliana*
ga1-1	gibberellin-deficient mutant in *Arabidopsis thaliana*, isolation NG5
ga1-2	gibberellin-deficient mutant in *Arabidopsis thaliana*, isolation 659
gai	gibberellin-insensitive mutant in *Arabidopsis thaliana*
GC	gas chromatograph
GC MS(D)	gas chromatography mass spectrometry (detection)
gib1	gibberellin-deficient mutant in tomato
[H]	applied dose concentration of ligand
$[H]_{50}$	dose concentration required for half-maximum response
HRec	ligand-receptor complex
(RP) HPLC	(reversed-phase) high performance liquid chromatography
k_1, k_{-1}	kinetic association and dissociation constants
K_D	dissociation constant of a ligand-receptor complex
k_r	rate constant for the rate-limiting step in the series of events leading from the formation of HRec to Response
$[KNO_3]_{50}$	KNO_3 concentration required for half-maximum response

KRI	kováts retention indices
LD	long day
LFR	low-fluence-range
LNR	low-nitrate-range
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
nd	not determined
p	Hill-coefficient
Pfr	farred-absorbing form of phytochrome
Pr	red-absorbing form of phytochrome
PPP	pentose phosphate pathway
PVP	polyvinylpyrrolidone
QAE	quaternary ammonium ether
R	response
R_{max}	maximum response
R_{min}	minimum response in the absence of exogenous [H]
Rec	receptor
RQ	respiratory quotient
SD	standard deviation
SHAM	salicylhydroxamic acid
V_{alt}	capacity of the alternative pathway
v_{alt}	activity of the alternative pathway
v_{cyt}	activity of the cytochrome pathway
v_{res}	residual O_2 uptake in the presence of KCN and BHAM
v_{tot}	total rate of O_2 uptake in the absence of inhibitors
VLFR	very-low-fluence-range
VLNR	very-low-nitrate-range
ρ	fraction of a pathway that is engaged
ρ_{alt}	fraction of the capacity of the alternative pathway that is engaged.
ρ_{cyt}	fraction of the cytochrome pathway that is engaged

Chapter 1

General introduction

The emergence of many annual weed species is often restricted to certain periods of the year. The timing differs from species to species. In temperate regions, emergence often occurs within one or two months in spring, sometimes followed by some additional flushes in summer. Strict spring-germinating species are for instance *Ambrosia artemisiifolia* (Baskin and Baskin, 1980) and *Polygonum persicaria* (Bouwmeester and Karssen, 1992). In a number of species, for example *Chenopodium album* (Baskin and Baskin, 1977a; Bouwmeester, 1990), *Portulaca smallii* (Baskin and Baskin, 1987) and *Spergula arvensis* (Karssen *et al.*, 1988; Bouwmeester, 1990) additional germination flushes were observed during summer. Species that originate from climates with a hot dry summer and a cool humid winter like *Arabidopsis thaliana* (Baskin and Baskin, 1983) and *Lamium purpureum* (Baskin and Baskin, 1984) mainly germinate in autumn. They often survive the winter as rosette plants and start to elongate in spring. Some autumn-germinating species, like *Veronica arvensis* (Roberts and Boddrell, 1983), *Veronica hederifolia* (Roberts and Lockett, 1978a) and *Aphanes arvensis* (Roberts and Neilson, 1982) show a second germination flush in spring.

This periodicity of seasonal emergence may be due to seasonal fluctuations in the number of seeds in the soil. This is particularly true for grasses that form transient seed banks in which none of the seeds remain alive in the soil for more than one year (Thompson and Grime, 1979). However, dicotyledonous annual species form large persistent seed banks of which the size outnumbers the annual input of seeds. Well-documented studies indicate that survival of seeds in these seed banks may be as long as decades to centuries (Chippindale and Milton, 1934; Baskin and Baskin, 1977b; Ødum, 1978; Kivilaan and Bandurski, 1981; Priestley and Posthumus, 1982). Emergence from such seed banks is strongly stimulated by frequent soil cultivation. The periodicity of emergence, however, is not essentially affected by soil disturbance (Roberts and Feast, 1973; Roberts and Lockett, 1978b), indicating that variability in the number of seeds in soil cannot account for the observed emergence patterns.

It has been demonstrated for many species that changes in seed dormancy can explain the timing of emergence (Karssen, 1982; Baskin and Baskin, 1985). Some of the first direct evidence that buried seeds are not constantly ready to germinate came from studies on germination periodicity in *Polygonum aviculare* (Courtney, 1968) and several other species (Taylorson, 1970, 1972). Since then, a wealth of information became available about changes in germination capacity in buried seeds (e.g. Baskin and Baskin, 1977a, 1983, 1984, 1990; Roberts and Lockett, 1978a, b; Karssen, 1980/81b; Bouwmeester and Karssen, 1989, 1992). In all these studies comparable methods were used. Seeds packed in envelopes that permit good contact with soil, were planted in soil outdoors and after several intervals germination capacity was determined under laboratory conditions.

By testing germination capacity at a range of temperatures and in the absence and presence of germination-stimulating factors like for instance light and nitrate, it became clear that the range of conditions over which germination can proceed, becomes wider during alleviation of dormancy whereas it narrows during dormancy induction, indicating that dormancy is not an all-or-nothing property (Vegis, 1964; Karssen, 1980/81a, 1982; Baskin and Baskin, 1985; Bouwmeester, 1990; Bouwmeester and Karssen, 1992).

Definitions of dormancy

An excessive number of terms has arisen over the years to classify various aspects of dormancy (Roberts, 1972; Nikolaeva, 1977; Bewley and Black, 1982b; Karssen, 1982; Lang, 1987; Lang *et al.*, 1987). The abundance of terminology can easily give rise to quite some confusion. Classification can be based on (a) the apparent origin (e.g. environmental, genetic, metabolic, physiological or structural), (b) the depth of dormancy (true or relative) and (c) timing of dormancy (innate, primary, secondary). Also definitions of dormancy are numerous (e.g. Amen, 1968; Taylorson and Hendricks, 1977; Salisbury and Ross, 1978; Bewley and Black, 1982b). The statement by Hobson (1981) that '*there may be as many definitions of dormancy as there are investigators concerned with the subject*' clearly demonstrates the confusion of this term and the lack of knowledge about underlying physiological mechanisms.

Unfortunately, many definitions of dormancy fully neglect the existence of a distinction

between breaking of dormancy and the eventually following process of germination. Similarly, a distinction between inhibition of germination and induction of dormancy is often not recognized. Simpson (1990), however, made this distinction and defined dormancy as *'Temporary failure of a viable seed to germinate, after a specified length of time, in a particular set of environmental conditions that later evoke germination when the restrictive state has been terminated by either natural or artificial means.'*

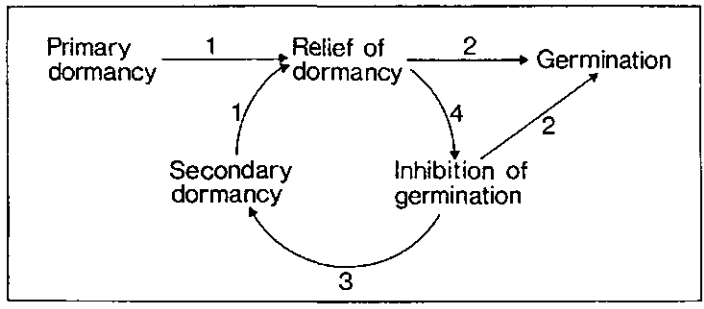

Fig. 1.1 Schematic presentation of changes in seed dormancy. Numbers are referred to in the text. After Karssen (1982).

So long as knowledge about dormancy mechanisms is so limited, a terminology based on timing or degree of dormancy is preferred above one based on the origin. Therefore, the terminology used in this thesis is based on Karssen (1982) (Fig. 1.1). Primary dormancy develops during seed maturation on the mother plant. It prevents precocious germination when the seeds are still on the mother plant. Primary dormancy can be relieved by dry storage, often referred to as dry after-ripening or by imbibition at certain temperatures over certain time periods (1). Germination can now proceed, provided that environmental conditions allow germination (2). These conditions involve basic requirements for water, oxygen and an appropriate temperature, and depending on the species, other factors like light and nitrate may be highly stimulatory. If these requirements are not fulfilled, germination is inhibited (4). This state is sometimes referred to as 'enforced dormancy' (Roberts, 1972).

Because germination can occur as soon as the environmental restrictions are terminated, Hilhorst and Karssen (1992) proposed the term of 'pseudo-dormancy' for this state. Prolongation of the environmental restrictions can result in re-induction of dormancy (3) (Karssen, 1980/81a). Because it develops after dispersal in seeds that have emerged from primary dormancy or in seeds that were non-dormant at harvest, this state of dormancy is called secondary dormancy, that can be relieved and re-induced. Three successive annual cycles have been demonstrated in seeds of *Polygonum aviculare* L. (Baskin and Baskin, 1990) and *Polygonum persicaria* L. (Bouwmeester and Karssen, 1992). Therefore, it is likely that this cycle can be repeated for numbers of years until the seeds germinate or die.

It is evident that dormancy is of indubitable value for the reduction of the germination chances under environmental conditions that are unfavourable for seedling establishment. Spring-germinating species are dormant in summer and autumn, dormancy is relieved during winter and they can germinate in spring, provided environmental conditions are suitable. Species that germinate in autumn are dormant in winter and spring, dormancy is alleviated in summer allowing the seeds to germinate in autumn under suitable conditions.

The ability of plants to produce dormant seeds presumably evolved more than 30 million years ago (Mapes *et al.*, 1989). From the observation that palaeozoic fossil conifer cones bore mature seeds with well-developed cotyledonary embryos, the authors concluded that there was a significant delay between fertilization and seed germination in the earliest conifers, which indicates that quiescence before seed germination may have initiated the evolution of seed dormancy. This emphasizes the importance of dormancy for successful survival of species. Dormancy obviously is part of a strategy that ensures survival in an everchanging environment.

In temperate regions, temperature seems to be the main regulatory factor in the control of dormancy. It has been postulated that breaking of primary dormancy and induction of secondary dormancy are simultaneous processes. Totterdell and Roberts (1979) stated that only induction of secondary dormancy was temperature dependent in *Rumex crispus* and *R. obtusifolius* seeds. Alleviation of dormancy occurred equally well at all temperatures below 15 °C. Cone and Spruit (1983), however, demonstrated in *Arabidopsis thaliana* seeds that both processes are temperature dependent. The net result of both processes determines

whether a certain exposure to a fluctuating temperature regime or to constant temperatures can be classified as dormancy breaking or -inducing.

Apart from temperature, dormancy can also be modified by some other environmental conditions, like nitrate (Karssen, 1980/81a; Hilhorst, 1990a), oxygen- and carbon dioxide levels in soil (Karssen, 1982) and dehydration (Mayer and Poljakoff-Mayber, 1982; Karssen *et al.*, 1988; Bouwmeester, 1990). In desert regions dehydration may even play a dominant role in dormancy regulation.

Germination

Light

After the alleviation of dormancy seeds are capable to germinate, but often still absolutely depend on certain environmental conditions. Light can be regarded as the most extensively studied germination-stimulating factor. It is generally accepted that light acts through the photoreversible protein phytochrome (Taylorson, 1987). In darkness the pigment is in an inactive form with an absorption maximum at 660 nm, that can absorb red light and is therefore designated as Pr. Upon red light irradiation Pr is quickly converted into the active Pfr form that has an absorption maximum at 730 nm. Absorption of farred irradiation by Pfr causes reversion to Pr. This reversion can also occur in darkness, although at a strongly reduced rate.

Because many phytochrome-mediated responses did not correspond with measured levels of Pfr, especially when very low fluence levels induced a significant response, it became apparent that phytochrome does not simply act in the monomeric Pfr form (Raven and Shropshire, 1975). Evidence is growing that phytochrome behaves as a dimer (Brockmann *et al.*, 1987) that exists in three forms, namely Pr:Pr, Pr:Pfr and Pfr:Pfr. The first form is inactive, the second form is induced by very low fluence values, resulting in a very-low-fluence-response (VLFR) and the third form results from irradiation by low fluence values, resulting in a low-fluence-response (LFR) (VanderWoude, 1985). The fluence requirements of the VLFR and LFR differ by approximately 10000 fold. The VLFR is not farred

reversible and can be saturated with red light as well as with farred irradiation (Small *et al.*, 1979; Mandoli and Briggs, 1981). Also green safelight (520 nm) may induce a VLFR (Blaauw-Jansen and Post, 1985; Kendrick and Cone, 1985).

Sensitivity to low amounts of Pfr has been induced by several treatments like chilling (VanderWoude and Toole, 1980; Cone *et al.*, 1985; VanderWoude, 1985), imbibition at high temperatures (Cone *et al.*, 1985; Kendrick and Cone, 1985; Taylorson and Dinola, 1989), treatment with anaesthetics (VanderWoude, 1985) and treatment with gibberellins (De Petter *et al.*, 1985; Rethy *et al.*, 1987). Because in addition to this VLFR a response was induced in the LFR as well, the fluence response curves exhibited a biphasic character.

Light and gibberellins

Although the stimulatory effect of light on germination has been studied extensively, a black box exists between the perception of the light stimulus and the final germination response. An early event may be the association of Pfr with a membrane (Taylorson, 1982). However, immunocytochemical studies give equivocal answers to the question whether Pfr is indeed associated with membranes. Even if so, it has to be answered whether this binding is relevant for its function. In oat cells most of the sequestered Pfr was associated with structures that have no morphologically identifiable membranes surrounding them. There is no convincing evidence that the inactive Pr form associates with any particular membrane or organelle. Pr is distributed uniformly throughout the cytosol (Pratt, 1986; Taiz and Zeiger, 1991).

Since gibberellins (GAs) can replace light in several species, it was proposed that Pfr may act by inducing the synthesis of GAs within the seed. Several attempts have been made to correlate promotion of germination by Pfr with increased levels of GAs in seeds. Taylor and Wareing (1979) for example reported rapid increases in GA levels shortly after irradiation of seeds of *Picea sitchensis* and *Pinus sylvestris*. In lettuce seeds an increase in GA_9 was observed following exposure to light (Bianco and Bulard, 1981). Recently, the isolation of *Arabidopsis thaliana* mutants that no longer require light for germination (*det*) has permitted the identification of some genes that may be involved in GA biosynthesis

(Chory *et al.*, 1989; Chory and Peto, 1990). These *det* mutants were obtained from a population of mutagenized dark-grown seedlings, that displayed many phenotypic characteristics of light-grown wild-type plants. Both *det1* and wild-type plants were transformed by introducing photoregulated promoters fused to screenable marker genes. It was demonstrated that the *DET1* gene may encode a transcriptional repressor. Thus, GA biosynthetic genes may be negatively controlled by the *DET1* gene at the level of transcription. The positive effect of light on wild-type seeds may result from the Pfr-inhibited *DET1* synthesis or action. Nambara *et al.* (1991) demonstrated that the *det* mutants still require GA for germination, that is synthesized independently of light.

The role of GA biosynthesis in Pfr-induced germination of lettuce seeds has been challenged by Hazebroek and Coolbaugh (1991). From the observation that kaurene oxidation is not an obligate requirement for germination coupled with the fact that kaurene metabolism occurs in light, it was concluded that kaurene oxidation occurs after germination has been induced, and is not directly involved in Pfr-dependent processes associated with induction of germination. Also in pea, there is no evidence that germination requires *de novo* synthesis of GAs (Sponsel, 1985).

It has also been suggested that Pfr releases the available GA from a compartment to its site of action without an increase in amount (Taylorson, 1982). In spinach plants, in which exposure to long days (LD) results in stem elongation, it was demonstrated that LD conditions result in an increase of GA_1 and GA_{20} and a decrease in GA_{19} and GA_{53} (Talon *et al.*, 1991).

However, it is not likely that GA biosynthesis or availability can alone account for the light-induced germination. Because GA action of several species was dependent on light (Taylorson and Hendricks, 1976; Fredericq *et al.*, 1983), it was concluded that light also increased sensitivity to GAs.

Light effects on both GA biosynthesis and GA sensitivity were found in *Sisymbrium officinale* seeds (Hilhorst *et al.*, 1986). By use of the GA-biosynthesis inhibitor tetcyclasis (Rademacher and Jung, 1981) it was demonstrated that germination of *S. officinale* seeds was preceded by *de novo* synthesis of GAs.

GA action in seeds

The mechanism of GA action in germination has been extensively studied in barley and other cereals. In the aleurone layer GAs stimulate the production of hydrolases, chiefly α-amylase, that digest the contents of the starchy endosperms (e.g. Bewley and Black, 1982a). Calcium is required for this action (Bush *et al.*, 1989). Both cytosolic and endoplasmic reticulum calcium levels increased as a result of GA action (Bush, 1992). It was demonstrated that GAs influence the pattern of gene expression and protein synthesis. The expression of some genes was up-regulated, whereas others were down-regulated (Jacobsen and Gubler, 1992; Rogers *et al.*, 1992). The relation to hormone receptors has still to be resolved.

In tomato seeds GAs induced the enzymatic hydrolysis of the galacto-mannan-rich endosperm cell walls by increasing the activity of endo-ß-mannanase, mannohydrolase and α-galactosidase. It was hypothesized that GAs are synthesized in the embryo and transported to the endosperm, where they induce the activity of endosperm-weakening enzymes (Groot *et al.*, 1988). The GA-deficient *gib1* mutant of tomato did not germinate in the absence of applied GAs. However, removing of the embryo-surrounding layers overcame the block to germination, as applied GAs also did. The mechanical resistance of the endosperm cells around the radicle tip could be decreased by exogenous GAs, both in *gib1* and wild-type seeds (Groot and Karssen, 1987).

Nitrate

The stimulatory action of nitrate on germination has been described for many graminaceous and dicotyledonous weed species (Hendricks and Taylorson, 1972; Roberts and Smith, 1977; Vincent and Roberts, 1977). So far, there is no general agreement as to its mechanism of action. It is not likely that the enhancement effect of nitrate is an unspecific nutrition effect, because otherwise ammonium should also increase germination, which often is not the case (Haas and Scheuerlein, 1990). Roberts and Smith (1977) proposed that nitrate acts as an alternative electron acceptor. As a result of increased re-oxidation of NADPH to

$NADP^+$ the operation of the pentose phosphate pathway (PPP) should be stimulated at the expense of glycolysis, citric acid cycle and terminal oxidation reactions. A possible involvement of the PPP on the stimulation of germination was based on observations that inhibitors of glycolysis and following reactions can stimulate germination. Hendricks and Taylorson (1975) proposed that nitrate inhibits catalase activity which spares hydrogen peroxide for reconversion of NADPH to $NADP^+$, thus indirectly promoting the activity of the PPP. Bewley and Black (1982b), however, raised pertinent questions about both proposals because the role of the PPP in regulating the breaking of dormancy and the stimulation of germination is still in question. This will be dealt with later on.

Both theories about nitrate action involve the reduction of nitrate to nitrite by nitrate reductase. Whether or not this reduction is required for germination has been studied in *Sisymbrium officinale* seeds by Hilhorst and Karssen (1989). It was demonstrated that nitrate levels remained constant during the first 8 hours following a red light irradiation, in which induction of germination occurred. During the next 8 hours nitrate levels declined, indicating the presence of nitrate reductase. The authors concluded that this enzyme activity was related to the onset of seedling growth rather than to the induction of germination and that therefore nitrate in the unreduced state may evoke an effect, as was already proposed by Hendricks and Taylorson (1972). On the contrary, nitrate action in fern-spore germination has been shown to be mediated by nitrate reductase (Haas and Scheuerlein, 1991).

The actual mechanism of nitrate action is still unclear. Hilton and Thomas (1986) suggested that nitrate stimulates germination by an increased oxygen uptake. However, it appears more likely that the increased oxygen uptake was the result and not the cause of the germination process. Hilhorst and Karssen (1988) proposed that nitrate acts somehow as an indispensable co-factor for Pfr action. Hilhorst (1990c) suggested that the small nitrate ion may alter phytochrome receptors, thereby allowing the binding of Pfr:Pfr dimers to the activated receptors. Haas and Scheuerlein (1990, 1991) assumed in *Dryopteris filix-mas* spores a specific effect on one of the early steps in the signal-transduction chain initiated by the formation of Pfr. Since the action of nitrate in these spores can be substituted by Pfr action, nitrate is not absolutely required for germination (Haas and Scheuerlein, 1991), in contrast to *S. officinale* seeds (Hilhorst and Karssen, 1989).

When nitrate exerts a stimulatory effect on germination in the absence of light, as was found for *Polygonum persicaria* seeds after 3-5 weeks chilling in darkness (Karssen, 1980/81b), regulation independent of Pfr action may be proposed. However, the capacity for dark germination may also be caused by the threshold levels of 'pre-existing' Pfr in the seeds (Cone and Kendrick, 1985).

Light and nitrate

Interactions between light and nitrate are not exclusive for *S. officinale* seeds (Karssen and De Vries, 1983; Hilhorst *et al.*, 1986) but are common in many species (Vincent and Roberts, 1977, 1979; Roberts and Benjamin, 1979; Williams, 1983; Hilton, 1984, 1985; De Petter *et al.*, 1985; Probert *et al.*, 1987; Grubišić and Konjević, 1990; Singh and Amritphale, 1992). Although both light and nitrate stimulated germination in *Echinocloa colonum* seeds, no interaction between both factors was detected in this species (Ellis *et al.*, 1990).

In *S. officinale* seeds nitrate was required for the Pfr-induced GA biosynthesis, whereas its action in Pfr-stimulated GA sensitivity was not needed (Hilhorst *et al.*, 1986). In *Chenopodium album* seeds, however, nitrate increased the response to applied GAs (Saini *et al.*, 1985b). An increase in sensitivity to Pfr due to nitrate has been reported for *Kalanchoë blossfeldiana* seeds (De Petter *et al.*, 1985).

Other germination stimulating factors

Besides light and nitrate several other environmental and chemical factors are known to stimulate germination, like alternating temperatures (Steinbauer and Grigsby, 1957), ethylene (Schonbeck and Egley, 1981; Egley, 1984; Saini *et al.*, 1985a, b; Kępczyński, 1986) and several other nitrogenous compounds like nitrite, thiourea, hydroxylamine, cyanide and azide (Major and Roberts, 1968; Roberts and Smith, 1977; Upadhyaya *et al.*, 1982; Adkins *et al.*, 1984a). Interactions between these factors, either additive or synergistic, have also been frequently reported and are reviewed by Karssen and Hilhorst (1992) and Probert (1992).

Dormancy

Although mechanisms of action of germination-stimulating factors like light and nitrate are only poorly understood, knowledge about regulation of dormancy even seems to be more limited.

Different categories of dormancy can be recognized, possibly each with a different dormancy mechanism. In addition, it seems likely that different dormancy mechanisms may operate simultaneously or after each other within one species. Until now, only few dormancy mechanisms are known in detail, like embryo immaturity and seed coat hardiness. Embryos of for instance *Fraxinus nigra* (Vanstone and LaCroix, 1975) and many Umbelliferae species like *Heracleum sphondylium* (Stokes, 1952) and *Apium graveolens* (celery) (Van der Toorn and Karssen, 1992) are morphologically immature when the dispersal unit is released, and require a period of further development before they become able to germinate.

Mechanical restraint by the seed coat has been reported for several *Syringa* species (Junttila, 1973) and for *Xanthium pennsylvanicum* seeds (Esashi and Leopold, 1968). In these species the embryo cannot generate sufficient thrust to overcome the mechanical barrier of the seed coat. In *X. pennsylvanicum* seeds other dormancy mechanisms operate simultaneously (Bewley and Black, 1982b). Dormancy in many *Leguminosae* species can be attributed to seed coat imperviousness to water, which is so effective that germination may be delayed for many years (Rolston, 1978) or even centuries as was the case in *Nelumbo nuciferea* seeds that were found buried in China in 1952 (Priestley and Posthumus, 1982).

Most studies of dormancy have concentrated on breaking of dormancy only. Because dormancy in many species is a reversible process, it is evident that a satisfactory explanation of the control of dormancy in these species has to include this reversible character.

Various hypotheses have been proposed to account for the possible mechanism of dormancy. These include hormonal regulation, involvement of membranes and metabolic regulation.

Hormonal regulation

The idea that the regulation of seed dormancy may involve interaction between natural promotive hormones, such as GAs and cytokinins and the inhibitory hormone abscisic acid (ABA), was developed 40 years ago by Luckwill (1952). The abundant quantities of hormones in seeds at certain stages of development and effects of applied growth regulators resulted in widespread support for the balance theory, in which the onset, maintenance and termination of dormancy were regulated by simultaneously operating promoters and inhibitors of germination (Amen, 1968). However, the theory has also been criticized by several authors, mainly because it is too simplistic (Wareing and Saunders, 1971; Taylorson and Hendricks, 1977; Black, 1980/81; Bewley and Black, 1982b; Wareing, 1982; Roberts and Hooley, 1988). It is evident that experiments with applied growth regulators can never provide final proof that endogenous hormones are indeed involved in dormancy regulation and germination. On the other hand, absence of effects of applied growth regulators is not an argument against their regulatory role. Lack of penetration, inactive forms or low sensitivity may account for the absence of an effect (Wareing, 1982; Karssen *et al.*, 1989).

ABA

Karssen *et al.* (1983) convincingly demonstrated that in *A. thaliana* the onset of primary dormancy on the mother plant involves the action of ABA. In wild-type seeds dormancy developed during seed maturation but not in the ABA-deficient mutant (*aba*). Dormancy is also much reduced in the ABA-insensitive mutants (*abi1, abi3*) (Koornneef *et al.*, 1984). Reciprocal crosses between wild type and *aba* mutant showed that the onset of dormancy correlated well with the presence of embryonic ABA rather than with maternal ABA (Karssen *et al.*, 1983). In *Helianthus annuus* (Le Page-Degivry *et al.*, 1990) and apple (Le Page-Degivry, 1990) embryo dormancy was initiated immediately after endogenous ABA reached its highest value. Application of fluridone to *H. annuus* seeds prevented both ABA biosynthesis and the development of dormancy. However, fluridone did not prevent the development of dormancy when it was applied after the ABA level in the seed declined. On the contrary, there appears to be no correlation between ABA content and the level of

dormancy in wheat. ABA concentrations in a sprouting-resistant (highly dormant) and a sprouting-susceptible (low dormant) cultivar were closely similar (Walker-Simmons, 1987).

The involvement of ABA in the maintenance and termination of dormancy in the mature seed is probably minimal in several species, as there is little or no carry-over of ABA into the mature seed, as was shown for instance in *A. thaliana* seeds (Karssen *et al.*, 1983). In apple, however, dormancy seems to be maintained by the continued presence of ABA (Le Page-Degivry, 1990). During a dormancy-terminating chilling treatment of apple seeds the level of ABA in the axis even increased (Singh and Browning, 1991), indicating that loss of dormancy is not caused by a decline in ABA levels. A role of ABA in the maintenance of dormancy was also suggested in wheat, as dormant seeds exhibited prolonged expression of ABA-responsive genes (Morris *et al.*, 1991). In *Corylus avellana* seeds ABA levels fell equally during chilling and high temperature treatment, although only the former was effective in breaking of dormancy (Williams *et al.*, 1973). Mature lettuce seeds may contain considerable amounts of ABA. It is supposed that this ABA is required to suppress reserve mobilization before germination commences (Dulson *et al.*, 1988).

The mechanism of ABA action in dormancy induction and eventually in its maintenance is not known (Black, 1991). The expression of certain genes might be suppressed by ABA. Alternatively, the expression of other genes might be up-regulated. Black (1991) further raised the possibility that ABA caused the formation of a spectrum of proteins. Namely, in the non-dormant recombinant mutant seeds of *A. thaliana* (*aba/abi3*) there is impaired synthesis of late abundant storage proteins (Koornneef *et al.*, 1989). Moreover, embryonic axes of dormant wheat seeds form some heat-stable proteins in response to ABA which are not similarly effective in non-dormant seeds (Ried and Walker-Simmons, 1990).

GAs

Although developing seeds are rich sources of GAs (Sponsel, 1987), no evidence is presented that GAs are somehow involved in the onset of dormancy on the mother plant. Karssen and Laçka (1986) demonstrated in *A. thaliana* that whether or not seeds are able to synthesize GAs, is not relevant for the induction of dormancy. Both wild-type and GA-deficient (*ga1*) seeds develop dormancy. Studies with GA-deficient mutants of *A. thaliana*

(Barendse *et al.*, 1986) and tomato (Groot *et al.*, 1987) showed that GAs are also not required for seed growth. The rate of fruit growth, however, was clearly influenced by GAs.

Because exogenous GAs often replace the requirement for a dormancy-breaking chilling treatment, it has been frequently hypothesized that chilling elevates GA levels and thereby breaks dormancy. However, there have been no consistent demonstrations that chilling precipitates an increase in GA level in a pattern related to breaking of dormancy. Many measurements of GA levels have been carried out using bio-assays. Modern gas chromatography mass spectrometry (GC MS) techniques will definitely enlarge the knowledge about the involvement of GA levels in breaking of dormancy. There are however good arguments to assume that the *capacity* to synthesize GAs, rather than GA levels themselves, is regulatory. Chilling of hazel seeds, for instance, resulted in no appreciable increase in GA levels. However, when the now non-dormant seeds were transferred to high temperatures the capacity to synthesize GA_1 and GA_9 rose dramatically (Williams *et al.*, 1974). Analogously, Metzger (1983) concluded in *Avena fatua* that after-ripening does not cause a direct change in the levels of GAs during dry storage but induces a greater capacity during the following imbibition period. Further evidence that GA levels themselves are not regulatory in breaking of dormancy comes from studies with a GA-deficient *ga1* mutant of *A. thaliana*. A short chilling treatment enhanced the responsiveness of both *ga1* and wild-type seeds in darkness. Thus, dormancy breaking is not dependent on GA biosynthesis (Karssen *et al.*, 1989).

The sensitivity concept

The lack of correlation between hormone levels and a developmental event that might be hormonally regulated, may indicate that sensitivity to a hormone rather than the hormone level itself is regulatory. The concept of sensitivity has received much attention during the past ten years (Trewavas, 1981, 1982, 1991; Guern, 1987; Roberts and Hooley, 1988). It was indeed demonstrated that embryo responsiveness to exogenous ABA was highly correlated with the induction of dormancy during seed development in wheat (Walker-Simmons, 1987). Similarly, embryos of high-temperature-induced dormant and non-dormant *Avena sativa* seeds varied in their sensitivity to exogenous ABA (Poljakoff-Mayber *et al.*,

1990). An increase in sensitivity to applied GAs as a result of a short chilling pre-incubation treatment was reported for otherwise GA_3-insensitive aleurone tissue of wheat (Singh and Paleg, 1984) and *ga1* seeds of *A. thaliana* (Karssen *et al.*, 1989).

Conclusions on hormone sensitivity are mainly based on the interpretation of applied hormone response curves. Actually, the term sensitivity is too imprecise. It is obvious that it includes the number of hormone binding sites or more specifically the number of hormone receptors. Moreover, the affinity of the receptor for the hormone and the overall capacity of the responding system to respond to the number of occupied receptors may contribute to the observed sensitivity (Firn, 1986). In contrast with mammalian physiology, attempts to quantify hormone sensitivity have only started recently (Weyers *et al.*, 1987; Fitzsimons, 1989; Weyers and Paterson, 1992). This delay may be attributed to the regenerative, developmental and organizational plasticity of plant cells related to the sessile life-style of plants (Trewavas, 1981). The complexity of responses to environmental and hormonal signals that provide the basis for plant growth and development has long been recognized (Learned, 1992). Whereas a limited number of hormones may be involved in a large variety of developmental events in plants, well-defined systems control physiological activities in well-defined animal organs. In plants coordination between tissues and cells is much less than in animals, and plants also lack the pronounced differentiation between sensory and responding cells and tissues (Trewavas and Gilroy, 1991).

Involvement of membranes

Evidence that dormancy is causally related to some property of cell membranes came from studies in which dormancy was released by anaesthetic-like substances like n-propanol and ethanol (Taylorson and Hendricks, 1980/81; Adkins *et al.*, 1984c; Larondelle *et al.*, 1987). Prevention of this increase by application of external pressure presents an argument for membrane action (Hendricks and Taylorson, 1980; Taylorson, 1988). However, Taylorson (1991) recently reported that stimulation of germination of *Echinochloa crus-galli* seeds by n-butanol could not be reversed by pressure, indicating that pressure does not simply prevent the action of alcohols as previously thought, but may have an action of its

own. The quantification of leakage components after imbibition of *Rumex crispus* seeds at temperatures that either allow or disallow red light-induced germination after transfer to 20 °C did also not present circumstantial evidence that membranes are involved in the release from dormancy (Taylorson and Dinola, 1990). In addition, the determination of transition temperatures could not prove the involvement of membranes in dormancy breaking (Taylorson and Dinola, 1990).

In the same laboratory the composition of proteins in membranes was monitored in dormant and non-dormant *E. crus-galli* seeds (Di Nola and Taylorson, 1989; Di Nola *et al.*, 1990). It was demonstrated that the transition from a dormant to a non-dormant state as a result of brief high temperature exposure or treatment with n-propanol was associated with synthesis of specific proteins and a decrease in content of others in the plasma membrane. These changes may influence the physical nature of lipid-bilayers in membranes. Also phase-transitions may depend on protein composition. No changes in the content and composition of lipids and lipid fatty acids were detected.

Thus, it can be concluded that although some evidence is presented that membrane behaviour is associated with dormancy, the evidence needs to be more convincing. However, the assumption that GA receptors (Hooley *et al.*, 1991) as well as Pfr receptors (Gallagher *et al.*, 1988) are located in membranes, makes a role for membranes in the control of dormancy very likely. The fact that temperature seems to be the main regulatory factor in dormancy provides additional evidence for the involvement of membranes, as temperature is known to affect physical properties of membranes (Raison *et al.*, 1980; Di Nola and Mayer, 1986).

Metabolic regulation

An increase in oxygen availability, stimulation of the alternative pathway of respiration, increased metabolism of carbohydrates and activation of the pentose phosphate pathway (PPP) have all been proposed as possible dormancy-breaking mechanisms.

Increase in oxygen availability

Limited uptake of oxygen due to covering structures has been regarded as a possible cause of dormancy. This was based on some observations. Firstly, removal of covering structures or cutting, puncturing or abrading the tissues enclosing the embryo allowed germination. This effect could be the result of an increased availability of oxygen but may also result from increased water uptake and leaching out of inhibitors. Secondly, in some species dormancy of intact seeds could be removed by exposing them to oxygen tensions higher than that of air. Low oxygen penetration may be attributed to the presence of a mucilage layer as for instance in *Sinapis arvensis* seeds (Edwards, 1968) or to the consumption of oxygen by the coat itself as described in *Pyrus malus* (Côme and Tissaoui, 1973).

Even when covering structures limit the amount of oxygen available to the embryo, it can be questioned whether this is related to dormancy. The simplest explanation is that germination is inhibited because there would be insufficient oxygen to support the oxygen-requiring germination process. A lack of correlation between the availability of oxygen and dormancy came from studies with the dimorphic seeds of *Xanthium pennsylvanicum* that differ in the degree of dormancy. No differences in oxygen availability were observed between the upper deep-dormant seed and the lower low-dormant seed (Porter and Wareing, 1974). Oxygen uptake was lower than permeability of the seed coat would maximally allow. The isolated embryo of the upper seed, however, required more oxygen for germination than that of the lower seed. This difference is not fully understood. Involvement of inhibitors and interacting effects with ethylene have been proposed but also opposed (Bewley and Black, 1982b).

Because temperature influences the availability of oxygen, Vegis (1964) suggested that effects of temperature on dormancy may be explained by fluctuations in oxygen availability. However, this theory has never been proven.

The question whether or not dormancy breaking requires an increased oxygen uptake has often been investigated by comparing effects of dormancy-breaking chilling or after-ripening treatments on germination capacity and oxygen uptake. Data are quite conflicting. Chen and Varner (1970) reported that dormant seeds of *Avena fatua* consume less oxygen than after-

ripened seeds during the first 12 hours of imbibition. On the contrary, Simmonds and Simpson (1971) found no difference in oxygen uptake rates of excised embryos from dormant and non-dormant seeds of the same species during the first 10 hours of imbibition. A rise in respiratory capacity was observed during stratification of *Prunus cerasus* (Pollock and Olney, 1959), *Pyrus communis* (Alscher-Herman *et al.*, 1981) and *Malus domestica* (Bogatek and Rychter, 1984). The increase in oxygen uptake at the end of the 80 days stratification period in apple was, however, attributed to the partially broken seed coats. Oxygen uptake rates after 20 days of stratification were similar to those at the start of the stratification period and the authors concluded that respiratory processes do not play any decisive role in dormancy breaking of apple seeds (Bogatek and Rychter, 1984).

From these observations it is evident that the onset of the germination process during a dormancy-breaking treatment is quite a nuisance in studies on a possible causal relationship between increased oxygen uptake and breaking of dormancy. This aspect has also been neglected in studies in which effects of applied nitrate on both germination capacity and oxygen uptake rate were compared. In *Avena fatua* nitrate dose response curves for the stimulation of germination and oxygen uptake were similar, from which the authors concluded that nitrate may stimulate germination by promoting oxygen uptake (Adkins *et al.*, 1984b). Although oxygen uptake increased four days before visible germination, it can be questioned whether the increase in oxygen uptake rate cannot simply be attributed to early germination events rather than being causally related to breaking of dormancy. Hilton and Thomas (1986) compared germination and oxygen uptake rates before visible germination of five weed species in the presence or absence of potassium nitrate. Rates of oxygen uptake in KNO_3 were higher than those in water when KNO_3 promoted germination. When KNO_3 was inhibitory, rates were decreased and were similar when KNO_3 had no effect. Therefore, the authors concluded that respiration is a key metabolic event involved in the transition from the dormant to the non-dormant state. However, also from these results it can be questioned whether a causal relationship exists between the action of nitrate and oxygen uptake, because the observed differences in oxygen uptake may equally well result from differences in early germination events preceding visible germination. A crucial experiment would be to follow oxygen uptake rates under conditions that break dormancy but do *not* allow germination,

since a germination-stimulating factor is missing.

Stimulation of the alternative pathway of respiration

The existence of both the cytochrome- and the alternative pathway of mitochondrial electron transport has been demonstrated in seeds. During imbibition the contribution of both pathways may change as reported for chick pea (Burguillo and Nicolás, 1977), soy bean (Yentur and Leopold, 1976; Siedow and Girvin, 1980), mung bean (Siedow and Girvin, 1980), tomato (Gui *et al.*, 1991) and maize (Leprince *et al.*, 1992).

Inhibitors of the cytochrome pathway of respiration have frequently been reported to stimulate germination (Hendricks and Taylorson, 1972; Roberts and Smith, 1977; Esashi *et al.*, 1979; Zagórski and Lewak, 1983; Adkins *et al.*, 1984a; Cohn and Hughes, 1986; Tilsner and Upadhyaya, 1987). This observation led to the suggestion that the alternative pathway, induced by such treatment, is involved in breaking of dormancy. In a series of investigations of *Xanthium pennsylvanicum* seeds (Esashi *et al.*, 1979, 1981a, b, 1982a, b), an appropriate balance of fluxes through both pathways and a large capacity for the alternative path appeared to be necessary for the termination of dormancy. Esashi *et al.* (1979) also stated that induction of secondary dormancy arises from inactivation of the alternative pathway. Other authors, however, concluded that although both respiratory pathways are operational during dormancy breaking, the alternative pathway is not an obligate requirement for it (Alscher-Herman *et al.*, 1981; Adkins *et al.*, 1984a, b; Bogatek and Rychter, 1984; Brooks *et al.*, 1985; Tilsner and Upadhyaya, 1985, 1987; Upadhyaya, 1986).

Increased metabolism of carbohydrates

In *Avena fatua* dormancy-breaking treatments, like after-ripening and soluble sugars influenced the metabolism of carbohydrates, particularly the raffinose-family oligosaccharides (Foley *et al.*, 1992). However, it is not known whether these changes in carbohydrate metabolism are a cause or an effect of dormancy breaking.

Activation of the pentose phosphate pathway

The regulatory role of the PPP in the breaking of dormancy, as firstly introduced by Roberts (1969) has been investigated by several authors, either by determining C_6/C_1 ratios, $NADP^+/NADPH$ ratios or activities of the regulatory enzymes of the PPP. The C_6/C_1 ratio technique is based on the premise that $^{14}CO_2$ will be released in equimolar quantities from [6-^{14}C]glucose and [1-^{14}C]glucose when glucose is metabolized via glycolysis Krebs cycle pathway but will be released only from [1-^{14}C]glucose when glucose is metabolized via the PPP. Data are quite conflicting. A correlation between the activity of the PPP and dormancy breaking has been reported for *Avena fatua* by Simmonds and Simpson (1971, 1972) and by Gahan *et al.* (1986), whereas a lack of correlation in the same species has been reported by Adkins and Ross (1981), Upadhyaya *et al.* (1981) and Fuerst *et al.* (1983). Gahan *et al.* (1986) attributed this difference to the way enzyme measurements were made, either *in vivo* (cytochemical) (Gahan *et al.*, 1986) or *in vitro* (relatively crude extracts) (e.g. Upadhyaya *et al.*, 1981). The correlation found by Simmonds and Simpson (1971, 1972) could be attributed to postgerminative events (Upadhyaya *et al.*, 1981). No correlation between the activity of the PPP and dormancy breaking was found in seeds of *Xanthium pennsylvanicum* (Satoh and Esashi, 1980) and *Spergula arvensis* (Jones and Hall, 1981). However, in peanut seeds Swamy and Sandhyarani (1986) concluded that the operation of the PPP appears to be a prerequisite for dormancy breaking.

From these observations it is clear that the PPP theory of dormancy breaking as proposed by Roberts (1969) has been weakened throughout the years, without fully ruling out its contribution in the control of dormancy.

Outline of the thesis

Studies on seasonal dormancy patterns have been mainly descriptive. Virtually nothing is known about the mechanisms by which (soil) temperature regulates this seasonal periodicity.

In this thesis two concepts of dormancy control were investigated, a **hormonal** concept and a **metabolic** concept.

Hormonal regulation of dormancy

Control may be exerted via changes in hormone levels and/or via changes in hormone sensitivity. With respect to the germination-stimulating hormone GA both possibilities were extensively studied.

The experiments were performed with seeds of two related cruciferous species *Arabidopsis thaliana* (L.) Heynh. and *Sisymbrium officinale* (L.) Scop.

Due to the availability of hormone mutants that lack the capacity to synthesize GAs (*ga1* mutant) (Koornneef and Van der Veen, 1980) or are insensitive to GAs (*gai* mutant) (Koornneef *et al.*, 1985), seeds of the first species are a very powerful tool to investigate whether GA biosynthesis is required for changes in dormancy or whether GA sensitivity is the main regulatory principle. The mutation in the *ga1* mutant blocked early steps in the GA-biosynthetic pathway prior to *ent*-kaurene (Zeevaart and Talon, 1992). The biochemical nature of the mutation in *gai* seeds is not known. Scott (1990) suggested the involvement of transcriptional repressor proteins in the control of plant-gene expression. Recombinants of *ga1* and *gai* are also available. In wild-type seeds changes in GA sensitivity without the interference of GA biosynthesis can be studied by performing GA dose response experiments in darkness, since light is supposed to be required for GA biosynthesis (Hilhorst and Karssen, 1988; Karssen *et al.*, 1989).

In *S. officinale* seeds a situation without GA biosynthesis can be created by depriving the seeds from light and nitrate since it was indirectly demonstrated that a combination of both factors is required for GA biosynthesis (Hilhorst *et al.*, 1986). However, 'pre-existing' Pfr and endogenous nitrate in the seeds may already somewhat overcome these requirements allowing some germination in water in darkness in extremely sensitive seeds.

Whether or not changes in sensitivity to light and nitrate participate in regulating reversible annual dormancy patterns was additionally investigated in both species. It was already demonstrated by Hilhorst (1990a, b) that sensitivity to light and nitrate decreased in *S. officinale* seeds during induction of secondary dormancy at a constant temperature of 15 °C.

In **Chapter 2** it is shown that studies on germination and dormancy in *A. thaliana* seeds are seriously hampered by the large variability in seed material due to harvest time and conditions of dry storage.

Chapter 3 deals with dose responses of wild-type and *ga1* seeds to several pure GAs. Pure GAs were also applied in combination to investigate whether different GA receptors are involved in the GA-induced germination. Combined GC MS in combination with deuterated GAs were used to qualify and quantify GAs in these seeds. Some preliminary results on the effects of a dormancy-releasing chilling pretreatment and a germination-stimulating irradiation on GA levels in wild-type and *gai* seeds of *A. thaliana* are also presented.

Chapter 4 describes the interaction between light and GA biosynthesis and -sensitivity in four genotypes of *A. thaliana*: wild type, *ga1*, *gai* and *gai/ga1*. Effects of dormancy-breaking and -inducing pre-incubation treatments at constant temperatures on GA dose response curves in darkness and light were compared in these four genotypes to study the role of GA biosynthesis and GA sensitivity in dormancy and germination. Moreover, tetcyclasis, an inhibitor of GA biosynthesis was used to elucidate the role of GA biosynthesis in dormancy control.

In **Chapter 5** the role of sensitivity to light, GAs and nitrate and GA biosynthesis in regulating annual dormancy patterns and germination of *A. thaliana* seeds was investigated. Seeds of wild type and *ga1* mutant were imbibed at outside temperatures under absolutely light-tight conditions. At regular intervals germination capacity of part of the seeds was determined under laboratory conditions. Detailed dose response analyses were used to interpret the observed sensitivity changes. The time span of one experiment was 18 months and of another experiment 9 months.

Chapter 6 deals with the involvement of sensitivity to light, nitrate and GAs in regulating seasonal dormancy patterns and germination of *S. officinale* seeds. Portions of seeds were buried in the field and at regular intervals some portions were exhumed in order to perform detailed dose response analyses to light, nitrate and GAs. The time span of burial was 15 months.

Metabolic regulation of dormancy

The objective of this part of the study was to investigate whether changes in dormancy are controlled by changes in metabolic activity. Moreover, the involvement of the alternative pathway of respiration and shifts in respiratory pathways during changes in dormancy were investigated.

In these experiments seeds of *S. officinale* were used. This species allows conclusive separation between processes concerning breaking of dormancy and those concerning actual germination. The intensity of dormancy can namely vary by incubating seeds in darkness without resulting in germination. The degree of dormancy (= germination capacity) can be tested by providing light and nitrate.

In **Chapter 7** effects of temperature-induced changes in dormancy on oxygen uptake rate of *S. officinale* seeds were studied. Rates of oxygen uptake were measured with the use of oxygen electrodes.

Chapter 8 describes changes in the contribution of different respiratory pathways during prolonged incubation of *S. officinale* seeds at 24 °C in darkness. Rates of oxygen uptake measured with oxygen electrodes were also compared to rates determined gas chromatographically.

Finally, all results are integrated and discussed in **Chapter 9.**

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Chapter 2

Variability in light-, gibberellin- and nitrate requirement of *Arabidopsis thaliana* seeds due to harvest time and conditions of dry storage

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Summary

Freshly harvested seeds of *Arabidopsis thaliana* harvested in different seasons showed considerable variability in requirement for light (wild type), gibberellins (GAs) (wild type, GA-deficient, *ga1-2* mutant) and nitrate. Generally, sensitivity to light was high in seed lots harvested between September and March and significantly lower in those harvested between April and June. Sensitivity to GAs varied in a nearly parallel way. These differences in sensitivity can be attributed to conditions during seed development, like temperature and nitrate availability. Endogenous nitrate levels varied enormously amongst seed lots (about a factor 600) and were weakly correlated to the germination capacity at 10 °C after 15 min of red light irradiation. In addition, differences in sensitivity to nitrate may be responsible for the enormous differences in light requirement between seed lots. Other unknown factors may contribute as well. At a germination temperature of 10 °C seeds were much more sensitive to GAs and light than at 24 °C.

The degree of dormancy rapidly declined upon dry storage of the seeds. Loss of dormancy was accelerated with increasing storage temperature; the requirements for light and GAs became less strict. Sensitivity to GAs increased and seeds that initially only responded to continuous irradiation with white fluorescent light, responded to a single red light pulse after 15 months of dry storage at 2 °C.

It is demonstrated that studies on germination and dormancy of *A. thaliana* are hampered by the large variability of seed material and the relatively high rate of after-ripening.

Introduction

The small crucifer *Arabidopsis thaliana* (L.) Heynh. has recently become an important model species in plant physiology, biochemistry, molecular biology and genetics. Numerous developmental mutants have been isolated and characterized. In seed physiology, hormone mutants that are either deficient in or insensitive to particular hormone(s) have been used to elucidate mechanisms of hormone action in seed dormancy and the development of desiccation tolerance (see reviews by Karssen *et al.*, 1987; Finkelstein *et al.*, 1988). It has been demonstrated that during seed development abscisic acid (ABA) is responsible for dormancy induction (Karssen *et al.*, 1983) and the development of desiccation tolerance (Koornneef *et al.*, 1989), and that gibberellins (GAs) are required for germination (Karssen *et al.*, 1989). However, in these studies it is problematic that considerable variability exists in the degree of dormancy of freshly harvested seeds.

Both summer- and winter-annual races occur in *A. thaliana*, which may possess different degrees of dormancy (Laibach, 1951). However, also when grown under more or less controlled conditions in a greenhouse, the season of ripening can affect the degree of dormancy. Cone and Spruit (1983) found that seeds harvested in winter, 1981 were more sensitive to light than seeds harvested in summer, 1979. The spectral quality of light during development of *A. thaliana* plants is reported to influence the light requirement of freshly harvested seeds (McCullough and Shropshire, 1970; Hayes and Klein, 1974). Also temperature during development may affect the light requirement (McCullough and Shropshire, 1970). In other species, conditions like photoperiod (Gutterman, 1992) and nitrate availability (Fawcett and Slife, 1978; Saini *et al.*, 1985a, b; Bouwmeester, 1990; Fenner, 1991) are known to have an influence on the germination requirements of the progeny.

Apart from the conditions during seed development, conditions during dry storage after harvest can also affect the dormancy characteristics of the seeds. Upon dry storage of *A. thaliana* seeds at room temperature (20-25 °C) the germination requirements became less stringent, in the sense that germination became possible at a broader range of temperatures (Baskin and Baskin, 1972) and that the light requirement was reduced (McCullough and

Shropshire, 1970; Baskin and Baskin, 1972; Karssen and Laçka, 1986). This loss of dormancy under dry conditions, often referred to as dry after-ripening, is dependent on temperature, seed moisture content and oxygen availability (Roberts and Smith, 1977). Dormancy of *A. thaliana* seeds can also be relieved by imbibition, which is also temperature dependent (Cone and Spruit, 1983). The rate of dormancy release in imbibed seeds was much faster than under dry conditions, namely in days instead of weeks (Karssen and Laçka, 1986).

It was the aim of the present study to further characterize differences in dormancy of seed lots of *A. thaliana* harvested in different seasons. The analysis involves studies of the differences in sensitivity to GAs, light, nitrate and temperature. Responses to GAs were compared in wild type and GA-deficient (*ga1-2*) mutant. Of all seed lots endogenous nitrate levels were determined to investigate a possible link with sensitivity to light, since interactions between light and nitrate are known for several species (e.g. Vincent and Roberts, 1977, 1979; Hilhorst *et al.*, 1986; Grubišić and Konjević, 1990). In addition, the requirement for GAs and light was followed during dry storage of the seeds and the role of the temperature of dry storage was investigated. Implications of harvest time and after-ripening on dormancy studies are presented.

Materials and methods

Production of seeds

A. thaliana seeds of wild type and GA-deficient mutant *ga1-2* were propagated in different seasons in 1988, 1989, 1990 and 1991. For this purpose, seeds were sown in 9 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll, Dassel, Germany) to which 4 ml of either pure water (wild type) or 10 μM GA_{4+7} (*ga1-2*) was added. Pure water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France). Water of this quality has been used in all experiments. After 5 days at 2 °C to break seed dormancy, the seeds were allowed to germinate at 24 °C under continuous white fluorescent light (Philips TL 57, Eindhoven, The Netherlands). Photon fluence rate at seed level was

11 μmol m^{-2} s^{-1}. After 3 days the seedlings were transplanted to 5.5 cm plastic pots, filled with potting soil (Trio, number 17 special, Naturado, Veenendaal, The Netherlands) and grown in an air-conditioned greenhouse. The plants were fertilized every two weeks, alternatively with 2 g l^{-1} N-P-K-Mg (19+6+20+3) and 2 g l^{-1} N-P-K (18+18+18) (Kristalon, DSM, Sittard, The Netherlands). When required, additional light was given (Philips, SONT 400W, Eindhoven, The Netherlands) from 6.00 a.m. to 10.00 p.m. Photon fluence rate at plant height was 97 μmol m^{-2} s^{-1}. Day temperature was 22 °C and night temperature 18 °C. Relative humidity was around 70 %.

When the rosettes of *ga1-2* plants were fully expanded, they were sprayed with 10 μM GA_{4+7} once a week during 2-3 successive weeks. When seeds were ripe, they were collected, allowed to dry at room temperature for about 4 days, cleaned by sieving and blowing, and thereafter stored dry at +2 °C in darkness in closed glass vials. For one experiment seeds were also stored dry at -20 and +20 °C.

Germination conditions

Triplicates of 50-100 seeds were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll, Dassel, Germany) and moistened with 1.5 ml of water or the test solution. Germination was tested at 10 or 24 °C in temperature-controlled incubators (Bosch, KSR 2511/01, Van Rijn, Amsterdam, The Netherlands). Light conditions were darkness, a single red light pulse of 15 min or continuous irradiation with white fluorescent light (Philips, TL57, Eindhoven, The Netherlands). Photon fluence rate at seed level under red and white light was 11 μmol m^{-2} s^{-1}. A single red light pulse was given after 2 h of imbibition at 24 °C in darkness. After the irradiation the seeds were returned to darkness. Red light (620-700 nm) was obtained from six red fluorescent tubes (Philips TL 20W/15, Eindhoven, The Netherlands) filtered through one layer of 3 mm plexi-glass (Red 501, Röhm & Haas, Darmstadt, Germany).

Germination occurred at a range of GA_{4+7}- or KNO_3 concentrations. GA_{4+7} (ICI, Yalding, U.K.) was dissolved in a few drops of 1N KOH and diluted with water; pH values of all concentrations were around 7. In one experiment GA_{4+7} was diluted with a phosphate

citrate buffer containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. The pH of the buffer was 5.0. In Chapter 3 it will be demonstrated that the commercial GA_{4+7} mixture contained 54 % GA_4 and 46 % GA_7-isolactone, whereas GA_7 was not detected. As a result of short exposure of the mixture to KOH most GA_7-isolactone was converted to GA_7, whereas GA_4 was not affected (Chapter 3). KNO_3 (Suprapur, Merck, Darmstadt, Germany) was dissolved in water.

In one experiment seeds were pre-incubated in darkness at 2 °C for 7 days. After pre-incubation in water the seeds were transferred to a range of GA_{4+7} concentrations. The adjacent water was removed from the seeds with a Büchner funnel and the filter paper with seeds brought to fresh GA_{4+7} solution.

To determine germination percentages, both germinated and non-germinated seeds were counted 7 (24 °C) or 14 (10 °C) days after the start of the germination test. Radicle protrusion was taken as the criterion for germination.

All manipulations of incubated seeds were conducted in dim green light, obtained by filtering one green fluorescent tube (Philips TL 40W/17, Eindhoven, The Netherlands) with two layers of yellow no. 46 and two layers of blue no. 62 Cinemoid filters (Strand Electric, London, U.K.).

Nitrate measurements

Of several wild-type seed lots endogenous nitrate contents were measured, essentially according to Hilhorst (1990). Until measurement seeds were stored dry at 2 °C. Portions of about 50 mg seeds were frozen and thawed in 0.2 ml water. After thawing, the seeds were homogenized for 1 min in 1.5 ml water with a stainless steel rod (IKA-RW 15, Janke & Kunkel KG, Staufen im Breisgau, Germany) after addition of some purified sea sand (Merck, Darmstadt, Germany). The homogenate was kept on ice for 1 h and regularly shaken. After centrifugation for 15 min at 14000 rpm 1 ml of the supernatant was transferred to a new Eppendorf tube on ice. Thereafter, 500 μl was put on top of a 3 mm layer of Lichroprep RP-8 (particle size 25-40 μm, Merck, Darmstadt, Germany) in a 2 ml plastic column supported by a MA 25 pre-filter (Millipore, Etten-Leur, The Netherlands). The column had been pre-

washed with 3 portions of 500 μl methanol followed by 200 μl of the supernatant. After filtration samples of 20 μl were injected into a HPLC system (model 3500 B, Spectra Physics, Santa Clara, California, U.S.A.) equipped with a spectrophotometric detector set at 210 nm (model C-R1B, Shimadzu, Kyoto, Japan). The column was a stainless steel Lichrosorb 10 NH_2 column (Chrompack, Middelburg, The Netherlands). The mobile phase was 10 mM KH_2PO_4, pH 3.7; the flow rate was 1.5 ml min^{-1}. Nitrate levels were calculated on the basis of a linear relationship between concentration and peak height of pure nitrate standards. Nitrate content was measured in three independent replications and expressed in μmol g^{-1} seed on a fresh weight basis.

Results

Effects of harvest date

Responses to light

Wild-type seeds of *A. thaliana* harvested in various seasons showed directly after harvest considerable differences in their response to a single red light pulse of 15 min or to continuous irradiation with white fluorescent light (Table 2.1). Responses to light were much larger at 10 °C than at 24 °C. At the latter temperature differences in light sensitivity between seed lots were only observed when germination was tested in continuous white light and not when a single red light pulse was given. At 10 °C clear differences in light sensitivity were also visible after a single red light irradiation. In continuous white light, all seed lots, with the exception of the May, 1991 seeds, showed more than 75 % germination at 10 °C. Generally, light sensitivity was high in seed lots harvested in any month between September and March and was significantly lower in seeds harvested in the period April until June. The high light sensitivity of seed lots harvested between September and March was accompanied by substantial dark germination at 10 °C. The germination tests at 24 °C showed that light sensitivity was highest in seeds harvested in September, 1990.

Table 2.1 Germination capacity of several wild-type seed lots of *A. thaliana* directly after harvest and endogenous nitrate contents of the seeds. Germination was tested at 10 or 24 °C in water in darkness (D), after a 15 min red light irradiation followed by darkness (Red) or in continuous white fluorescent light (WL). Before irradiating the seeds for 15 min, they were imbibed during 2 h at 24 °C in darkness. sd = standard deviation, nd = not determined.

Harvest	Germination, %						$[NO_3^-]$ $\mu mol\ g^{-1}$	
	10 °C			24 °C				
	D	Red	WL	D	Red	WL	mean	sd
April, 1988	10	45	87	0	0	1	0.16	0.02
October, 1988	10	81	97	1	3	52	9.51	0.19
February, 1989	67	86	98	2	3	53	9.55	0.12
May, 1989	6	25	82	1	3	10	10.44	0.75
June, 1989	3	11	nd	0	1	nd	1.02	0.09
December, 1989	38	76	84	3	5	38	28.95	2.36
May, 1990	1	29	76	0	0	5	0.28	0.06
September, 1990	83	99	nd	22	68	nd	95.46	9.46
March, 1991	19	68	87	1	3	62	4.42	0.11
May, 1991	0	2	27	0	0	1	0.19	0.06

Responses to nitrate

Sensitivity to nitrate was determined at 24 °C in four different seed lots (Fig. 2.1). Directly after harvest, seeds of the May, 1990 and 1991 seed lots did not respond to KNO_3 at concentrations up to 40 mM. Seeds harvested in October, 1988 and stored dry during 2 months responded slightly to applied KNO_3, whereas seeds from the April, 1988 seed lot,

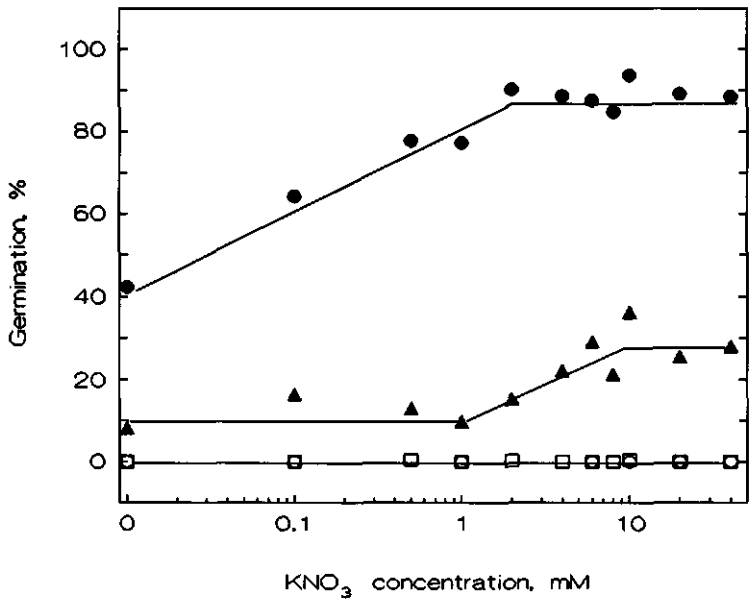

Figure 2.1 The effect of KNO_3 on the red light-induced germination of *A. thaliana* wild-type seeds, harvested in April, 1988 (●), October, 1988 (▲), May, 1990 (○) or May, 1991 (□). In both May seed lots the response was determined directly after harvest; in the October, 1988 seed lot it was determined after 2 months of dry storage at 2 °C and in the April, 1988 seed lot after 9 months of dry storage. A red light irradiation of 15 min was given 2 h after the start of imbibition in KNO_3 at 24 °C, and thereafter the seeds were transferred to 24 °C in darkness.

which were stored dry for 9 months, were quite sensitive to KNO_3. Germination increased from 40 % in water to 90 % in KNO_3 concentrations above 1 mM. The influence of dry storage at 2 °C is seen from the comparison between the data in Table 2.1 and Fig. 2.1. Without dry storage, seeds from the April, 1988 seed lot did not germinate in water at 24 °C after 15 min of red light irradiation (Table 2.1), whereas after dry storage germination was 40 % (Fig. 2.1).

In order to determine whether the observed differences in light sensitivity, as shown in Table 2.1, could be explained by differences in the endogenous nitrate content of the seeds,

endogenous nitrate contents were measured. As shown in Table 2.1, nitrate levels varied enormously amongst seed lots, for instance seeds harvested in September, 1990 contained nearly 600 times as much nitrate as seeds harvested in April, 1988. With the exception of the seed lot harvested in May, 1989 the data support the hypothesis that seeds harvested in the period with low light sensitivity (April-June) contained considerably less nitrate than seeds harvested in the period with high light sensitivity (September-March). However, in the latter part of the year differences in nitrate content were also substantial, maximally extending at a factor of about 20. When germination at 10 °C in response to a short red light pulse is plotted against the endogenous nitrate content of all seed lots a weak correlation ($R = 0.76$) was seen. Some seed lots gave lower or higher percentage germination than could be expected from their endogenous nitrate content (data not shown).

Responses to GAs

Freshly harvested seeds also showed variable responses to GAs in darkness (Fig. 2.2). In a similar way as with light sensitivity, the seeds were more sensitive to GA_{4+7} at 10 °C than at 24 °C, both in wild-type and *ga1-2* seeds. Moreover, at both germination temperatures *ga1-2* seeds were more sensitive to applied GA_{4+7} than wild-type seeds. At 24 °C wild-type seeds in darkness were fully GA dependent, whereas at 10 °C seeds of several harvests could germinate without GA application. Exogenous GA further increased the response. Seeds of the *ga1-2* mutant were fully dependent on exogenous GA at both temperatures. It can be noticed that seeds harvested in the period October-February were more sensitive to GAs than seeds harvested in spring, with the exception of *ga1-2* seeds harvested in December, 1989 and tested at 24 °C and *ga1-2* seeds harvested in April, 1988. Seeds harvested in May of three successive years had different degrees of dormancy; the one harvested in 1991 was extremely insensitive to GAs.

Effects of dry storage

The effect of dry storage at 2 °C on responses to light and GAs was investigated in the October, 1988 seed lot. Germination was tested at 24 °C. Dry storage for 15 months

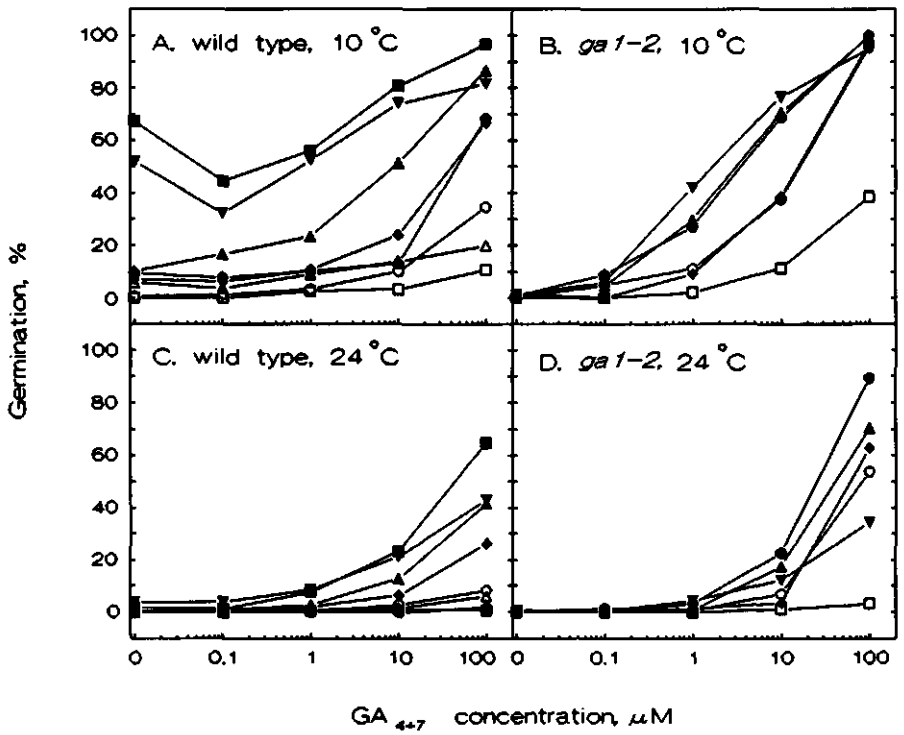

Figure 2.2 The influence of harvest time on the GA dose responses of *A. thaliana* wild-type (A, C) and *ga1-2* (B, D) seeds directly after harvest. Germination was tested at a range of non-buffered GA_{4+7} concentrations in darkness, either at 10 °C (A, B) or at 24 °C (C, D). Seeds were harvested in April, 1988 (●), October, 1988 (▲), February, 1989 (■), May, 1989 (◆), December, 1989 (▼), May, 1990 (○), March, 1991 (△) or May, 1991 (□).

increased the response of both wild-type (Figs. 2.3A, C) and *ga1-2* (Figs. 2.3B, D) seeds to applied GAs in darkness. In addition, light sensitivity increased: whereas an effect of a single red light irradiation on GA dose responses was absent in freshly harvested seeds of both genotypes (Figs. 2.3A, B), a significant response to a single red light irradiation was observed after 15 months of dry storage (Figs. 2.3C, D). Again, *ga1-2* seeds remained fully dependent on applied GA_{4+7}. However, part of the wild-type seeds germinated without GA

application after this period of dry storage, provided that they were irradiated. Continuous irradiation with white fluorescent light also stimulated germination in freshly harvested seeds (Figs. 2.3A, B). After 15 months of dry storage it was still more stimulatory than a single red light pulse (Figs. 2.3C, D). At 10 °C a single red light irradiation induced more than 80 % germination of the wild-type seeds in water (cf. Table 2.1), whereas its effect on *ga1-2*

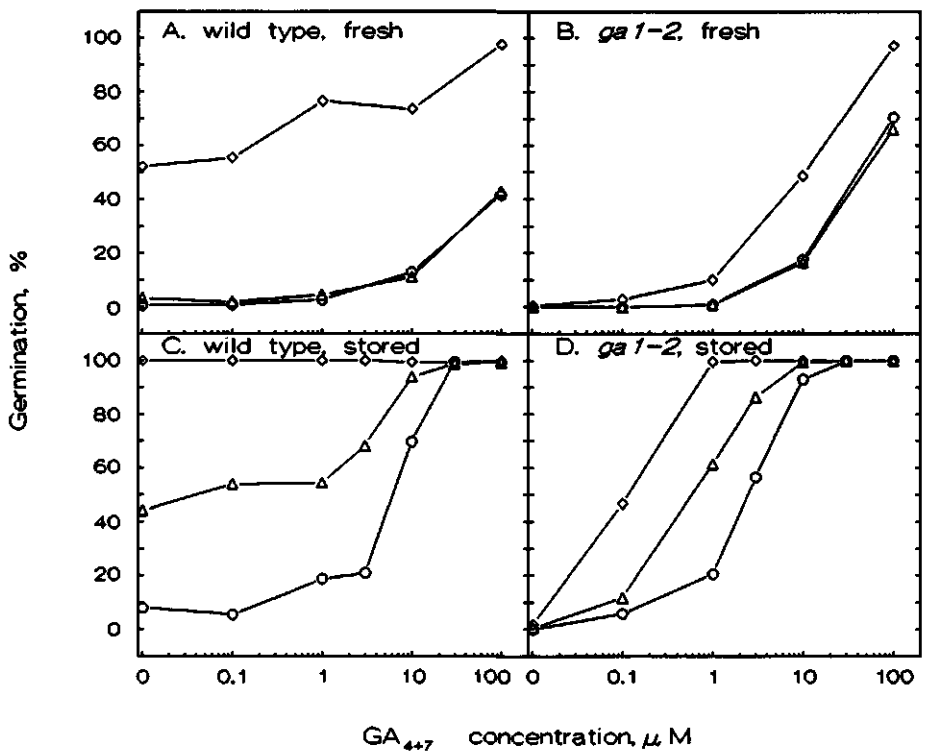

Figure 2.3 The effect of dry storage on the responses to GA_{4+7} and light of *A. thaliana* wild-type (A, C) and *ga1-2* (B, D) seeds. After a period of dry storage at 2 °C for 15 months (C, D) germination was compared with that of freshly harvested seeds (A, B). Germination was tested at 24 °C at a range of non-buffered GA_{4+7} concentrations in darkness (○), after 15 min of red light irradiation followed by darkness (△) or in continuous white fluorescent light (◇). Seeds were harvested in October, 1988.

seeds was minimal (data not shown).

The temperature conditions during dry storage of the seeds greatly influenced the rate of after-ripening and thus the degree of dormancy. This is illustrated in Fig. 2.4 for seeds of the October, 1988 seed lot that had been in dry storage for 9 weeks at temperatures of -20, +2 and +20 °C. With increasing temperatures of dry storage the response to applied GAs at a germination temperature of 24 °C increased (Figs. 2.4A, B). In wild-type seeds also the response to a 15 min red light irradiation became larger (Fig. 2.4A). After a storage period as short as 9 weeks, the response to 100 μM GA_{4+7} was already somewhat increased in seeds stored at -20 °C. A storage period of 9 weeks at +20 °C made wild-type seeds almost independent of exogenous GA when irradiated with red light, whereas red light did not have an effect in freshly harvested seeds or in seeds stored at -20 °C for 9 weeks (Fig. 2.4A). Also in *ga1-2* seeds a single red light irradiation did not have a significant effect in comparison with dark-treated seeds, both immediately after harvest and after 9 weeks of dry storage at all temperatures tested (Fig. 2.4B).

Influences of harvest time and storage conditions on responsiveness to GAs and light have several implications on dormancy studies in *A. thaliana*, as illustrated in Fig. 2.5 on seeds harvested in May, 1990. In freshly harvested seeds (closed symbols), a chilling pretreatment of 7 days at 2 °C reduced the requirement for applied GAs (compare circles with triangles). A similar shift was also caused by dry storage of the seeds, in this case 8 months of dry storage at 2 °C (compare open with closed circles). However, when the stored seeds were given a chilling pretreatment, the requirement for GAs was not further reduced. Thus, a treatment of 7 days at 2 °C, which breaks dormancy in freshly harvested seeds, had no effect in after-ripened seeds.

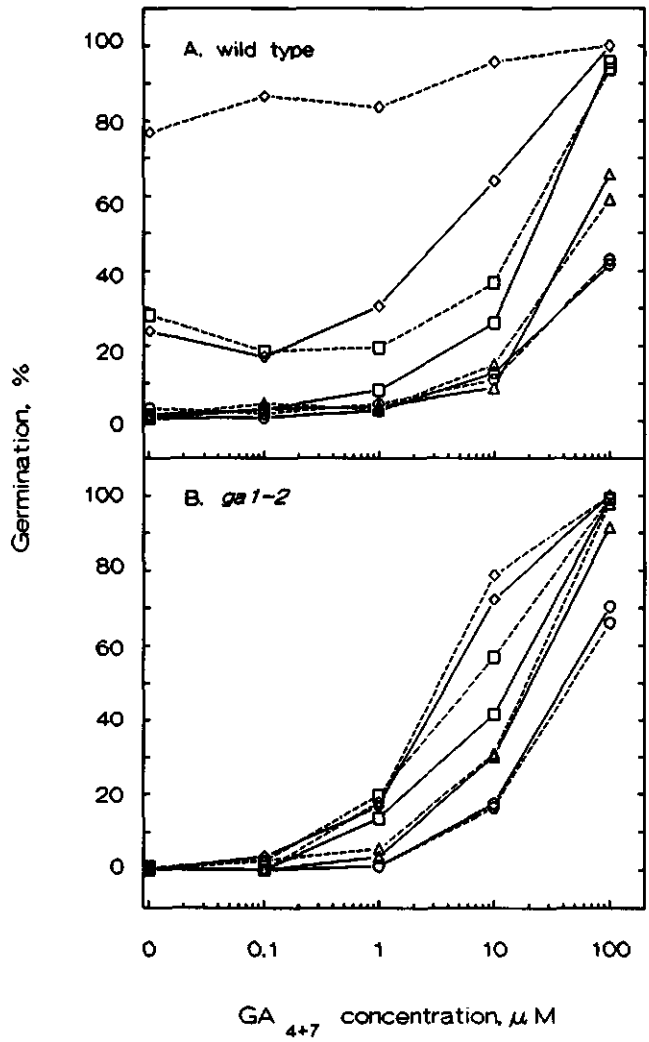

Figure 2.4 The effect of the temperature of dry storage on GA dose responses of *A. thaliana* wild-type (A) and *ga1-2* (B) seeds. Directly after harvest seeds were stored dry at -20 °C (△), +2 °C (□) or +20 °C (◇) for 9 weeks, and germination at 24 °C was compared with that of freshly harvested seeds (○), either in darkness (solid lines) or after 15 min of red light irradiation followed by darkness (broken lines). Seeds were harvested in October, 1988.

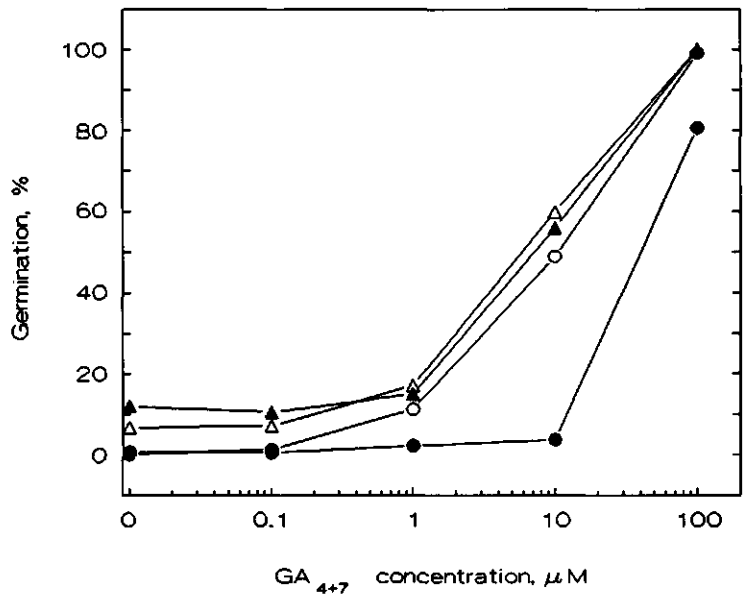

Figure 2.5 The effect of a chilling pretreatment on the GA dose response of *A. thaliana* wild-type seeds. Germination was tested either directly after harvest (closed symbols) or after 8 months of dry storage at 2 °C (open symbols). After pre-incubation in water in darkness for 7 days at 2 °C (triangles) the seeds were transferred to a range of GA_{4+7} concentrations, buffered at pH 5, and germination was compared with that of non-pre-incubated seeds (circles). Germination was tested at 24 °C in darkness. Seeds were harvested in May, 1990.

Discussion

The present results clearly show that studies on germination and dormancy in *A. thaliana* seeds are seriously hampered by large variations in responsiveness of the seeds to factors that stimulate germination. Seeds harvested at different times of the year are often in quite variable states of dormancy as expressed by the considerable variation amongst seed lots in the sensitivity to light (Table 2.1), applied GAs (Fig. 2.2) and to a lesser extent to applied nitrate (Fig. 2.1). Much variability in dormancy due to harvest time and storage conditions

may easily cause a rather antagonistic characterization of the germination behaviour of *A. thaliana*. For instance, seeds harvested in May, 1991 were extremely insensitive to light during tests at 24 °C, whereas seeds from September, 1990 hardly required light when tested at 10 °C (Table 2.1). If the response to GAs had only been determined with seeds harvested in May, 1991, *A. thaliana* seeds would have been characterized as highly GA insensitive, whereas seeds from other harvest dates were clearly responsive to GAs (Fig. 2.2). The temperature at which germination was tested also determined whether responses to light and to GAs were visible (Table 2.1, Fig. 2.2). Also storage conditions influenced the reaction of the seeds in germination studies. Effects of a normally dormancy-breaking chilling pretreatment may be overlooked in seeds that had been in dry storage (Fig. 2.5).

Effects of harvest date

The variability in seed material did not affect the absolute GA requirement of *ga1-2* seeds, neither in darkness nor in light (Figs. 2.2, 2.3, and 2.4), as was observed before (Karssen *et al.*, 1989). In darkness, wild-type seeds of several seed lots were also totally dependent on exogenous GA_{4+7}. However, some seed lots could germinate in darkness without GA application, especially at 10 °C. This may be a result of a satisfaction of the requirement for farred-absorbing phytochrome (Pfr) by 'pre-existing Pfr' (Cone and Kendrick, 1985) or of some other factor that is independent of the phytochrome system (Duke, 1978). It is not very likely that the dim green light was responsible for the observed dark germination, because seeds were only shortly exposed at the start of imbibition before they were fully imbibed. However, an extremely light-sensitive seed may also respond to the low amounts of Pfr generated by dim green light under such conditions (Kendrick and Cone, 1985).

Irrespective of the variability in seed material, it can be concluded that *ga1-2* seeds of all seed lots were more sensitive to applied GAs than wild-type seeds of the same harvest. This receptor-up regulation has been described previously for *A. thaliana* seeds (Karssen *et al.*, 1987).

Although an unequivocal correlation between season of harvest and degree of dormancy

was missing, we conclude that seeds harvested in the period September-February were more sensitive to GAs than those harvested in the period March-June (Fig. 2.2). In addition, seeds harvested between September and March exhibited a higher light sensitivity than seeds harvested between April and June (Table 2.1). Thus, sensitivity to GAs and to light varied in a more or less parallel way, which, however, does not simply mean that both share the same response mechanism, as studies with *A. thaliana* and *Sisymbrium officinale* clearly demonstrated an independent co-action of GA and light (Hilhorst and Karssen, 1988; Derkx and Karssen, 1993; Chapter 6).

Variation in sensitivity to GAs and to light was not only found from month to month, but also from year to year. Seeds harvested in May, 1991 were much more dormant than seeds harvested in May of the preceding two years (Table 2.1, Fig. 2.2).

Differences in the degree of dormancy from year to year are difficult to understand. However, variation from month to month may be attributed to several environmental factors such as the spectral quality of the light during seed maturation (McCullough and Shropshire, 1970; Hayes and Klein, 1974), photoperiod (Gutterman, 1992), temperature (McCullough and Shropshire, 1970) and nitrate availability (Fawcett and Slife, 1978; Saini *et al.*, 1985a, b; Bouwmeester, 1990; Fenner, 1991). In general, a higher germinability is obtained when seeds mature under light with a high red/farred ratio and when plants are grown under short day conditions (Gutterman, 1992). In some species, like for instance *Amaranthus retroflexus*, higher temperatures result in seeds with higher germinability, whereas in other species, for example soybean, an inverse relationship was found between temperature and germinability (Gutterman, 1992).

It is not likely that the photoperiod can account for the observed differences in light- and GA requirement of the seeds, because in the greenhouse day length was always maintained at 16 h by providing additional light throughout the year in case required. Also the spectral quality of the light is probably not responsible for the observed differences, because the additional light had a high red/farred ratio.

The maximum temperature could not be controlled in the greenhouse. Thus, it is possible that this factor was partly responsible for the observed differences in dormancy. Temperature conditions during the ripening period of the seeds, being as short as 20 days,

may have had an influence on the rate of after-ripening and the degree of dormancy.

Endogenous nitrate levels varied as much as maximum a factor 600 amongst seed lots (Table 2.1). The *A. thaliana* plants were fertilized with nitrate every 2 weeks. Nitrate accumulation in the seeds depends on the nitrate uptake by the mother plant and the rate of water transport, and therefore depends on all factors that influence these processes, like nitrate level in soil, temperature, water content in soil and relative humidity. Seeds with low endogenous nitrate content were indeed deeply dormant, in that sensitivity to light was low (Table 2.1). The seed lot with the highest endogenous nitrate content (September, 1990) was extremely sensitive to light (Table 2.1). However, some other seed lots showed a lower or higher response to light than could be expected from their endogenous nitrate content. Because a weak correlation exists between the endogenous nitrate content of the seeds and its germination capacity ($R = 0.76$) it is concluded that endogenous nitrate may contribute to the degree of light sensitivity, but is not the only decisive factor.

The potential influence of endogenous nitrate is supported by the effect of applied nitrate. KNO_3 concentrations above 1 mM enhanced the light germination of seeds harvested in April, 1988, which had been stored dry for 9 months, from 40 % in water to 90 % (Fig. 2.1). In the May, 1990 and 1991 seed lots, which were both low in endogenous nitrate content, KNO_3 did not stimulate germination in concentrations up to 40 mM (Fig. 2.1). Water uptake measurement (0.8 g per g seed) indicates that 1 g of *A. thaliana* seeds takes up 30 μmol KNO_3 during imbibition in a 40 mM KNO_3 solution. This level equals the endogenous level in the December, 1989 seed lot, which also did not respond to a single red light irradiation at 24 °C directly after harvest. Only the September, 1990 seed lot that contained 95.46 μmol $NO_3^- g^{-1}$ seed germinated at 24 °C in water after a single red light pulse. Although endogenous nitrate may contribute to the observed differences in response to light, it cannot be excluded that *sensitivity* to nitrate is even more important. Sensitive seeds require less nitrate than relatively insensitive seeds; thus, even when the endogenous nitrate content is high, as for instance in the May, 1989 seeds, the response may be low as a result of an extremely low nitrate sensitivity. Whether endogenous nitrate or sensitivity to nitrate are responsible for the observed differences in GA sensitivity is questionable, because effects of applied nitrate on GA dose response curves were negligible (data not shown).

It is more likely that variation in GA requirement is related to variable action of endogenous ABA in developing seeds. ABA is known to be responsible for induction of dormancy in *A. thaliana* seeds, while there is little or no carry-over into the mature seed (Karssen *et al.*, 1983). During seed development ABA levels determine the GA requirement for germination of mature seeds (Karssen and Laçka, 1986). Until now, no indication was found that environmental factors control the level of ABA during seed development of *A. thaliana* seeds. However, in wheat temperature strongly affects the sensitivity to ABA during seed development (Walker-Simmons, 1987, 1988).

Effects of dry storage

The degree of dormancy of *A. thaliana* seeds gradually declined upon dry storage of the seeds after harvest, a well-known phenomenon especially in cereals and grasses but also in dicotyledonous species (Bewley and Black, 1982). When compared with rates mentioned for other species, dry after-ripening in *A. thaliana* is quite rapid, as can be derived from the increase in light- and GA sensitivity after a dry storage period as short as 9 weeks (Fig. 2.4). Storage of *Rumex crispus* seeds for 5 years at 2-4 °C, for example, results in no appreciable change in dormancy (Cavers, 1974), and dormancy of *Rumex obtusifolius* seeds was only somewhat affected by storage at 1.5 °C for 9 months (Totterdell and Roberts, 1979). During storage the conditions for germination became gradually less stringent: in freshly harvested seeds a single red light pulse was ineffective in increasing the response to applied GA_{4+7} and continuous irradiation increased the response significantly (Figs. 2.3A, B), whereas in 15-month after-ripened seeds a single red light pulse was sufficient to increase the response to applied GA_{4+7} (Figs. 2.3C, D).

The mechanism whereby after-ripening promotes the capacity to germinate is still unclear. One possible mechanism would involve an alteration in permeability of seed coat membranes (Bewley and Black, 1982).

Although not proven yet, it is proposed that GA-binding sites, suggested to be located in membranes (Hooley *et al.*, 1991), may be exposed in a temperature-dependent way, as temperature is known to affect physical properties of membranes (Raison *et al.*, 1980; Di

Nola and Mayer, 1986). It can also be speculated that the availability of receptors is altered by temperature, for instance by a temperature-dependent disintegration of inhibitors blocking the receptors. The rate of receptor synthesis may also be temperature dependent. The Pfr-binding component is also assumed to be located in a membrane (Taylorson, 1988). The reduced sensitivity to light and to GA_{4+7} at 24 °C in comparison with 10 °C may be explained by membrane properties that result in a decreased availability of phytochrome- and GA receptors at 24 °C.

In conclusion, responses of *A. thaliana* wild-type and *ga1-2* seeds to GAs, light and nitrate are strongly influenced by time of harvest, storage period, storage temperature and germination temperature. Differences in the degree of dormancy of several seed lots may imply that some can react to stimulatory factors like GAs, light and nitrate, whereas others do not or to a lesser extent (Table 2.1, Figs. 2.1, 2.2). Cultivation of *A. thaliana* plants for seed production under well-defined, controlled conditions is, thus, required to minimize seasonal influences as much as possible. During dry storage responsiveness increases (Figs. 2.3 and 2.4). This increase may, however, implicate that a treatment like chilling, which is effective in breaking dormancy in freshly harvested seeds, is no longer effective in after-ripened seeds (Fig. 2.5). The degree of sensitivity reached after some months of dry storage cannot be further enhanced by a chilling treatment. Thus, limitations occur at the receptor level.

The great variability in seed material and relatively rapid changes in dormancy during dry storage make *A. thaliana* a complicated species. However, the availability of hormone mutants deficient in the capacity to synthesize GAs or insensitive to applied GAs (Koornneef *et al.*, 1985), in addition to clear responses to GAs (wild type and *ga1-2* mutant) and light, compensate sufficiently for these shortcomings and makes it a unique species to study the role of hormones in dormancy control and germination.

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Chapter 3

Gibberellins in seeds of *Arabidopsis thaliana*: biological activities, identification and effects of light and chilling on endogenous levels

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Summary

The activities of several gibberellins (GAs) in stimulating germination of wild-type and GA-deficient *ga1* seeds of *Arabidopsis thaliana* were compared. Of the six compounds tested GA_4 and GA_7-isolactone had highest activity and GA_7 and GA_9 lowest, activities of GA_1 and GA_3 were intermediate. Combined application of pure GAs presented no indications that more than one GA receptor is involved. Four GAs were identified in extracts from wild-type and GA-insensitive *gai* seeds by combined gas chromatography mass spectrometry: GA_1, GA_3, GA_4 and GA_9. Effects of light and chilling on levels of GA_1, GA_4 and GA_9 were studied by using deuterated standards. Light increased both GA levels and germination in unchilled wild-type and *gai* seeds. As a result of irradiation GA levels in *gai* seeds were 7-10 times as high as in wild-type seeds. Whereas dark germination was 0 %, light germination of *gai* seeds was 14 % and of wild-type seeds 95 %. A chilling pre-treatment of 7 days 2 °C was required to further enhance light germination of *gai* seeds. Light did not increase GA levels of chilled seeds of both genotypes. Actually, levels of GA_4 and GA_9 of chilled *gai* seeds in light were respectively 7 and 12 times lower than levels of non-chilled seeds, whereas the chilled seeds germinated better. Slightly elevated levels of GA_4 were detected in darkness after chilling, germination capacity was still 0 %. These results strengthened the conclusion that GAs are required for germination of *A. thaliana* seeds, whereby GA_4 has intrinsic biological activity. However, it is not likely that light and chilling stimulate germination primarily by increasing levels of GA. Increased GA sensitivity is a possible alternative.

Introduction

The light requirement of germination can often be replaced by application of gibberellins (GAs). This has led to the suggestion that light induces GA biosynthesis. Inhibition of light-stimulated germination by synthetic inhibitors of GA biosynthesis and reversal of this action by applied GAs strengthened this conclusion (Hilhorst and Karssen, 1988; Derkx and Karssen, 1993; Chapters 4, 6). Additionally, light may cause a change in GA conversions such that specifically the level of bio-active GA(s) rises above a certain threshold and initiates radicle growth. A change of the inactive GA_{19} to the active GA_{20} and GA_1 was reported as a result of an exposure of spinach rosette plants to long days (Talon *et al.*, 1991).

Which GA is bio-active varies amongst species. In stem elongation of *Arabidopsis thaliana* GA_4 was bio-active (Talon *et al.*, 1990a), in *Thlaspi arvense* GA_9 (Metzger, 1990) and in *Brassica napus* and *Hordeum vulgare* GA_1 (Rood *et al.*, 1989; Boother *et al.*, 1991).

In contrast with vegetative tissues, identifications of GAs in mature seeds and determinations of level and composition are rather scarce. Immature seeds generally contain much higher GA levels than any other tissue, but GA levels often drop to zero at seed maturity (Graebe, 1987; Hutchison *et al.*, 1988). When compared to darkness, a twofold increase in GA activity, attributed to GA_7 and GA_9 was found after a short light exposure of seeds of *Picea sitchensis* and *Pinus sylvestris* (Taylor and Wareing, 1979). Chilling released dormancy of hazel seeds, but hardly increased GA levels. However, after transfer to germination conditions, levels of GA_1 rose 40-fold and levels of GA_9 at least 300-fold (Williams *et al.*, 1974). It was hypothesized that the actual breaking of dormancy involves the activation of GA production. Similarly, Metzger (1983) showed that in wild oat seeds the release of dormancy by an after-ripening period is not accompanied by a direct change in the level of GAs, but results in a greater capacity for GA biosynthesis during imbibition. It was suggested that this increase is not a necessary prerequisite for germination.

Germination of *A. thaliana* seeds is absolutely dependent on GAs. GA-deficient *ga1* seeds only germinate after application of the commercially available GA_{4+7} mixture, both in darkness and in light (Karssen *et al.*, 1989; Chapter 4). Irradiation lowers the requirement for applied GAs. In wild-type seeds irradiation also increases germination in the absence of

applied GAs. Tetcyclasis, an inhibitor of the first three steps of *ent*-kaurene oxidation (Rademacher and Jung, 1981), antagonizes the stimulative effect of light on germination. Therefore, *de novo* synthesis of GAs might be involved. A short chilling treatment sometimes enhances the light effects on both GA sensitivity and GA biosynthesis, but it can also directly increase GA sensitivity, without the interference of light (Karssen *et al.*, 1989; Chapter 4).

It is the aim of the present study to determine the activity of various GAs in stimulating germination of *A. thaliana* seeds. Interaction with endogenous GAs will be excluded by using *ga1* seeds and limited by the determination of dose responses of wild-type seeds in darkness. Endogenous GAs of wild-type seeds will be analysed by combined gas chromatography mass spectrometry (GC MS). Similar analyses will be done with GA-insensitive *gai* seeds. Shoots of this mutant were reported to contain increased levels of C_{19}-GAs when compared with wild type (Talon *et al.*, 1990b). Furthermore, preliminary results are presented of the effects of light and chilling on endogenous GA levels in seeds by using labelled internal standards.

Materials and methods

Production of seeds

Seed lots of wild type, GA-deficient mutant *ga1-2* (isolation No. 659) and GA-insensitive mutant *gai* (isolation No. 37.630) of *Arabidopsis thaliana* (L.) Heynh. were harvested in October, 1988, May, 1989, 1990 or 1991 from plants grown in an air-conditioned greenhouse. The cultivation of plants is described in Chapter 2. When seeds were ripe, they were collected, allowed to dry at room temperature for about 4 days, cleaned by sieving and blowing and stored dry at 2 °C in darkness in glass vials.

Germination experiments

Triplicates of 50-100 seeds were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll, no 595, Dassel, Germany) and moistened with 1.5 ml of the test solution. Germination occurred at a range of GA concentrations, either a commercial mixture

of GA_{4+7} or pure GAs (see below). GAs were dissolved in a small volume of either ethanol or 1N KOH and further diluted with a phosphate citrate buffer containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. Unless otherwise mentioned, pH of the buffer was 5.0. Solutions were kept at 2 °C. Germination tests were performed at 24 °C in a temperature-controlled incubator (see Chapter 2).

Light conditions were darkness or continuous irradiation with white fluorescent light (see Chapter 2).

To determine germination percentages, both germinated and non-germinated seeds were counted 7 days after the start of the germination test. Radicle protrusion was taken as the criterion for germination.

In one experiment the germination test was preceded by a pre-incubation treatment of 7 days at 2 °C. Pre-incubation was in milli-Q water in darkness.

All manipulations of incubated seeds were done in dim green light (see Chapter 2).

Extraction and purification of gibberellins

The determination of GAs followed that of Croker *et al.* (1990) with modifications. Portions of 5.0 g of wild-type or *gai* seeds were sown in a 19 cm Petri dish on a single layer of filter paper and moistened with 20 ml of milli-Q water. Seeds were either pre-incubated in darkness at 2 °C during 7 days or not pre-incubated. After pre-incubation or directly after the start of imbibition seeds were imbibed at 24 °C during 8 h either in darkness or in white light. The seeds were then transferred to a mortar, immediately frozen in l-N_2, ground and homogenized in 150 ml 80 % MeOH together with deuterated standards of GA_1, GA_4 and GA_9 (100 ng each) for quantification of their endogenous levels. The homogenate was stirred overnight at 4 °C. After centrifugation and filtration, the residue was extracted ultrasonically for 1 h with 100 ml 80 % MeOH. Small amounts of tritiated GA_1, GA_4 and GA_9 were added to check recoveries during further purification. Methanol was removed under reduced pressure at 40 °C. The aqueous residue was adjusted to pH 7.5-8.0 and washed with light petroleum. It was then filtered through a water-prewashed PVP (about 1 g) column with 2 x 2.5 ml water at pH 8.0. The eluate was adjusted to pH 2.5 (6 N HCl) and partitioned

against ethyl acetate (3 x equal volume). The combined organic phases were partitioned against 5 % (w/v) sodium bicarbonate (3 x 1/5 volume). The combined aqueous phases were acidified to pH 3.0 (6 N HCl) and partitioned against diethyl ether (3 x equal 1/3 volume), that was reduced to dryness *in vacuo* at 30 °C.

The residue was applied with 4 portions of 10 ml water at pH 8.0 onto a QAE Sephadex A-25 (Pharmacia) anion exchange column (5 cm long, 1 cm i.d.) pre-equilibrated with 1 % sodium formate pH 8.0. The column was washed with water at pH 8.0 (3 x 5 ml) and GAs were eluted with 0.2 M formic acid (4 x 5 ml) onto a pre-equilibrated C18 Sep-Pak cartridge (Waters Associates). After washing with 2 mM acetic acid + 1 % methanol (2 x 5 ml), GAs were eluted with 5 ml 80 % methanol. The sample was then reduced to dryness *in vacuo* at 40 °C.

Samples were fractionated by reversed-phase (RP) HPLC, using a 250 x 10 mm column packed with Chromspher C18 (Chrompack, Bergen op Zoom, The Netherlands) and a linear gradient of increasing methanol in 0.01 % acetic acid (10 % methanol to 70 % methanol over 25 min) at a flow rate of 4 ml min^{-1}. Samples were dissolved in 300 μl methanol, made up to 1000 μl with water and injected onto the column using a 1 ml loop. Putative GA-containing fractions of 12 ml each (3 min) were collected and taken to dryness. These fractions were methylated with excess ethereal diazomethane and each fractionated once more using the same RP HPLC system and gradient. Putative Me-GA containing fractions were collected. The dried fractions were trimethylsilylated with 15 μl Deriva-Sil (Chrompack) at 20 °C for 10 min. For GA measurements a Hewlett Packard GC MS system was used. Samples (4-5 μl) were injected splitless into a Hewlett Packard Ultra-1 fused silica capillary column (cross-linked methyl silicone; 25 m x 0.2 mm x 0.33 μm film thickness) at an oven temperature of 70 °C. After 2 min the temperature was increased to 250 °C at 30 °C min^{-1} and thereafter to 310 °C at 2.5 °C min^{-1}. The injector- and interface temperature were 275 °C and 310 °C, respectively. Mass spectra were acquired after 13 min, full scan from 600 to 200, or using selected ion monitoring.

For identification the KRI values (Kováts Retention Indices) were determined and the spectra were compared to pure standards or to spectra as published by Gaskin and MacMillan (1991). Quantitative amounts were calculated from the ratio of peak areas of specific masses

of the deuterated and non-deuterated GAs.

Gibberellins

The composition of a commercial GA_{4+7} mixture; ICI, Lot 69-694-CD (Yalding, U.K.) was determined. Fractions were separated by RP HPLC, collected, pooled and weighed.

Pure GA_1, GA_3, GA_4, GA_7, and GA_7-isolactone were partitioned and isolated by RP HPLC from commercially available chemicals. GA_1 and GA_3 were obtained from gibberellic acid A_3; Sigma # G3250, Lot 12F-0335 (St. Louis, U.S.A.). GA_4 and GA_7-isolactone were obtained from GA_{4+7}. GA_7 was obtained from GA_7; USBC # NDC12894 (Cleveland, Ohio, U.S.A.). RP HPLC was done as described above. GA_9 was a gift from the Department of Plant Cytology and Morphology of the Agricultural University, Wageningen, The Netherlands.

Results

Commercial GA_{4+7} mixture

Table 3.1 Gibberellins identified by GC MS of their Me Esters or MeTMSi derivatives in the commercial mixture GA_{4+7}.

GA	KRI	Principal ions and relative abundance (% base peak)
4	2518	M^+ 418(17), 386(15), 328(28), 289(44), 284(100), 233(44), 225(84), 224(83), 201(32), 129(62)
7-isolactone	2506	M^+ 416(11), 384(38), 356(67), 311(27), 298(56), 281(56), 244(32), 223(61), 222(100), 193(46)

It was demonstrated by RP HPLC and full-scan GC MS that the commercial GA_{4+7} mixture contained GA_4 and GA_7-isolactone, whereas GA_7 was not detected (Table 3.1). Weighing of the fractions after separation by HPLC showed that 54 % of the mixture was GA_4 and 46 % GA_7-isolactone.

Solubility of GAs in water is low, even under ultrasonic conditions. Solubility is enhanced by first dissolving GAs in a small volume of either 1 N KOH or ethanol and then diluting with aqueous solutions. The latter method dissolves GA up to concentrations of 0.2 mM, whereas the former method dissolves GA up to concentrations of at least 1 mM.

It was demonstrated by RP HPLC that as a result of the short exposure of the mixture of GA_4 and GA_7-isolactone to KOH, most GA_7-isolactone was converted to GA_7, whereas

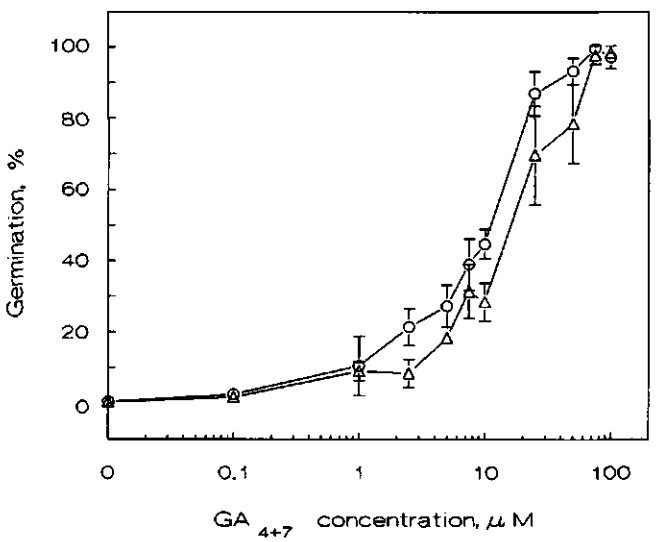

Figure 3.1 The effect of the way of dissolving a commercial GA_{4+7} mixture on GA dose responses of *A. thaliana* wild-type seeds. The GA mixture was dissolved in a small volume of ethanol (○) or 1N KOH (△) and further diluted with a buffer containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. The pH of the buffer was 5.0. Germination was tested at 24 °C in darkness. Seeds were harvested in May, 1989. Vertical bars represent SD.

GA_4 was not affected. On the contrary, ethanol did not alter the composition of the GA_4/GA_7-isolactone mixture. Responses of wild-type seeds to the GA mixture dissolved in either KOH or ethanol were compared (Fig. 3.1). Calculation of GA doses required for half-maximum response, obtained by fitting the germination data as logistic dose response curves, showed that GAs dissolved in ethanol were 1.8 times more active than the KOH-dissolved mixture.

Effect of pH on GA dose responses

Uptake and transport of GAs depend on the degree of dissociation. At lower pH values the undissociated state becomes more predominant and as a result uptake is facilitated. In

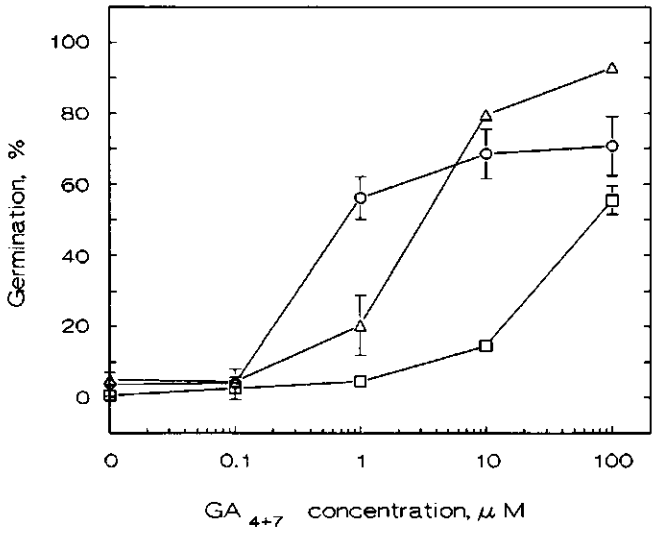

Figure 3.2 The effect of pH on GA dose responses of wild-type seeds of *A. thaliana*. A commercial GA_{4+7} mixture was dissolved in a small volume of 1 N KOH and further diluted with phosphate citrate buffer (3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid). The pH of the buffer was 3.0 (○), 5.0 (△) or 7.0 (□). Germination was tested at 24 °C in darkness. Seeds were harvested in October, 1988. Vertical bars represent SD.

order to determine the optimal pH, dose responses to the commercial GA_{4+7} mixture buffered at various pH values with a phosphate citrate buffer were tested for wild-type seeds of *A. thaliana* at 24 °C in darkness (Fig. 3.2). At pH 5.0 the requirement for the applied GA mixture was approximately ten times lower than at pH 7.0. Further acidification influenced seedling growth negatively, especially in the presence of high exogenous GA concentrations. Consequently, applied GAs were buffered at pH 5.0 in further experiments.

Effects of pure GAs

Biological activities of various GAs were compared. To prevent conversion of GAs by KOH, all GAs were dissolved in small volumes of ethanol before they were further diluted with phosphate citrate buffer at pH 5.0. The low amount of ethanol did not affect germination (data not shown).

In both genotypes the response to various pure GAs differed considerably (Fig. 3.3). The threshold concentration required to stimulate germination in darkness at 24 °C was lowest in GA_4 and GA_7-isolactone and highest in GA_7 and GA_9. Whereas 10 μM GA_7-isolactone induced a maximal physiological response in both genotypes, the same concentration of GA_7 hardly evoked any effect. Responses to GA_1 and GA_3 were intermediate. Differences in GA doses required for half-maximum response, calculated from fitted logistic dose response curves, varied between 0.6 and 90 μM for *ga1* seeds (Table 3.2). All curves were parallel as can be derived from the similarity in the slopes of the curves at half-maximum response. Values for wild-type seeds are not included because most curves did not reach a plateau. However, comparison of the curves in Figs. 3.3A and B shows that wild-type seeds always required somewhat higher levels of a certain GA.

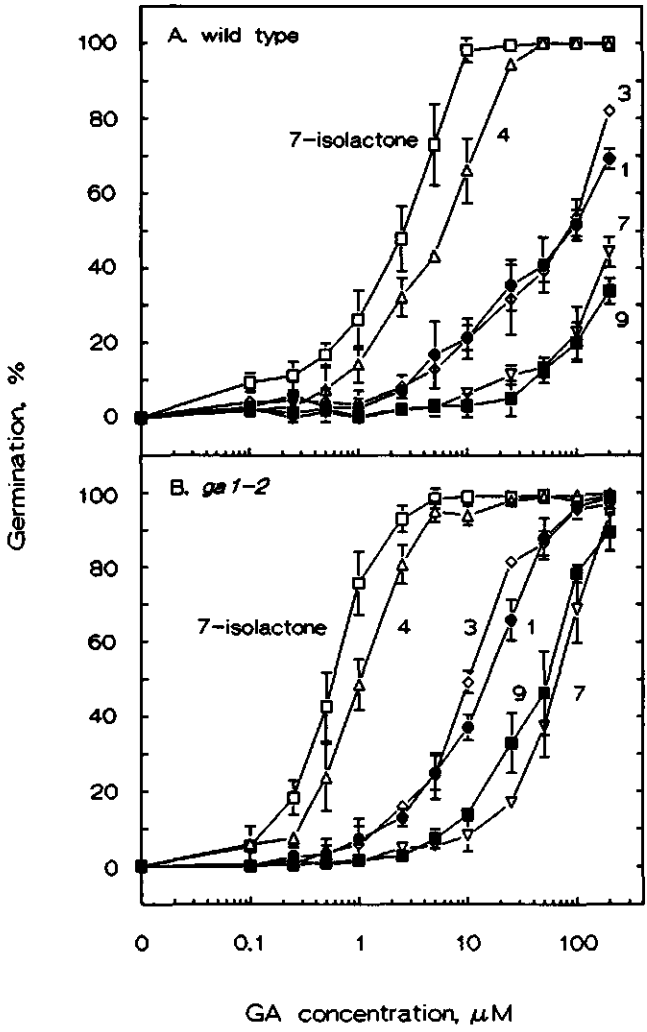

Figure 3.3 GA dose responses of wild-type (A) and *ga1-2* (B) seeds of *A. thaliana* to GA_1 (●), GA_3 (◇), GA_4 (△), GA_7 (▽), GA_7-isolactone (□) and GA_9 (■). GAs were dissolved in a small volume of ethanol and further diluted with a buffer containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. The pH of the buffer was 5.0. Germination was tested at 24 °C in darkness. Seeds were harvested in May, 1990. Vertical bars represent SD.

Table 3.2 GA doses of *ga1-2* seeds of *A. thaliana* required for half-maximum response ($[GA]_{50}$) and slopes at $[GA]_{50}$ ($\log(\partial Response/\partial \log[GA]_{50})$) calculated from fitting the dose response data in Fig. 3.3B as logistic dose response curves.

GA	$[GA]_{50}$	slope
1	15.9	1.8
3	10.0	1.9
4	1.0	2.0
7	90.1	2.0
7-isolactone	0.6	2.0
4/7-isolactone (ICI)	1.0	2.0
9	59.2	1.9

Combined application of several pure GAs was studied in order to see whether the response to a certain GA is affected by the simultaneous presence of another GA. This experiment was only performed with wild-type seeds. Increasing the concentration of the relatively inactive GA_7 somewhat decreased the response to simultaneously present GA_7-isolactone (Fig. 3.4A). Reversely, increasing concentrations of GA_7-isolactone slightly affected the small response to GA_7 (Fig. 3.4B). When the two relatively active GAs GA_4 and GA_7-isolactone were combined, stimulation of germination was also not completely additive (Fig. 3.5).

GA determinations

The influence of light and chilling on germination and GA levels was investigated in wild-type and *gai* seeds. After pre-incubation in darkness at 2 °C during 7 days, seeds were transferred to 24 °C for 8 h either in darkness or in white light. Subsequently, extracts were

made. Unchilled seeds were similarly imbibed at 24 °C for 8 h.

GA_1, GA_3, GA_4 and GA_9 were identified by full-scan GC MS in extracts from *A. thaliana* wild-type and *gai* seeds (Table 3.3). The amounts of GA_1, GA_4 and GA_9 were quantified by means of deuterated standards. Unchilled seeds that were kept in darkness contained GA_1 (wild type) and GA_9 (wild type, *gai*), whereas GA_4 was absent. GA_3 was present but not quantified. Germination capacity of these seeds was zero. Irradiation only slightly increased germination of unchilled *gai* seeds but caused almost full germination of wild-type seeds. Light increased in unchilled seeds of *gai* the levels of GA_1, GA_4 and GA_9, and of wild type the GA_4 level, whereas the levels of GA_1 and GA_9 remained fairly constant. As a result of irradiation of unchilled seeds levels of GA_1 and GA_4 became seven times, and of GA_9 ten times higher in seeds of *gai* than in wild type.

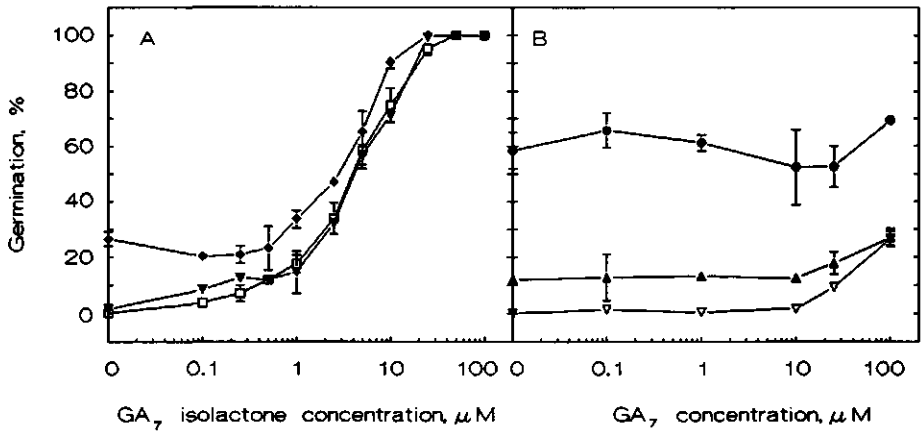

Figure 3.4 The effect of combined application of GA_7 and GA_7-isolactone on GA dose response curves of *A. thaliana* wild-type seeds. **A.** Range of GA_7-isolactone concentrations with 0 μM GA_7 (□), 10 μM GA_7 (▾) or 100 μM GA_7 (◆). **B.** Range of GA_7 concentrations with 0 μM GA_7-isolactone (▿), 0.5 μM GA_7-isolactone (▲) or 5 μM GA_7-isolactone (●). GAs were dissolved as in Fig. 3.3. Germination was tested at 24 °C in darkness. Seeds were harvested in May, 1990. Vertical bars represent SD.

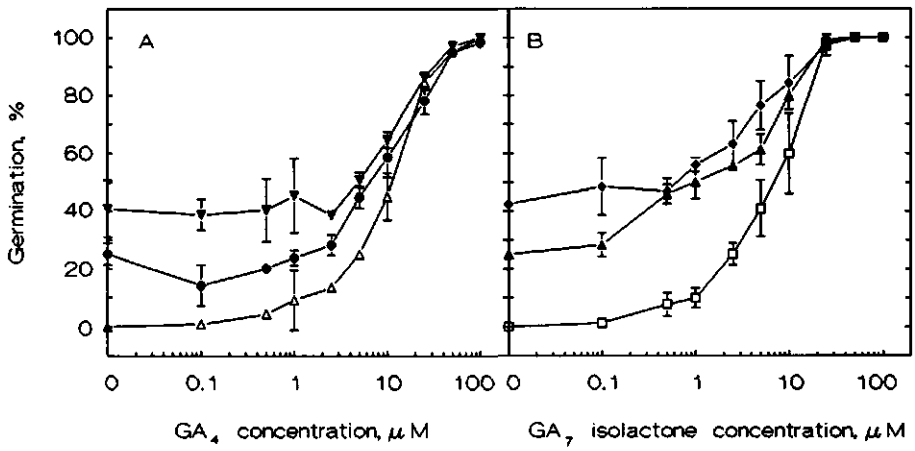

Figure 3.5 The effect of combined application of GA_4 and GA_7-isolactone on GA dose response curves of *A. thaliana* wild-type seeds. **A.** Range of GA_4 concentrations with 0 μM GA_7-isolactone (▵), 2.5 μM GA_7-isolactone (●) or 5 μM GA_7-isolactone (▾). **B.** Range of GA_7-isolactone concentrations with 0 μM GA_4 (□), 5 μM GA_4 (▲) or 10 μM GA_4 (◆). GAs were dissolved as in Fig. 3.3. Germination was tested at 24 °C in darkness. Seeds were harvested in May, 1990. Vertical bars represent SD.

In comparison with unchilled seeds, chilled seeds of both genotypes had in darkness somewhat reduced levels of GA_9 and slightly elevated levels of GA_4. Also after chilling, seeds of both genotypes did not germinate in darkness. When chilled seeds were irradiated, germination capacity of both genotypes was high. However, the increase in germination capacity was not accompanied by increases in GA levels.

Discussion

The high activity of applied GA_4 and the presence of considerable amounts of GA_4 in seeds of wild type and *gai* indicates that GA_4 has intrinsic biological activity in *A. thaliana* seeds. Other GAs that were detected in both genotypes were GA_9, GA_3 and GA_1 (Table 3.3).

Table 3.3 Effects of light and chilling on GA levels and germination of *A. thaliana* wild-type and *gai* seeds. Seeds were pre-incubated at 2 °C in darkness during 7 days or not pre-chilled. Seeds were then imbibed at 24 °C during 8 h in darkness or in white light, before extracts were made. To test germination capacity of chilled and non-chilled seeds, radicle emergence was scored after 7 days at 24 °C in darkness or continuous white light.

+ = identified, but not quantified, - = not detected.

pre-incubation	dark/ light	gibberellin content, ng g^{-1}				germination, %
		GA_1	GA_3	GA_4	GA_9	
wild type						
0d 2 °C	dark	1.6	+	-	5.0	0 ± 0
0d 2 °C	light	1.0	+	6.1	4.5	95.3 ± 3.7
7d 2 °C	dark	1.0	+	5.2	-	0.4 ± 0.7
7d 2 °C	light	1.3	+	6.1	4.4	94.2 ± 2.9
gai						
0d 2 °C	dark	-	+	-	6.0	0 ± 0
0d 2 °C	light	7.1	+	41.0	43.9	14.4 ± 5.5
7d 2 °C	dark	0.8	+	8.0	3.5	0 ± 0
7d 2 °C	light	0.6	+	5.9	3.5	73.9 ± 7.6

Activities of these GAs were respectively about 60, 10 and 15 times as low as that of GA_4 (Fig. 3.3, Table 3.2). At the limit of detection, no mass spectra were obtained of other GAs.

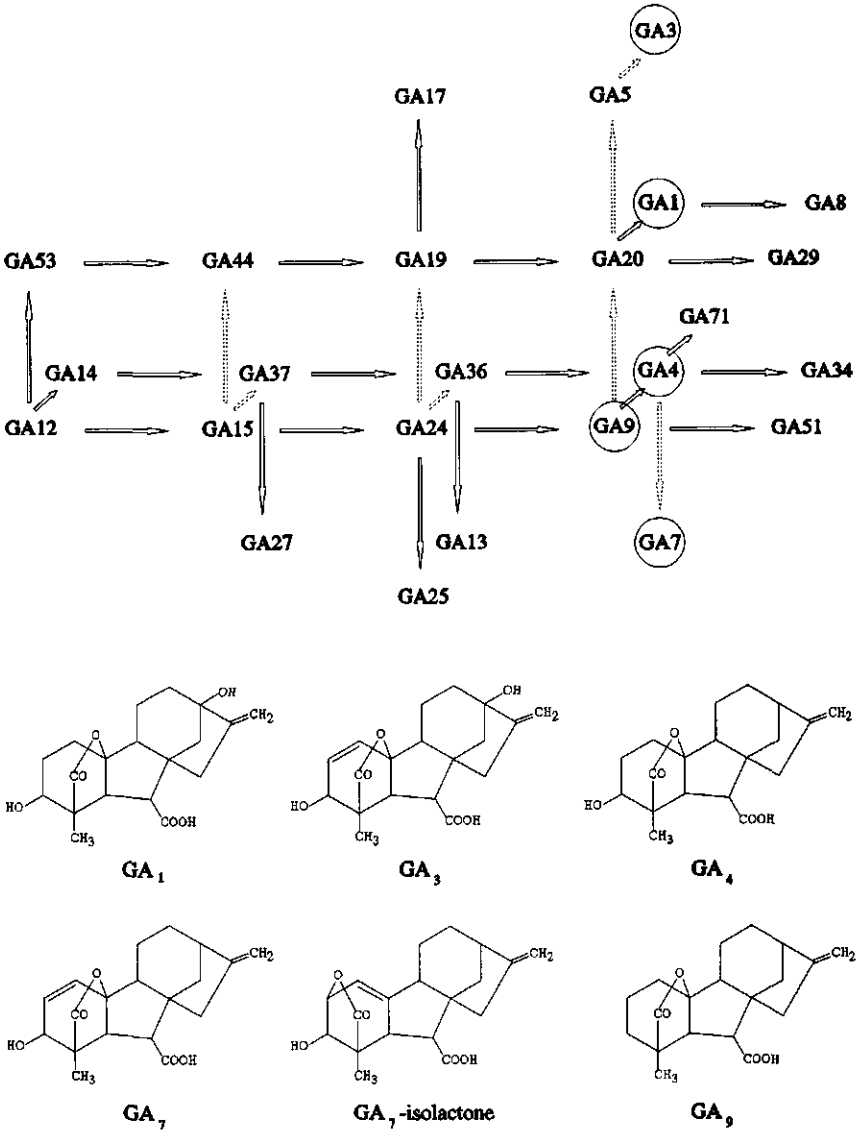

Figure 3.6 Hypothetical pathways for GA biosynthesis in shoots of *A. thaliana* as proposed by Talon *et al.* (1990a). Dotted arrows indicate GA conversions in other systems (Graebe, 1987; Smith *et al.*, 1991). Chemical structures of the used GAs.

Activities of GAs

The detected GAs are members of three different pathways that were hypothesized to exist in shoots of *A. thaliana* wild-type plants (Talon *et al.*, 1990a). GA_4 is a member of the early-3-hydroxylation pathway, GA_9 of the non-3,13-hydroxylation pathway and GA_1 and GA_3 of the early-13-hydroxylation pathway (Fig. 3.6). GA_3 was not detected in shoots. At various positions these pathways can be connected via 13-hydroxylation or 3ß-hydroxylation. In *A. thaliana* shoots GA_9 has to be converted to GA_4 via 3ß-hydroxylation to become active (Talon *et al.*, 1990a). Therefore, the activity of GA_9 depends on the degree of metabolism to GA_4. It is well known that graded series of activities exist in GA-biosynthetic pathways, the lowest being for the intermediates early in the pathway (Phinney and Spray, 1982). Assuming that at pH 5.0 penetration is not limiting for GA_9, it is proposed that conversion of GA_9 to GA_4 is very restricted in seeds of both genotypes. On the contrary, GA_9 stimulated stem elongation almost as effective as GA_4 in wild-type plants of *A. thaliana* (Talon *et al.*, 1990a). The less active GAs in seed germination (GA_9, GA_3 and GA_1) may control separate processes during other phases of the life cycle. In *Thlaspi arvense* GA_9 is the endogenous mediator of thermo-induced stem growth (Metzger, 1990). GA_9 was more active than GA_1, which in turn was more active than GA_4. GA_1 and GA_4 were also detected as native GAs in extracts from shoots (Metzger and Mardaus, 1986). However, in the control of petiole growth GA_1 was the most active GA (Metzger and Hassebrock, 1990).

GA_9 can also be metabolized to GA_1 via the sequence $GA_9 \rightarrow GA_{20} \rightarrow GA_1$ (Graebe, 1987; Metzger, 1990) or to GA_3 via the sequence $GA_9 \rightarrow GA_{20} \rightarrow GA_5 \rightarrow GA_3$ (Smith *et al.*, 1991). However, these conversions were not found in shoots of *A. thaliana* (Talon *et al.*, 1990a) (Fig. 3.6). Therefore, it is unlikely that the reduced activity of GA_9 in comparison with GA_1 and GA_3 in seeds of *A. thaliana* is due to earlier appearance in the same biosynthetic pathway. GA_1 and GA_3 are synthesized independently of GA_9 in *A. thaliana* seeds.

GA_7 could not be detected in wild-type and *gai* seeds of *A. thaliana*. Applied GA_7 had an activity comparable to that of GA_9 in wild-type and *ga1* seeds. GA_7 was also not detected in shoots of *A. thaliana* (Talon *et al.*, 1990a). In the fungus *Gibberella fujikuroi* GA_7 is

formed via GA_4 (Graebe, 1987).

The high activity of GA_7-isolactone in the present study was in contrast to low activity in the stimulation of seedling growth of dwarf pea, cucumber and lettuce (Serebryakov *et al.*, 1984) and in the prevention of fruit surface russet in apple (Looney *et al.*, 1992).

Major differences in activity of natural GAs in several systems were already described 30 years ago, when only nine GAs were known (Brian *et al.*, 1962). It is hypothesized that active GAs have several characteristics in common: a 10-19 lactone bridge below the plane of the A-ring and a hydroxyl group at the C_3-position. Effectiveness is further enhanced when the C_{13}-position is also substituted (Stoddart, 1986). The former characteristic is present in all tested GAs, whereas substituents at C_3 and C_{13} vary amongst the tested GAs, none being present in GA_9 and both in GA_1 and GA_3 (Fig. 3.6). The presence of hydroxyl groups cannot simply explain activity in *A. thaliana* seeds because the less substituted GA_4 was more active than GA_1 and GA_3 (Fig. 3.3). Although the exact nature is not known, it is postulated that the difference in activity of GA_7 and GA_7-isolactone may be due to the stereochemistry of the lactone bridge (Fig. 3.6). As a result of exposure to KOH GA_7-isolactone was converted to GA_7. Due to lower activity of GA_7, dissolving the commercial mixture of GA_4 and GA_7-isolactone (Table 3.1) in a small volume of KOH before further diluting with buffer, gave somewhat reduced germination of wild-type seeds when compared with dissolving the mixture in a small volume of ethanol (Fig. 3.1). The reduction was only minimal because GA_4 in the mixture of 54 % GA_4 and 46 % GA_7-isolactone was not affected by KOH and therefore a right-hand shift of the GA dose response curve as a result of dissolving the commercial mixture in KOH was less than a factor 2.

Differences in activity to various GAs illustrate the ability of binding sites to distinguish between subtle variations in molecular structure. Although virtually nothing is known about low-abundance proteins that possess genuine receptor characteristics, it can be proposed that the affinity of GA receptors for various GAs varies or that different GA receptors exist.

Differences in affinity are illustrated by parallel positions of GA dose response curves along the X-axis (Firn, 1986; Weyers *et al.*, 1987), as followed from fitting the dose response data as logistic dose response curves and calculating curve parameters (Table 3.2).

There are no indications that more than one GA receptor is involved. The combination

of a high concentration of the low-active GA_7 with a range of high-active GA_7-isolactone concentrations did not result in an additive response in wild-type seeds (Fig. 3.4). This indicates that both GAs compete for the same binding site. The response to the combined application of two active GAs (GA_4 and GA_7-isolactone) was clearly less than additive, indicating competition between GA_4 and GA_7-isolactone (Fig. 3.5). Similar slopes of GA dose response curves also do not present evidence against the existence of one GA receptor. However, it should be emphasized that great caution is required in the interpretation of these competition studies because nothing is known about the fate of the GA molecules after addition to the incubation medium of seeds.

Differences in unspecific binding and differences in uptake and transport to the site of action in the lipid environment of the cell may also influence the activity of applied GAs. Uptake and transport of GAs depend on the dissociation of these weak carboxylic acids which have pK_a values around 4.0. The ionized state militates against transport across membranes. The undissociated state becomes more predominant by lowering the pH of the medium, thereby enhancing uptake. Increased germination responses at pH 5.0 when compared to pH 7.0 can be explained in this respect (Fig. 3.2).

Seeds of the *ga1* mutant required less GAs for germination than wild-type seeds (Fig. 3.3), which can be regarded as some receptor-up regulation (Karssen *et al.*, 1987). The sequence of activity of different GAs was similar in both genotypes. Metzger and Hassebrock (1990) came to a similar conclusion in comparing the activity of various GAs in eliciting stem growth of wild-type and GA-deficient plants of *Thlaspi arvense*.

GA determinations

Quantification of GA_4, GA_9 and GA_1, by means of deuterated standards demonstrated that light increased levels of these GAs. This was best seen in unchilled *gai* seeds that contained in light elevated levels of GAs in comparison with wild-type seeds (Table 3.3). Also in shoots the *gai* mutant contained higher levels of GAs than wild type. It has to be realized however that shoots contain 100-1000 times as much GAs as seeds (Talon *et al.*, 1990a). Dwarf *gai* plants accumulated C_{19}-GAs such as GA_1, GA_4 and GA_9. Levels were

10-30 times as high as in wild-type plants. Dwarf *gai* plants of *Hordeum vulgare* also contained 10-fold higher GA_1 levels than wild-type plants (Boother *et al.*, 1991).

The importance for germination of the stimulation of GA biosynthesis by light may be questioned. Obviously, elevated endogenous levels of the highly active GA_4 can stimulate germination. The growth retardant tetcyclasis, which blocks the oxidation of *ent*-kaurene to *ent*-kaurenoic acid, inhibits light-induced germination. The inhibition is alleviated by exogenous GA_4/GA_7-isolactone (Karssen *et al.*, 1989; Chapter 4). This indicates that the site of light action probably occurs subsequent to *ent*-kaurene in the pathway. However, the GA level is not the only decisive factor: *gai* seeds showed lower germination in light than wild-type seeds, in spite of higher GA levels. This emphasizes that sensitivity to GAs rather than GA level is limiting for *gai* seeds. After a chilling pretreatment GA levels in irradiated *gai* seeds were lower compared to non-chilled seeds, whereas germination was significantly enhanced (Table 3.3). It is known that a combination of chilling and light increases sensitivity to GAs, also in *gai* seeds (Chapter 4). Although elevated levels of the active GA_4 were found in darkness after chilling of both genotypes, light did not further increase this level, whereas light was absolutely required for germination. Thus, the role of light in stimulating germination of chilled seeds is still rather obscure.

Hazebroek and Coolbaugh (1991) demonstrated that kaurene oxidation is not an obligate requirement for phytochrome-mediated germination in lettuce, but occurs after germination has been induced. Such a mechanism is not very likely in seeds of *A. thaliana*, which absolutely require GAs for germination. GA-deficient mutants do not germinate in the absence of applied GAs, neither in darkness nor in light (Karssen *et al.*, 1989; Chapters 2, 4). Additionally, mutants that do not require light for germination, still require GA biosynthesis (Nambara *et al.*, 1991).

Subcellular studies of specific hormone binding sites and determination of hormone levels near its primary site of action are required to unravel the chain of events between the perception of an environmental stimulus and the final germination response.

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Chapter 4

Light- and temperature-induced changes in gibberellin biosynthesis and -sensitivity influence seed dormancy and germination in *Arabidopsis thaliana*: studies with gibberellin-deficient and -insensitive mutants

M.P.M. Derkx and C.M. Karssen

Summary

The influence of light and temperature on gibberellin (GA) biosynthesis and sensitivity to applied GA_{4+7} and their role in the regulation of seed dormancy and germination were studied with the use of GA-deficient (*ga1*) and GA-insensitive (*gai*) mutants of *Arabidopsis thaliana* (L.) Heynh. and with the recombinant *gai/ga1*. The results strongly depended on the temperature of germination. Temperature determined whether or not changes in response to applied GA_{4+7} could be detected. Breaking of dormancy could best be studied at a temperature of relatively low GA sensitivity (24 °C) and dormancy induction at a temperature of relatively high GA sensitivity (10 °C). Seeds of the *ga1* mutant did not germinate in the absence of applied GAs, neither in darkness, nor in light, indicating that GAs are absolutely required for germination of this species. Wild-type and *gai* seeds did not require applied GAs in light. The conclusion that light stimulates GA biosynthesis was strengthened by the antagonistic action of tetcyclasis, an inhibitor of GA biosynthesis, on the light-induced germination in water. In some wild-type seed lots GA biosynthesis also occurred in darkness, most probably as a result of 'pre-existing' phytochrome in the seeds. In wild-type, *ga1* and *gai/ga1* seeds light lowered the GA requirement, which can be interpreted as an increase in sensitivity to GAs. In *gai* and *gai/ga1* seeds light became only effective after dormancy was broken by either a chilling treatment of one week or a dry after-ripening period at 2 °C during some months. Seeds of *gai* and *gai/ga1* exhibited strongly reduced sensitivities to light and to GAs when compared to wild-type and *ga1* seeds.

The present genetic and physiological evidence strongly suggests that temperature

primarily regulates sensitivity to light in *A. thaliana* seeds. It increases during dormancy breaking, whereas the opposite occurs during induction of dormancy (8 days at 15 °C pre-incubation). As a result the capacity to synthesize GAs changes as well as the capacity to respond to GAs. GA responsiveness is also directly controlled by temperature, thus without the interference of light. It is concluded that both GA biosynthesis and sensitivity to GAs are not the primary controlling factors in dormancy, but are essential for germination. When light-sensitive seeds perceive a light stimulus and other environmental factors are not limiting, germination starts as a result of the interaction between GA receptors, present before light perception or newly synthesized or activated and *de novo* synthesized GA.

Introduction

It is generally accepted that plants have a flexible developmental programme that can be coordinated by environmental signals. Seeds, undoubtedly representing a precarious stage in a plant's life, require a flexible strategy for dormancy control and germination. Evidence is growing that hormones play an important role in the transduction of environmental signals. It has been thought for decades that changes in hormone levels are the main regulatory mechanism. In 1968 a model was proposed in which the onset, maintenance and termination of dormancy were regulated by a balance of simultaneously present promoters (gibberellins (GAs), cytokinins) and inhibitors (abscisic acid (ABA)) of germination (Amen, 1968). However, in many cases no relationship has been found between levels of hormones in the seed and its level of dormancy (Black, 1980/81; Cohn, 1987; Roberts and Hooley, 1988).

Discussions started by Trewavas (1981) resulted in a greater awareness of the importance of sensitivity and methods to measure sensitivity were developed, using several sensitivity parameters. Also theoretical backgrounds in relation to receptors were discussed (Firn, 1986; Nissen, 1985, 1988; Weyers *et al.*, 1987). Changes in sensitivity have been shown to contribute to the control of several processes (for ref. see Trewavas, 1991), including bud dormancy in potato tubers (Turnbull and Hanke, 1985) and seed dormancy (Karssen *et al.*, 1989). Care should be taken that regulation is not automatically attributed to changes in sensitivity when discrepancies exist between changes in response and in

endogenous hormone level. In addition, control exerted via hormone levels or through sensitivity needs not be considered as mutually exclusive possibilities. They may be aspects of the same mechanism of regulation. When the contribution of sensitivity is examined by application of the growth regulator under study, one has to realize that the level of endogenous hormone in the tissue always interferes with the sensitivity to the applied growth regulator (Roberts and Hooley, 1988). This can be circumvented by using either inhibitors of the biosynthesis of a certain hormone, which includes the risk of unknown side-effects, or hormone mutants that lack the capacity to synthesize a specific hormone. In several species, like rice (Murakami, 1972), pea (Potts and Reid, 1983), maize (Phinney and Spray, 1982), *Brassica rapa* (Rood *et al.*, 1989), *Thlaspi arvense* (Metzger and Hassebrock, 1990), lettuce (Waycott and Taiz, 1991), tomato (Koornneef *et al.*, 1981) and *A. thaliana* (Koornneef and Van der Veen, 1980) GA-deficient mutants have been isolated. In the last two species, selection was made for seeds that do not germinate without application of GAs. GA-treated seeds show dwarf growth that can be reverted to the phenotype of wild-type seedlings by GA spraying. For *A. thaliana* it was demonstrated by Zeevaart and Talon (1992) that the mutation in the GA-deficient mutant blocked early steps in the GA-biosynthetic pathway prior to *ent*-kaurene. The exact position of the block is not yet known. Also in *A. thaliana* GA-insensitive dwarfs were isolated that cannot be restored to the normal phenotype by GA spraying (Koornneef *et al.*, 1985).

Studies on hormone mutants of *A. thaliana* have demonstrated that the onset of dormancy on the mother plant correlated very well with the level of embryonic ABA (Karssen *et al.*, 1983), whereas GAs were not involved in the induction of dormancy in developing seeds (Karssen and Laçka, 1986). However, GAs were absolutely required for germination. The balance theory was replaced by a kind of 'remote control' in which the GA requirement of germination of ripe seeds is controlled by the ABA levels during seed development (Karssen and Laçka, 1986). However, the role of GAs in dormancy control is still obscure.

It was the aim of the present study to investigate whether release and induction of dormancy were regulated by changes in the level of GAs, changes in sensitivity to GAs or by a combination of both. For this purpose, GA dose response curves of germination were

determined after dormancy-breaking and -inducing pre-incubation treatments in seeds of GA-deficient (*ga1*) and GA-insensitive (*gai*) mutants, and in the recombinant (*gai/ga1*) of *A. thaliana* and compared to those of wild-type seeds. In addition, tetcyclasis, an inhibitor of GA biosynthesis, was used. Germination of wild-type seeds has been shown to be light dependent (Karssen *et al.*, 1989). Light can influence both GA biosynthesis and sensitivity to GAs (Hilhorst and Karssen, 1988; Karssen *et al.*, 1989). Therefore, dose response curves were determined in all genotypes both in darkness and in light to study the effect of light on the reaction to GA.

Materials and methods

Production of seeds

Seeds of wild type, GA-deficient mutants *ga1-1* and *ga1-2*, GA-insensitive mutant *gai* and recombinant seeds *gai/ga1-1* and *gai/ga1-2* of *Arabidopsis thaliana* (L.) Heynh. were propagated in different seasons in 1987, 1988, 1989, 1990 and 1991. To start cultivation seeds were sown in 9 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll, no 595, Dassel, Germany) to which 4 ml of either milli-Q water (wild type and *gai*) or 10 μM GA_{4+7} (*ga1* and *gai/ga1*) was added. After 5 days at 2 °C to break seed dormancy, the seeds were allowed to germinate at 24 °C under continuous white fluorescent light (Philips TL 57, Eindhoven, The Netherlands). Photon fluence rate at seed level was 11 μmol m^{-2} s^{-1}. After three days the seedlings were transplanted to an air-conditioned greenhouse, as described in Chapter 2.

When the rosettes of *ga1* and *gai/ga1* plants were fully expanded, they were sprayed with 10 μM GA_{4+7} once a week during 2-3 successive weeks. When seeds were ripe, they were collected, allowed to dry at room temperature for about 4 days, cleaned by sieving and blowing and stored dry at 2 °C in darkness in glass vials.

Pre-incubation and germination conditions

Triplicates of 50-100 seeds were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll, no 595, Dassel, Germany) and moistened with 1.5 ml of milli-Q water or the test solution. Pre-incubations and germination tests were performed in temperature-controlled incubators (see Chapter 2). After pre-incubation in water in darkness at different temperatures over varying periods, the seeds were transferred to germination conditions. Water was removed from the seeds on a Büchner funnel and the filter paper with seeds brought to fresh germination solution.

Germination occurred at a range of GA_{4+7} (ICI, Yalding, U.K.) concentrations at different constant temperatures. GA_{4+7} was dissolved in a few drops of 1N KOH, and diluted either with milli-Q water or with a phosphate citrate buffer containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. The pH of the buffer was 5.0. When diluted in water, pH values of all concentrations were around 7. Solutions were kept at 2 °C. By full-scan gas chromatography mass spectrometry it was demonstrated that the commercial GA_{4+7} mixture contained 54 % GA_4 and 46 % GA_7-isolactone, whereas GA_7 was not detected (Chapter 3). As a result of short exposure of the commercial mixture to KOH, part of GA_7-isolactone was converted to GA_7, whereas GA_4 was not affected (Chapter 3).

In some experiments tetcyclasis (BASF, Limburgerhof, Germany) was added to the GA_{4+7} solutions. A stock solution of 100 μM was made by dissolving tetcyclasis in a few drops of acetone and diluting with milli-Q water. The solution was stirred for 2 days until no further increase of absorbance at 240 nm was observed. The solution was filtered and kept at 2 °C.

Light conditions were darkness, 15 min red light irradiation or continuous irradiation with white fluorescent light (see Chapter 2).

To determine germination percentages, both germinated and non-germinated seeds were counted between 5 and 28 days after the start of the germination test, depending on the germination temperature. For germination temperatures of 2, 10, 15, 24 and 30 °C, germination was scored after 28, 14, 10, 7 and 5 days, respectively. Radicle protrusion was taken as the criterion for germination.

All manipulations of incubated seeds were done in dim green light (see Chapter 2).

Some experiments were performed in absolute darkness. No light was allowed to imbibed seeds by wrapping Petri dishes in aluminium foil and packing them in black plastic sheets. Also transferring seeds from pre-incubation conditions to germination conditions occurred in absolute darkness, as described above.

All experiments were repeated at least once with qualitatively similar results. Differences in absolute values between experiments could be attributed to different seed harvests or different periods of dry storage of the seeds after harvest (Derkx and Karssen, 1993a; Chapter 2).

Results

Germination is often strongly dependent on temperature. Therefore, the response to GAs of wild-type and *ga1* seeds was studied at different germination temperatures in darkness (Fig. 4.1). Germination of both genotypes depended on GA application. The sensitivity to GAs showed an inverse relationship to temperature. Two different alleles of the GA-deficient mutants, *ga1-1* and *ga1-2* showed more or less comparable behaviour (Figs. 4.1B, C). At all temperatures, the distribution of GA threshold levels for germination was wider in *ga1* seeds (Figs. 4.1B, C) than in wild-type seeds (Fig. 4.1A). This may be regarded as some receptor-up regulation of the GA receptor in *ga1* as compared to wild type.

Temperature also affects dormancy. Low temperatures often break dormancy and high temperatures (re-)induce it. However, in several species dormancy breaking is followed by induction of dormancy at the same constant temperature (Totterdell and Roberts, 1979; Cone and Spruit, 1983; Hilhorst *et al.*, 1986; Karssen *et al.*, 1988). The effect of pre-incubation at different constant temperatures was studied in wild-type seeds. After pre-incubation in water the seeds were irradiated with red light during 15 min because, as was shown in Fig. 4.1A, germination in water did not occur in darkness. Germination was tested both at 24 °C (Fig. 4.2) and at 10 °C (data not shown). The higher the pre-incubation temperature, the quicker the rate of dormancy breaking and the quicker the rate of re-induction of dormancy. Within the time period investigated, breaking of primary dormancy at 2 °C was

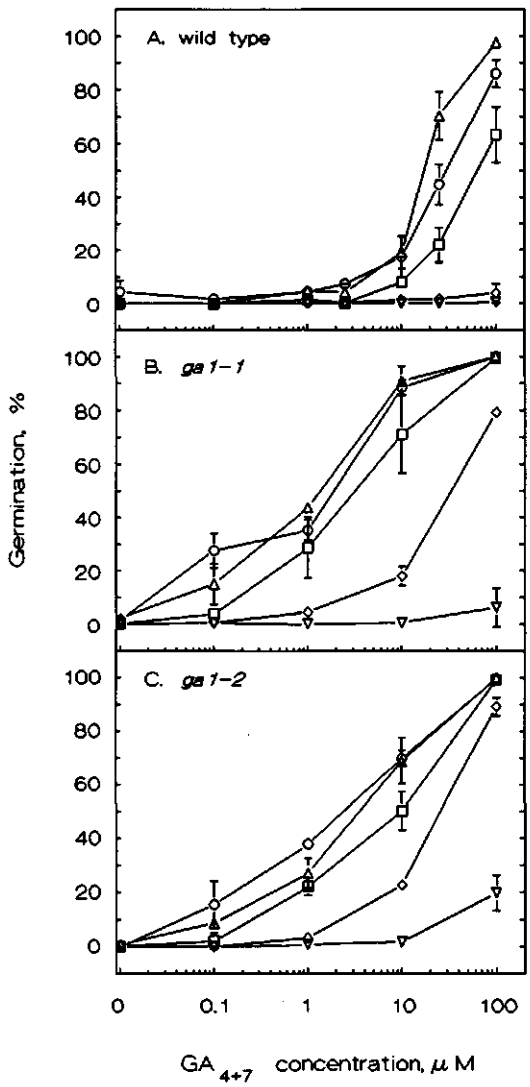

Figure 4.1 Germination of non-pre-incubated *A. thaliana* wild-type (A), *ga1-1* (B) and *ga1-2* (C) seeds at different constant temperatures. Germination was tested at a range of non-buffered GA_{4+7} concentrations in darkness at 2 (○), 10 (△), 15 (□), 24 (◇) or 30 °C (▽). Seeds were harvested in April, 1988. Vertical bars represent SD.

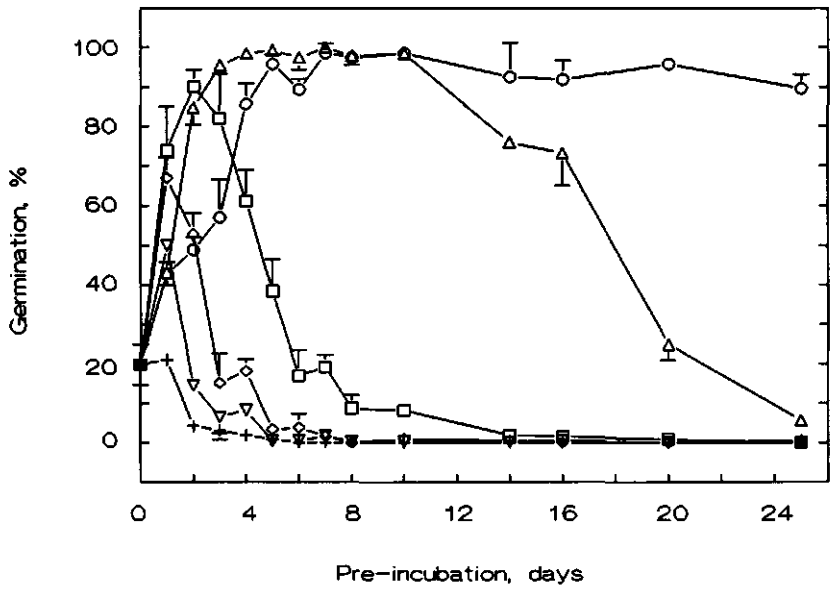

Figure 4.2 The effect of pre-incubation temperature on germination capacity of *A. thaliana* wild-type seeds. After pre-incubation in water in darkness over variable periods the seeds were irradiated with 15 min red light and thereafter transferred to 24 °C in darkness. Pre-incubation temperature was 2 (○), 10 (△), 15 (□), 20 (◇), 24 (▽) or 30 °C (+). Seeds were harvested in October, 1987. Vertical bars represent SD.

not followed by induction of secondary dormancy. During pre-incubation at 30 °C dormancy breaking was not visible. The germination tests at 24 °C showed the observed shifts slightly later than tests at 10 °C.

Based on the results presented in Fig. 4.2, in further experiments a pretreatment of 7 days 2 °C was chosen as dormancy-breaking treatment and 8 days 15 °C as dormancy-inducing treatment. The effects of these pretreatments on GA dose response curves of *ga1-1* seeds were tested in darkness at 10 and 24 °C. The temperature at which the effect of both pretreatments was tested, determined whether a shift in requirement for GAs was visible (Fig. 4.3). The lowering of GA requirement due to pre-incubation at 2 °C during 7 days was

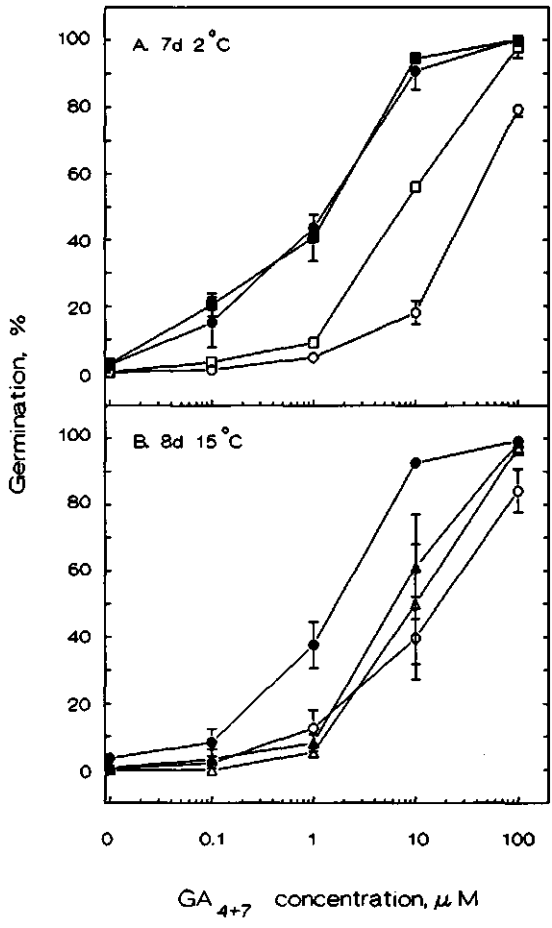

Figure 4.3 The effects of two different pre-incubation treatments on GA dose response curves of *ga1-1* seeds of *A. thaliana* tested at 10 °C (closed symbols) or 24 °C (open symbols). Seeds were pre-incubated in water in darkness for 7 days at 2 °C (A) (squares) or 8 days at 15 °C (B) (triangles) and thereafter transferred to non-buffered GA_{4+7}. Germination of these seeds was compared to germination of non-pre-incubated seeds, either freshly harvested in April, 1988 (A) or after 4 months of dry storage at 2 °C (B) (circles). Vertical bars represent SD.

apparent at a test temperature of 24 °C (open symbols) but not at 10 °C (closed symbols) (Fig. 4.3A). On the contrary, the increase in GA requirement that was caused by a pretreatment at 15 °C during 8 days was much better seen at 10 °C than at 24 °C (Fig. 4.3B).

Breaking of dormancy

To analyse the effects of a dormancy-breaking pretreatment at 2 °C in more detail, GA dose response curves of all four genotypes (wild type, *ga1-2*, *gai* and *gai/ga1-2*) were determined in darkness and continuous white fluorescent light (Fig. 4.4). Germination was tested at 24 °C. A pretreatment of 7 days 2 °C (squares) shifted the GA dose response curves in darkness (closed symbols) to lower concentrations, both in wild-type (Fig. 4.4A) and *ga1-2* seeds (Fig. 4.4B). Irradiation (open symbols) also lowered the GA requirement of these two genotypes. When chilling and light were combined, wild-type seeds became independent of GA application (Fig. 4.4A). The GA requirement of *ga1-2* seeds showed an extra decrease, compared to single treatments but the seeds remained absolutely dependent on exogenous GA in light (Fig. 4.4B).

GAs never induced any dark germination in seeds of *gai* (Fig. 4.4C) and *gai/ga1-2* (Fig. 4.4D), neither freshly harvested nor after a chilling pretreatment. In freshly harvested seeds light also had no effect. However, when light and chilling were combined, 90 % of *gai* seeds germinated in water. GA_{4+7} had no additional effect (Fig. 4.4C). In recombinant seeds, combination of chilling and light resulted in an increased response to applied GAs. Germination remained absolutely GA dependent (Fig. 4.4D).

To investigate whether the increase in GA sensitivity due to 7 days pre-incubation at 2 °C was reversible, pre-incubation time at 2 °C was prolonged. Tests were performed with wild-type (Fig. 4.5A) and *ga1-2* seeds (Fig. 4.5B). Germination was tested at 24 °C in darkness. Up to 13 weeks of pre-incubation GA sensitivity increased in both genotypes (Figs. 4.5A, B). This was followed by a decrease. After 27 weeks of pre-incubation GA sensitivity reached the original level at the start of imbibition (data not shown) and this level was still maintained after 36 weeks of pre-incubation.

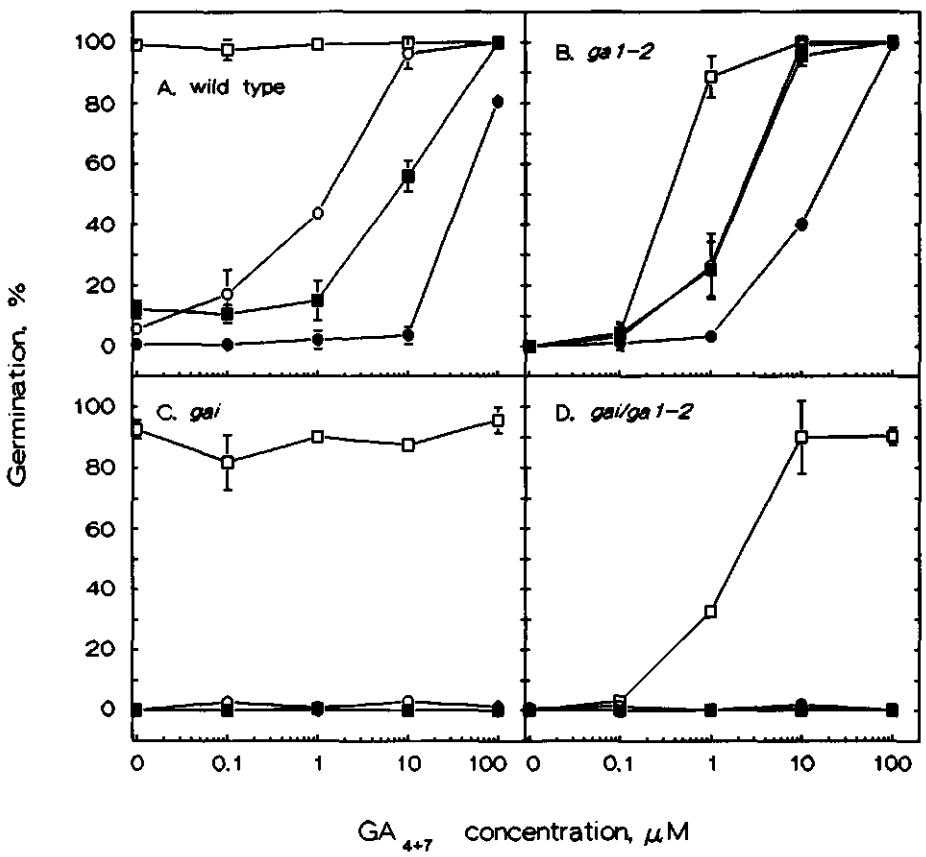

Figure 4.4 The effects of pre-incubation at 2 °C and light on GA dose response curves of wild-type (A), *ga1-2* (B), *gai* (C) and *gai/ga1-2* (D) seeds of *A. thaliana*. Pre-incubation occurred in water in darkness at 2 °C (squares), control seeds were not pre-incubated (circles). Germination occurred at 24 °C at a range of GA_{4+7} concentrations buffered at pH 5.0, in darkness (closed symbols) or in continuous white light (open symbols). Seeds were harvested in June, 1990. Vertical bars represent SD.

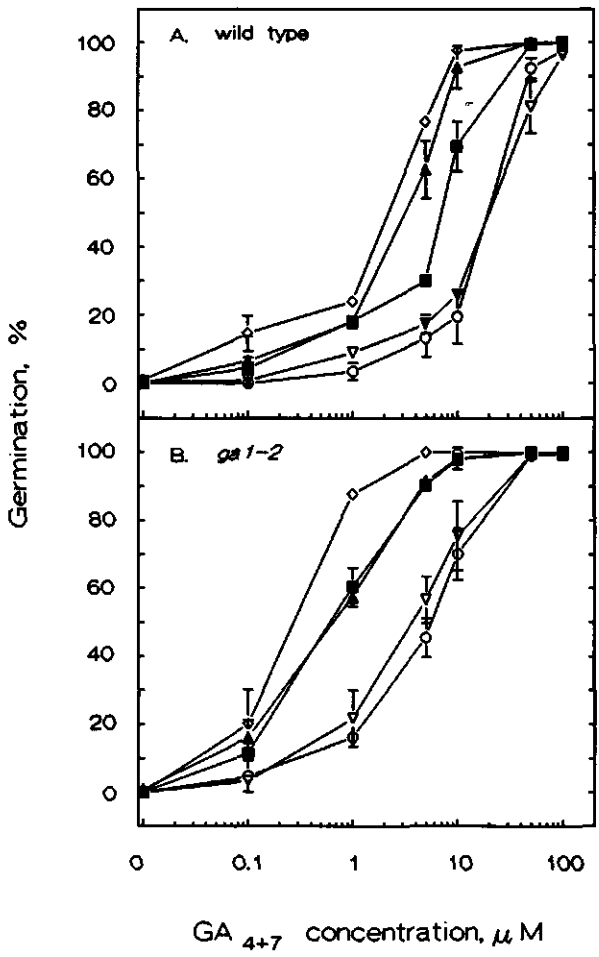

Figure 4.5 The effect of prolonged pre-incubation at 2 °C on GA dose response curves of wild-type (A) and *ga1-2* (B) seeds of *A. thaliana*. Seeds were pre-incubated in water in darkness for 0 (○), 4 (▲), 13 (◇), 19 (■) or 36 (▽) weeks. After pre-incubation the seeds were transferred to a range of GA_{4+7} concentrations, buffered at pH 5.0 and germination was tested at 24 °C in darkness. Seeds were harvested in June, 1989. Vertical bars represent SD.

Induction of dormancy

Dormancy induction was studied in seeds that had been in dry storage at 2 °C for 4 months. After-ripened seeds were chosen because of their considerably reduced dormancy (Karssen and Laçka, 1986; Derkx and Karssen 1993a; Chapter 2).

After 4 months of dry storage, germination in light of wild-type seeds was practically independent of exogenous GA (Fig. 4.6A). In darkness, the seeds exhibited a clear response to GA, however, also in water (0 μM GA_{4+7}) 20 % germinated. A pretreatment of 8 days 15 °C (triangles) lowered the germination in water to the same extent in darkness and light. In addition, the requirement for exogenous GA increased (Fig. 4.6A). This shift to higher GA concentrations was also visible in *ga1-1* (Fig. 4.6B) and *gai/ga1-1* seeds (Fig. 4.6D). Some germination was observed in *ga1-1* seeds in water.

The after-ripened seeds of *gai* germinated for 70 % in water. Exogenous GA_{4+7} did not further increase this response (Fig. 4.6C). A pretreatment of 8 days 15 °C resulted in a drop of germination to about 0 % over the whole GA-concentration range.

Effects of tetcyclasis on light-induced germination

The fact that germination of wild-type and *gai* seeds that both contain the dominant *GA1* locus, occurred in light without the presence of exogenous GA, strongly indicates that the seeds synthesize GAs under these circumstances. To further check this conclusion, tetcyclasis, an inhibitor of GA biosynthesis at the level of *ent*-kaurene synthetase (Rademacher and Jung, 1981), was added to chilled and non-chilled seeds. In wild-type seeds the increased germination in light and water caused by chilling was fully reversed by tetcyclasis (Fig. 4.7). Application of GA_{4+7} antagonized the inhibitory action of tetcyclasis. Thus, it seems that germination is preceded by *de novo* synthesis of GAs and that chilling increased the capacity of synthesis. Tetcyclasis-treated chilled seeds were more sensitive to GAs than non-chilled seeds. In *gai* seeds tetcyclasis had similar effects as in wild-type seeds (data not shown).

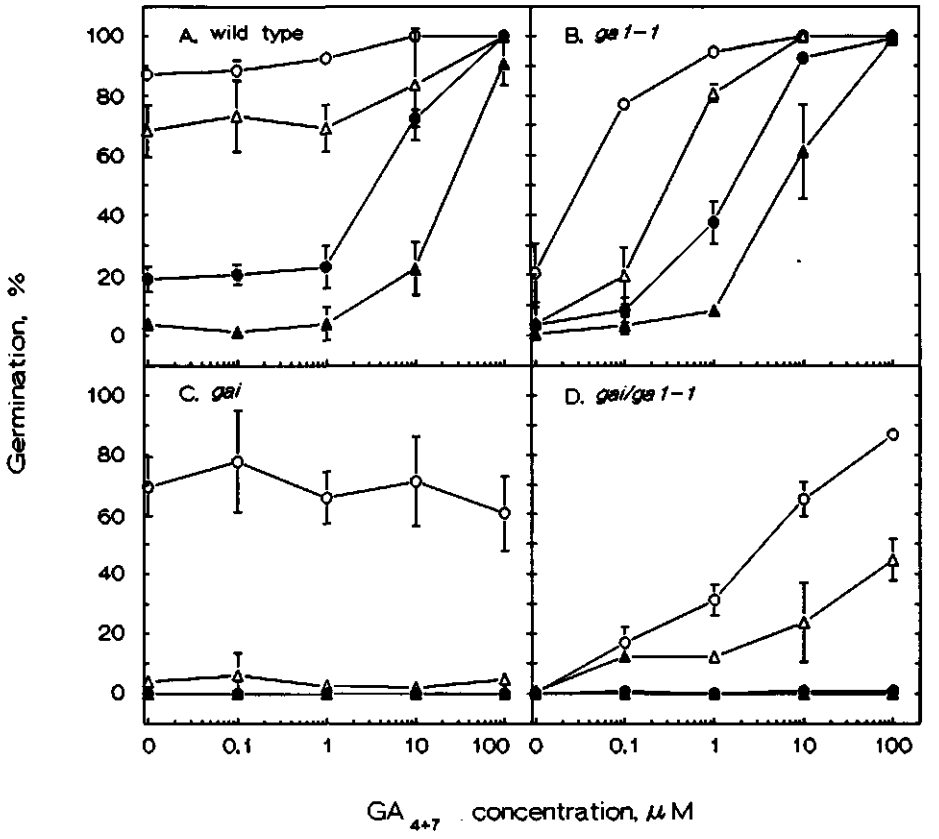

Figure 4.6 The effects of pre-incubation at 15 °C and light on GA dose response curves of wild-type (A), *ga1-1* (B), *gai* (C) and *gai/ga1-1* (D) seeds of *A. thaliana*. Pre-incubation occurred in water in darkness at 15 °C (triangles), control seeds were not pre-incubated (circles). Germination occurred at 10 °C at a range of non-buffered GA_{4+7} concentrations in darkness (closed symbols) or in continuous white light (open symbols). Seeds were harvested in April, 1988 and before the start of this experiment stored dry at 2 °C during 4 months. Vertical bars represent SD.

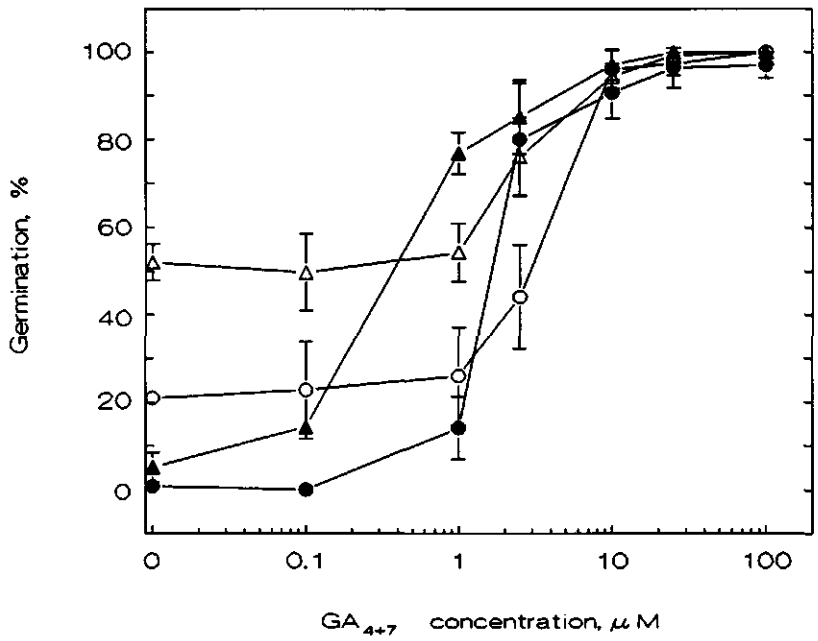

Figure 4.7 The effect of tetcyclasis on GA_{4+7}-induced germination of wild-type seeds of *A. thaliana* at 24 °C in continuous white light with (triangles) or without (circles) a pre-incubation in water in darkness at 2 °C during 7 days. Germination was tested at a range of GA_{4+7} concentrations, buffered at pH 5.0, in the absence (open symbols) or in the presence of 10 μM tetcyclasis (closed symbols). Seeds were harvested in May, 1991. Vertical bars represent SD.

GA dose responses in absolute darkness

The dependency on applied GA of wild-type seeds in darkness was not always absolute (Figs. 4.4A, 4.6A). This might be caused by 'pre-existing' phytochrome (Pfr) or an extremely high light sensitivity of some seed lots, such that the dim green light under which seed handling occurred, was sufficient to saturate a very-low-fluence-response. To investigate the role of 'pre-existing' Pfr and that of dim green light, seed handling under dim green light

was compared with absolute darkness in wild-type seeds harvested September, 1990. Seeds of that harvest were not fully GA dependent, also not when germination occurred in absolute darkness (Fig. 4.8). The role of the dim green light was minimal, germination was only slightly increased. A pretreatment of 7 days 2 °C increased water germination to a similar extent under both conditions. As with light-stimulated germination (Fig. 4.7), this increase under dim green light was fully reversed by 10 μM tetcyclasis. This seed lot did not show an increase in GA sensitivity due to the chilling pretreatment.

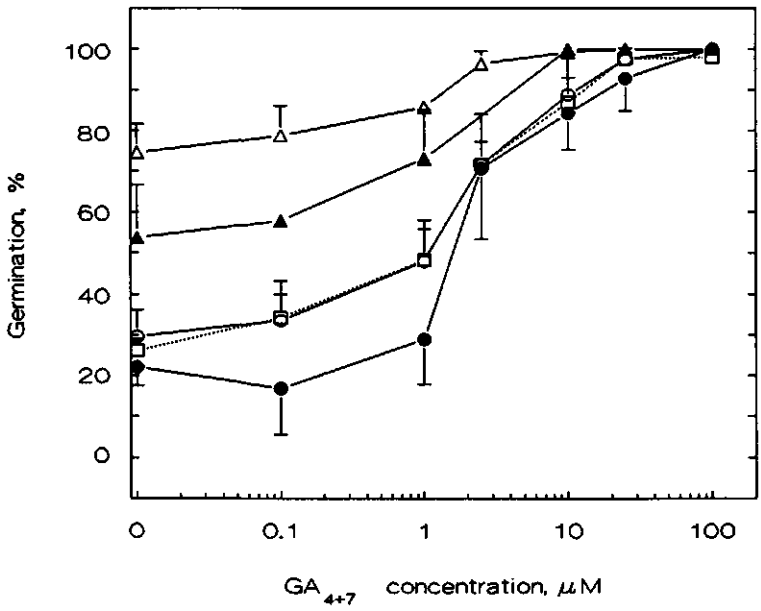

Figure 4.8 Germination of wild-type seeds of *A. thaliana* handled in absolute darkness (closed symbols) or under dim green light (open symbols). The seeds were pre-incubated at 2 °C during 7 days (triangles) in water in darkness or not pre-incubated (circles). Germination was tested at 24 °C at a range of GA_{4+7} concentrations, buffered at pH 5.0. To part of the chilled seeds 10 μM tetcyclasis was added during the germination test (dotted line, squares). Seeds were harvested in September, 1990. Vertical bars represent SD.

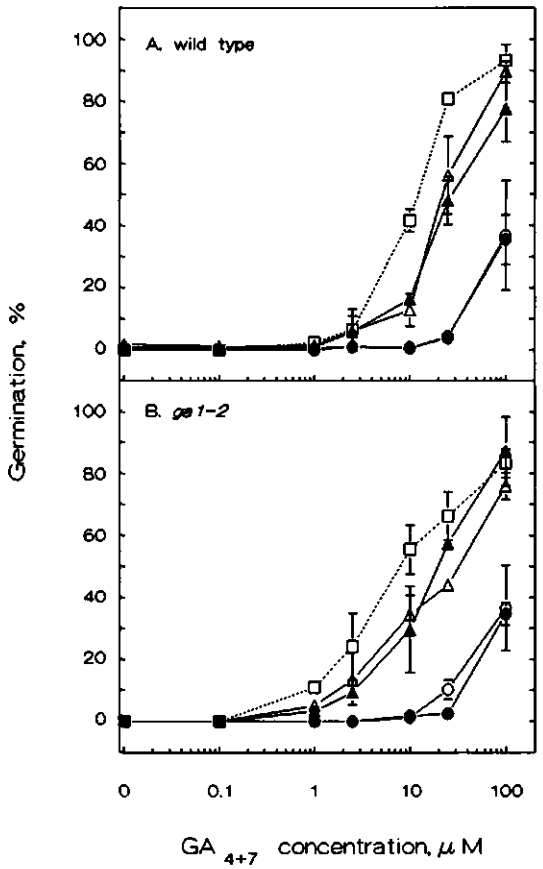

Figure 4.9 Germination of wild-type (A) and *ga1-2* (B) seeds of *A. thaliana* handled in absolute darkness (closed symbols) or under dim green light (open symbols). Pre-incubation and germination tests as in Fig. 4.8. Seeds were harvested in May, 1991. Vertical bars represent SD.

A similar experiment was carried out with seeds of wild type and *ga1-2* of a deeply dormant seed lot (harvested May, 1991). Both genotypes showed an increased response to applied GA_{4+7} after a chilling pretreatment, both under green light and in absolute darkness (Figs. 4.9A, B). Tetcyclasis further increased the sensitivity to applied GA. Also in the May, 1991 seed lot, the effect of green light could be neglected.

Discussion

The present results clearly show that in *A. thaliana* seeds changes in sensitivity to light are correlated to temperature-induced changes in dormancy. Since light stimulates the synthesis of GAs as well as GA responsiveness, this regulation ensures the synthesis of germination-stimulating factors, like GAs, and the synthesis or activation of receptors, when relatively low-dormant light-sensitive seeds perceive a light stimulus. On the contrary, it prevents these reactions when relatively dormant, light-insensitive seeds are irradiated. GA sensitivity is also directly influenced by temperature. These main conclusions can be deduced from the effects of dormancy-breaking and -inducing treatments on GA dose response curves of wild type, *ga1*, *gai* and *gai/ga1* seeds, both in darkness and in continuous white light (Figs. 4.4, 4.6). Whether or not shifts in requirement for GAs were visible strongly depended on the temperature at which GA dose responses were determined (Fig. 4.3). Therefore, dormancy breaking was studied at 24 °C and dormancy induction at 10 °C.

Shifts in requirement for GA_{4+7} and light in wild-type and ga1 seeds

GA biosynthesis

Germination of *A. thaliana* seeds is absolutely dependent on GAs, as was described previously by Karssen *et al.* (1989). Mutants that lack the capacity to synthesize GAs (*ga1* and *gai/ga1*) do normally not germinate in the absence of applied GAs, neither in darkness nor in light (Figs. 4.1B, C, 4.3, 4.4B, D, 4.5B, 4.6D, 4.9B). One exception was seen in *ga1-1* seeds in Fig. 4.6B. An explanation for this exception may be that this *ga1* mutant is somewhat leaky (Koornneef and Van der Veen, 1980). However, the possible presence of low ethylene concentrations in the incubator may also explain the absence of full GA dependency of *ga1-1* seeds, since ethylene is known to replace the need for GAs in *ga1* seeds, especially in light (Karssen *et al.*, 1989).

Applied GA was also required for germination of wild-type seeds in darkness (Figs. 4.1A, 4.4A, 4.5A, 4.6A). Irradiation with continuous white light in combination with

chilling (Fig. 4.4A) or with a period of dry after-ripening (Fig. 4.6A) made wild-type seeds independent of applied GAs, from which it can be concluded that light stimulates GA biosynthesis in wild-type seeds. The absence of such a light effect in *ga1* seeds (Fig. 4.4B) and the inhibitory action of tetcyclasis on the light-stimulated germination in wild-type seeds (Fig. 4.7) strengthened this conclusion. The inhibitory effect of tetcyclasis was reversed by application of GA_{4+7}. Thus, the germination process is preceded by *de novo* synthesis of GAs as a result of irradiation. A chilling treatment or a period of dry after-ripening acts by increasing the sensitivity to light and thereby the capacity to synthesize GAs, whereas the opposite occurs during a dormancy-inducing pretreatment of 8 days 15 °C. Therefore, GA levels do not themselves regulate dormancy, as was suggested before by Karssen and Laçka (1986). Although the presence of GAs is a prerequisite for germination, the actual control is at the level of phytochrome.

The use of tetcyclasis could also explain why wild-type seeds of some seed lots could germinate in the absence of light. Tetcyclasis fully reversed the germination in water after a chilling pretreatment of seeds handled under dim green light (Fig. 4.8). This germination in water could be attributed to GA biosynthesis, most probably as a result of 'pre-existing' Pfr. The response was namely not only observed when seed handling occurred under dim green light but also in absolute darkness (Fig. 4.8). Cone and Kendrick (1985) indeed demonstrated the presence of 'pre-existing' Pfr in *A. thaliana* wild-type seeds. Additionally, it is possible that the short exposures to dim green light generate a small amount of Pfr, being adequate to initiate a very-low-fluence-response (Kendrick and Cone, 1985). However, water germination in absolute darkness was only somewhat reduced in comparison with that obtained under dim green light (Fig. 4.8).

GA sensitivity

In addition to enhanced GA biosynthesis, light also increased the responsiveness to applied GAs in wild-type and *ga1* seeds (Figs. 4.4A, B, 4.6A, B). Chilling also increased the responsiveness to GAs, whereas a pretreatment of 8 days 15 °C decreased it. There is no doubt that the observed shifts in requirement for GAs can indeed be attributed to changes in sensitivity to GAs, rather than to changes in GA biosynthesis, because the shifts occurred in

the absence of GA biosynthesis, either due to a mutation (*ga1*) (Figs. 4.4B, 4.6B) or to the presence of tetcyclasis (Fig. 4.7, compare closed circles and closed triangles). Moreover, tetcyclasis did not reduce the GA-stimulated germination of both wild-type and *ga1* seeds in darkness (Fig. 4.9). The observed shifts in GA requirement due to chilling were not simply reflections in sensitivity to dim green light, as they were observed in absolute darkness as well in deeply dormant seeds of wild type and *ga1-2* mutant (Fig. 4.9).

Sensitivity to applied GAs was generally higher in *ga1* seeds than in wild-type seeds (Fig. 4.1), as was also described previously (Karssen *et al.*, 1987). In mammalian systems, this self-regulatory nature, whereby a hormone can regulate the function and number of its own receptor, e.g. up-regulation, is a well-known phenomenon (Hollenberg, 1985b).

Shifts in requirement for GA_{4+7} and light in gai and gai/ga1 seeds

The biochemical nature of the *gai* mutation is not known. Detailed molecular analysis has to learn whether the synthesis of a receptor is blocked or some other part of the signal-transduction pathway. The *gai* mutant is known to contain elevated levels of GAs in young shoots in comparison with wild-type plants (Talon *et al.*, 1990).

Seeds of *gai* and *gai/ga1* never showed more than a few percent germination in darkness (Figs. 4.4C, D, 4.6C, D). The reduced GA sensitivity of *gai* seeds is apparent from the absence of a GA effect on germination both after chilling and in light. A combination of light and chilling (Fig. 4.4C) or dry after-ripening (Fig. 4.6C) increased germination in water. Applied GAs did not further increase this response. In *gai/ga1* seeds, however, applied GAs increased germination when chilling or dry after-ripening and light were combined (Figs. 4.4D, 4.6D). This increased response can be attributed to an increased GA sensitivity. On the contrary, sensitivity to GAs decreased during a pretreatment of 8 days 15 °C (Fig. 4.6D). In comparison with the single-gene mutant *ga1*, the *gai/ga1* mutant showed a reduced GA sensitivity (Figs. 4.4B, D). The combination of a reduced biosynthesis of GAs and a reduced sensitivity prevents germination in water. Even if small amounts of endogenous GA are present due to 'leakage' of *ga1*, the seeds cannot respond because of the simultaneous presence of the *gai* mutation.

The different responses of *gai* and *gai/ga1* seeds to a range of GA_{4+7} concentrations (Figs. 4.4C, D, 4.6C, D) must be due to the capacity of the former mutant to synthesize GAs. The stimulation of GA biosynthesis required a combination of irradiation and chilling or dry after-ripening. Since the response to applied GA_{4+7} never rose above that in water, it has to be concluded that the amount of GAs that is synthesized in the *gai* mutant directly saturated the GA-response system, that additionally is affected by a combination of light and chilling or dry after-ripening. Extra evidence that both increased GA biosynthesis and -sensitivity are involved, came from the addition of tetcyclasis to *gai* seeds. This inhibitor reversed the germination in water. Moreover, in the presence of tetcyclasis chilled seeds were more sensitive to applied GAs than non-chilled seeds (data not shown).

During dormancy induction light sensitivity decreased, resulting in a reduced capacity to synthesize GAs and in a reduced capacity to respond to GAs.

Thus, the observed effects of light and temperature on GA biosynthesis and -sensitivity in *gai* and *gai/ga1* seeds confirmed the conclusions in wild-type and *ga1* seeds.

Regulation of dormancy and germination in A. thaliana seeds

In conclusion, the present data clearly demonstrate that studies with hormone mutants of *A. thaliana* often allow a good distinction between the role of GA biosynthesis and GA sensitivity in the regulation of dormancy and germination.

Evidence is presented that temperature primarily regulates sensitivity to light. Depending on temperature and the duration of the temperature pretreatment the response to light increases or decreases (Fig. 4.2). As a result of a change in sensitivity to light, the capacity to synthesize GAs will also change, as well as the capacity to respond to GAs. Additionally, the capacity to respond to GAs is also directly influenced by temperature, thus without the interference of light. When light-sensitive seeds perceive a light stimulus and other environmental factors are not limiting, germination starts as a result of the interaction between GA receptors, present before light perception or newly synthesized or activated and *de novo* synthesized GAs. Changes in light sensitivity are not always accompanied by changes in GA responsiveness. Consequently, it is proposed that sensitivity to GAs is not

primarily important for the control of dormancy. In the related species *Sisymbrium officinale* detailed analyses of dose responses to light and GAs demonstrated that dormancy control was indeed not at the level of the GA receptor whereas the phytochrome receptor was primarily involved in temperature-induced changes in dormancy (Hilhorst, 1990; Derkx and Karssen, 1993b; Chapter 6). Changes in sensitivity to GAs and light may consist of changes in the number of receptors, changes in the affinity of the receptors and changes in the response chain initiated by binding of GA or phytochrome to their receptors (Hollenberg, 1985a; Firn, 1986; Weyers *et al.*, 1987). The light-induced biosynthesis of GAs is ultimately required for germination, but does not participate in the control of dormancy. Also in the absence of GA biosynthesis due to the *ga1* gene or inhibition by tetcyclasis, changes in response to applied GAs occur.

The absence of direct dormancy control via GA levels confirmed suggestions by Trewavas (1981) and Metzger (1983). Trewavas based his conclusion on the shape of dose response curves in combination with measured endogenous concentrations of hormones: it is quite common that responses of plants to growth substances occur over a wide range of sometimes 5 to 6 orders of magnitude, thus relatively large changes in concentration initiate little response change (Kende and Gardner, 1976). Mostly, changes in endogenous concentrations in plants operate around the 2-5 fold range (Trewavas, 1981), from which the author concluded that control by change of concentration is improbable. The dose response curves presented in this paper generally spanned 2-3 orders of magnitude, sometimes even more. However, a very substantial change of response often occurred within one order of magnitude (see e.g. Fig. 4.6). Thus the shape of the presented dose response curves does not rule out the role of endogenous GAs in dormancy control, however, the use of hormone mutants and tetcyclasis proved that GA levels are not directly regulatory.

To our knowledge, these shifts in both the capacity to synthesize GAs as well as in GA sensitivity occurring in two directions and accompanying both breaking and induction of dormancy, have never been found before. Obviously, it provides a very flexible framework by which seeds can respond to their environment. Further analysis of sensitivity at the molecular level by isolating GA-binding sites in combination with measurements of endogenous GA levels are required to verify our conclusions.

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Chapter 5

Characterization of seasonal dormancy patterns in *Arabidopsis thaliana*: studies with gibberellin-deficient seeds

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Summary

Imbibed seeds of *Arabidopsis thaliana* passed annually through a seasonal pattern of changes in dormancy. Both primary and secondary dormancy were broken in summer. Seeds re-entered dormancy in autumn and winter. A second small flush occurred in early spring. The role of sensitivity to gibberellins (GAs), to light and to nitrate in regulating annual dormancy patterns and germination of this species was studied with the use of GA-deficient (*ga1-2*) and wild-type seeds. Dark-incubated seeds of both genotypes were exposed to a natural temperature regime for periods up to 18 months and at regular intervals germination capacity of portions of seeds was tested under laboratory conditions. Germination data were fitted as logistic dose response curves and curve parameters were calculated. Temperature during incubation outdoors regulated the light requirement of both wild-type and *ga1-2* seeds. These changes in light sensitivity were translated in changes in the capacity to synthesize GAs (wild-type seeds) as well as in changes in the capacity to respond to GAs (wild-type and *ga1-2* seeds). Additionally, temperature directly regulated GA sensitivity, thus without the interference of light. However, the significance of direct regulation of GA requirement seemed to diminish with prolonged incubation at outside temperatures, whereas temperature-induced reversible changes in light sensitivity clearly remained. Therefore, it is proposed that sensitivity to GAs is not limiting for germination at any time of the annual dormancy cycle. Sensitivity to light primarily determines whether light can initiate GA biosynthesis and increase GA sensitivity, thereby allowing germination when other environmental factors are not limiting. From interpretation of curve parameters, it is proposed that the observed changes in sensitivity to light and to GAs involve changes in the number of phytochrome-

and GA receptors, in the binding characteristics of the receptors or in the response chain initiated by the interaction of phytochrome and GA with their putative receptors. The GA requirement for germination is obvious as *ga1-2* seeds did not germinate at any time of the year when deprived from applied GAs. However, the capacity to synthesize GAs is not required for dormancy control, as a dormancy pattern was also observed in the absence of the capacity to synthesize GAs. Nitrate or sensitivity to nitrate did not contribute in the regulation of dormancy and germination of this species.

Introduction

It is well established that buried seeds of many species pass annually through a pattern of change in dormancy (Karssen, 1982; Baskin and Baskin, 1985; Bouwmeester, 1990). Although the adaptive significance of seasonal changes in dormancy is evident, the internal mechanisms by which these patterns are regulated, are not well understood. An adequate explanation of annual dormancy cycles has to involve its reversible character. Reversibility is nearly completely neglected in studies on dormancy mechanisms.

Studies with buried seeds have shown that temperature during burial plays a predominant role in the regulation of seasonal dormancy patterns. Changes in dormancy reflect themselves in the size of the temperature range at which germination can proceed at particular times. In general, the range is widened during alleviation of dormancy and narrowed during dormancy induction (Vegis, 1964; Karssen, 1982; Bouwmeester and Karssen, 1992). Thus, temperature controls both changes in dormancy and the actual germination process. Temperature during burial is also responsible for alterations in the light requirement of several species. Seeds that are light requiring on dispersal may lose light sensitivity during burial and may regain it after a further period of burial (Taylorson, 1972; Karssen, 1980/81).

The nature of the endogenous changes that are induced in the seeds by temperature during burial are still rather obscure. The abundant quantities of hormones in seeds at certain stages of development and the effects evoked by exogenous growth regulators on germination and dormancy led to the theory that dormancy is regulated by a balance of simultaneously operating promoters and inhibitors of germination (e.g. Amen, 1968). Studies on hormone

mutants of *Arabidopsis thaliana*, however, demonstrated that abscisic acid (ABA) was responsible for the onset of primary dormancy on the mother plant (Karssen *et al.*, 1983), whereas gibberellins (GAs) were not involved in this stage (Hilhorst and Karssen, 1992). On the contrary, GAs were required for germination (Karssen *et al.*, 1989). It was concluded that both hormones never act simultaneously in *A. thaliana* seeds.

Many attempts failed to correlate levels of hormones in the seed and its level of dormancy (Black, 1980/81; Wareing, 1982; Metzger, 1983; Roberts and Hooley, 1988). Correlations were also often missing in other developmental processes (Trewavas, 1981). Therefore, the balance theory has been challenged and it was argued by Trewavas (1981) that sensitivity to hormones is decisive in regulating several developmental processes. Although this view has been greeted with considerable criticism, the regulatory nature of sensitivity has been described for several developmental processes in the last decade (see Trewavas, 1991).

In previous studies we demonstrated that in *A. thaliana* seeds sensitivity to GAs increased during a dormancy-breaking temperature pretreatment (Chapter 4; Karssen *et al.*, 1989), whereas the opposite occurred during dormancy induction, both in seeds of the GA-deficient (*ga1-2*) mutant and in wild-type seeds (Chapter 4). It was concluded that also in the absence of GA biosynthesis, changes in the response to applied GAs could occur. Additionally, changes in sensitivity to a saturating red light pulse occurred during changes in dormancy, resulting in variable capacity to synthesize GAs in wild-type seeds and in variable responsiveness to GAs. As long as wild-type seeds, either sensitive or insensitive to GAs are imbibed in darkness, germination will not proceed, because GA biosynthesis is phytochrome dependent. But when sufficiently light-sensitive wild-type seeds are irradiated GA biosynthesis will start and GA responsiveness further increases and as a result germination will proceed. These conclusions are based on studies in which dormancy was alleviated or induced by pre-incubating seeds at constant temperatures in the laboratory for relatively short periods (Chapter 4).

In the present study dormancy regulation in *A. thaliana* is studied under outside conditions. A seasonal dormancy pattern in *A. thaliana* has been described by Baskin and Baskin (1983). At the time of seed dispersal, which occurs in Kentucky, U.S.A. around

May, seeds were dormant. During summer dormancy was released. Seeds re-entered dormancy during autumn and winter, resulting in dormant seeds in spring. In addition to this winter-annual behaviour, summer-annual races have been reported by Laibach (1951).

In the related species *Sisymbrium officinale* we demonstrated that sensitivity to light and nitrate increased when both primary and secondary dormancy were alleviated and this was reversed during induction of secondary dormancy. Sensitivity to GAs was not particularly related to changes in dormancy (Derkx and Karssen, 1993a; Chapter 6).

The aim of the present study was to monitor responses of *A. thaliana* seeds to GAs, to red light and to nitrate during dormancy changes under outside temperature conditions in order to elucidate the role of sensitivity to these factors and the role of GA biosynthesis in controlling seasonal dormancy patterns and germination. Responses to GAs and to light were determined in both wild-type and *ga1-2* seeds, to investigate whether eventual changes in light sensitivity exert an effect on GA sensitivity in addition to its postulated effect on GA biosynthesis. Nitrate may also stimulate germination of *A. thaliana* seeds (Derkx and Karssen, 1993b; Chapter 2), although not as pronounced as in *S. officinale* seeds (Derkx and Karssen, 1993a; Chapter 6). When nitrate was applied in combination with GAs in *ga1-2* seeds, no extra stimulatory action was evoked in comparison with single GA treatment. Because effects of nitrate on GA sensitivity were not to be expected, changes in response to nitrate were only studied in wild-type seeds. Dose response data to light and GAs were fitted as logistic dose response curves. These curves are equivalent to a function adapted from Michaelis-Menten kinetics modified by the Hill- or cooperativity coefficient, as described by Nissen (1985) and Weyers *et al.* (1987).

Seeds of both genotypes were imbibed outdoors in water under absolutely light-tight conditions. At regular intervals germination capacity of portions of seeds was detected under laboratory conditions.

Materials and methods

Production of seeds

Two seed lots of *Arabidopsis thaliana* (L.) Heynh. were used in this study. One seed lot was harvested in June, 1989 and the other in May, 1991. Seeds of wild type and GA-deficient mutant *ga1-2* were harvested from plants grown in an air-conditioned greenhouse as described in Chapter 2. Ripe seeds were allowed to dry at room temperature for about 4 days, cleaned by sieving and blowing and until the start of the experiments stored dry at 2 °C in darkness in glass vials. With the June, 1989 seeds the experiment started in August, 1989 and with the May, 1991 seeds in June, 1991.

Pre-incubation at outside temperatures

Portions of 0.09 g seeds (± 4500 seeds) (1989 experiment) or 0.16 g seeds (± 8000 seeds) (1991 experiment) were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll, Dassel, Germany) and moistened with 1.5 ml of milli-Q water. The Petri dishes were packed in plastic boxes lined with wet paper towels, that were rewetted several times. The boxes were wrapped in several layers of black plastic sheets to avoid any irradiation during incubation at outside temperatures. Under these conditions filter papers remained wet. Fungal contamination was incidental. Petri dishes with infected seeds were discarded. In the 1991 experiment germination during incubation at outside temperatures was very incidental, whereas in the 1989 experiment it was about 5-10 % for wild-type seeds. Before transfer to germination tests seedlings were removed. The boxes were placed at a height of 1.5 m under a roof. Air temperatures at this height were recorded at the Meteorological station in Wageningen. Means of decades were used.

Germination conditions

After several intervals of incubation seeds of one Petri dish of each genotype were

divided into smaller portions. The seeds were transferred to 150 (experiment 1989) or 200 ml (experiment 1991) milli-Q water in a beaker glass and carefully stirred. A pipette was used to suck volumes of 1.5 ml including seeds out of this beaker glass. In general, this volume contained 50-100 seeds. This volume was put on top of a filter paper that was sucked dry on a Büchner funnel. The filter paper with the seeds was finally transferred to the germination solution. All treatments were in triplicates.

To determine GA dose responses germination was tested at a range of GA_{4+7} concentrations. GA_{4+7} (ICI, Yalding, U.K.) was dissolved in a few drops of 1N KOH and diluted with a phosphate citrate buffer, containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. The pH of the buffer was 5.0. It was demonstrated by full-scan gas chromatography mass spectrometry that the commercial mixture contained 54 % GA_4 and 46 % GA_7-isolactone, whereas GA_7 was not detected (Chapter 3). As a result of short exposure of the mixture to KOH, most GA_7-isolactone was converted to GA_7, whereas GA_4 was not affected (Chapter 3). GA dose responses were determined in darkness.

Fluence responses were determined in buffer for wild-type seeds and in 0.25 and 1 μM GA_{4+7} for *ga1-2* seeds. Fluence response curves were obtained by irradiating single Petri dishes with light from a Leitz slide projector with a quartz-iodine lamp (Philips 250W, Eindhoven, The Netherlands) equipped with a narrow waveband interference filter (Balzers, Liechtenstein) of 667 nm (maximum transmission 47 %, bandwidth at 50 % of maximal transmission 10.9 nm). The fluence rate was varied by inserting neutral glass filters (NG, Schott u Gen., Mainz, Germany) behind the interference filter.

Irradiation time was 20 s but when fluence values higher than $1.06 * 10^{-3}$ mol m^{-2} were required, irradiation time was prolonged to maximally 4 min. Reciprocity was not affected at these longer times (H.W.M. Hilhorst, unpublished results). The fluence rate was calculated from the transmission characteristics of the neutral filters.

In the 1989 experiment germination of both genotypes was tested in buffer after 15 min of red light irradiation (620-700 nm), obtained from six red fluorescent tubes (see Chapter 2).

Responses to potassium nitrate (Suprapur, Merck, Darmstadt, Germany) were determined after 15 min red light irradiation.

Germination was tested at 10 or 24 °C in temperature-controlled incubators (see Chapter 2).

To determine germination percentages, both germinated and non-germinated seeds were counted 7 (24 °C) or 14 (10 °C) days after the start of the germination test. Radicle protrusion was taken as the criterion for germination.

All manipulations of incubated seeds were conducted in dim green light (see Chapter 2).

Calculation of dose response data

A quantitative description of the sensitivity of seeds to applied GA_{4+7} and to light was made by calculating logistic dose response curves from the individual data points, using a non-linear regression method (Tablecurve, Jandel Scientific, California, U.S.A.). The best fitting curves were produced and curve parameters were calculated. Nitrate dose response data were not subjected to this analysis.

Dose response curves are described by the following equation (Weyers *et al.*, 1987):

$$R = R_{min} + \frac{R_{max} - R_{min}}{1 + \left(\frac{[H]_{50}}{[H]}\right)^{p}} \qquad (1)$$

where:
- R = response
- R_{min} = minimum response in the absence of exogenous [H]
- R_{max} = maximum response
- $[H]$ = applied dose concentration
- $[H]_{50}$ = dose concentration required for half-maximum response
- p = Hill- or cooperativity coefficient

It is assumed that the receptors for one ligand are equivalent and independent, the response is proportional to the number of occupied receptors, the ligand can only exist in two

states: free or bound to its receptor and the identity or velocity of the rate-limiting step does not change during the response (Weyers *et al.*, 1987; Hilhorst, 1990).

The slope at half-maximum response ($[H] = [H]_{50}$) is calculated by:

$$\log\left(\frac{\partial R}{\partial \log[H]}\right) - \log\left(\frac{(R_{max}-R_{min})* p}{4} * \ln 10\right) \qquad (2)$$

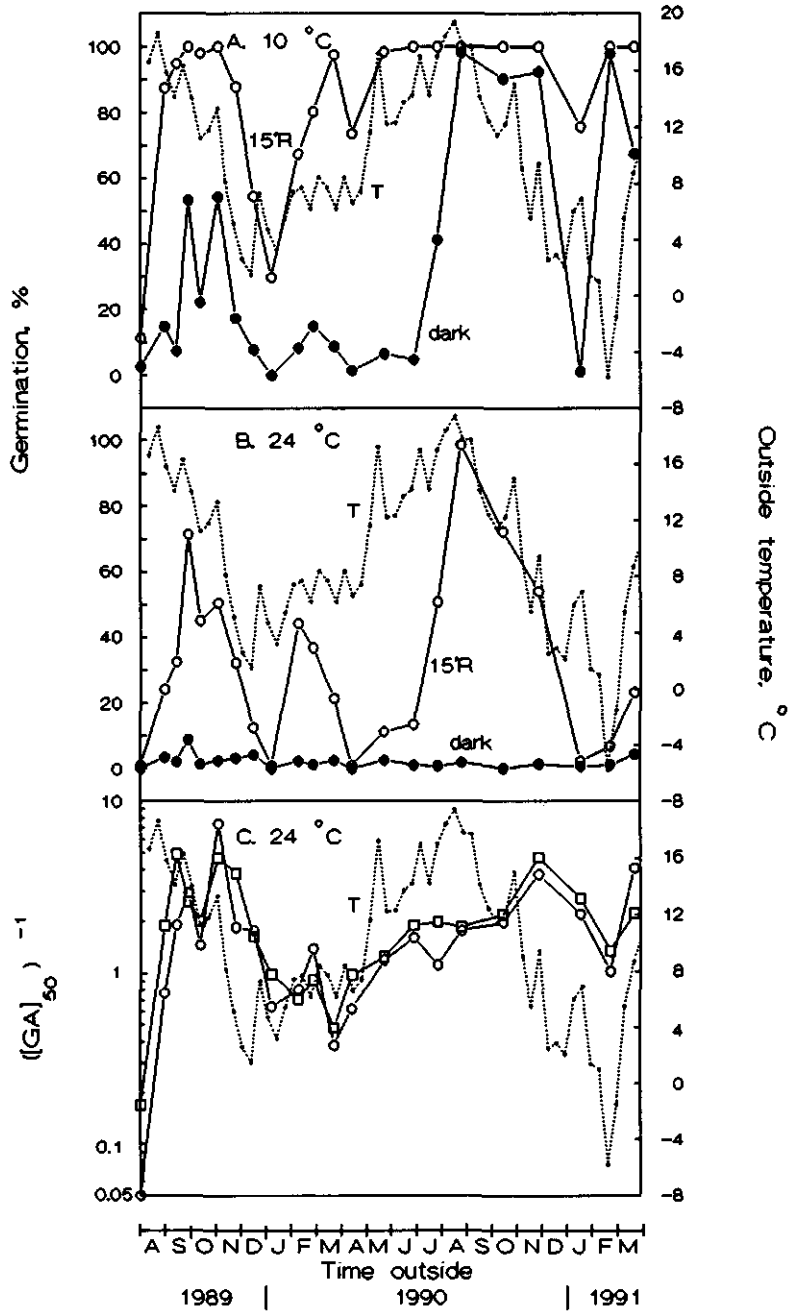
Results

To study the control of seasonal dormancy patterns *A. thaliana* seeds were incubated during 18 months (1989 experiment) or 8 months (1991 experiment) at outside temperatures while deprived from any light. At regular intervals portions of seeds were transferred to laboratory conditions and responses to GAs, to light and to nitrate were determined at 10 and 24 °C. Mainly data determined at the latter temperature are shown. Tendencies at both temperatures were more or less similar but in general more pronounced at 24 °C. Seeds germinated better at 10 °C than at 24 °C, as shown before (Derkx and Karssen, 1993b; Chapters 2, 4).

Figure 5.1A, B Seasonal variation in germination capacity of wild-type seeds of *A. thaliana* incubated under outside temperature conditions. Incubation occurred in Petri dishes that were packed in light-tight plastic boxes. Portions of seeds were transferred to the laboratory at the indicated times and germination was tested at 10 °C (A) or 24 °C (B) in a phosphate citrate buffer (see Materials and methods). Germination tests were in darkness (●) or after a 15 min red light irradiation (○).

C. Seasonal variation in GA requirement of wild-type (○) and *ga1-2* seeds (□). Germination capacity was tested at the indicated times at 24 °C in darkness at a range of GA_{4+7} concentrations. Inverse values of GA concentrations required for half-maximum response were calculated from the fitted GA dose response curves in Fig. 5.2 and Tables 5.1 and 5.2.

The dotted line indicates the air temperature at 1.50 m.


A. 10 °C
15'R
T
dark
B. 24 °C
T
15'R
dark
C. 24 °C
T
Germination, %
([GA]50)-1
Outside temperature, °C
A S O N D J F M A M J J A S O N D J F M
Time outside
1989
1990
1991

Responses to 15 min red light irradiation

Wild-type seeds hardly germinated in darkness at 24 °C in buffer, whereas a clear dormancy pattern was seen after a 15 min red light irradiation (Fig. 5.1B). Alleviation of dormancy occurred directly upon the start of incubation outdoors in August, 1989; it was soon followed by induction of secondary dormancy. A second small peak occurred in late winter, and from June, 1990 onwards breaking of secondary dormancy was very obvious. Re-induction of dormancy in 1990 occurred roughly around the same time as in 1989.

At 10 °C a seasonal pattern of dark germination was observed with peaks in autumn 1989 and 1990, whereas germination in light was high throughout the experiment with the exception of a few months in winter 1989-1990 (Fig. 5.1A).

Seeds of the *ga1-2* mutant incubated under natural temperature conditions and tested in buffer after regular intervals, did not show more than a few percent germination both at 10 and 24 °C, in light and darkness (data not shown).

Figure 5.2 Gibberellin dose response curves of *A. thaliana* wild-type (A-D) and *ga1-2* (E-H) seeds. Incubated seeds were transferred from outside temperature conditions to a constant temperature of 24 °C after several intervals of incubation outside. Incubation outside as described in Fig. 5.1 started in August, 1989 (●). Seeds were transferred to germination conditions in September, 1989 (▲), November, 1989 (■), December, 1989 (▼), January, 1990 (◆), March, 1990 (○), May, 1990 (△), July, 1990 (□), November, 1990 (▽), January, 1991 (◇) and February, 1991 (+). Germination was tested in darkness at a range of GA_{4+7} concentrations, buffered at pH 5. The germination data were fitted as logistic dose response curves. The best fitting curves were produced.

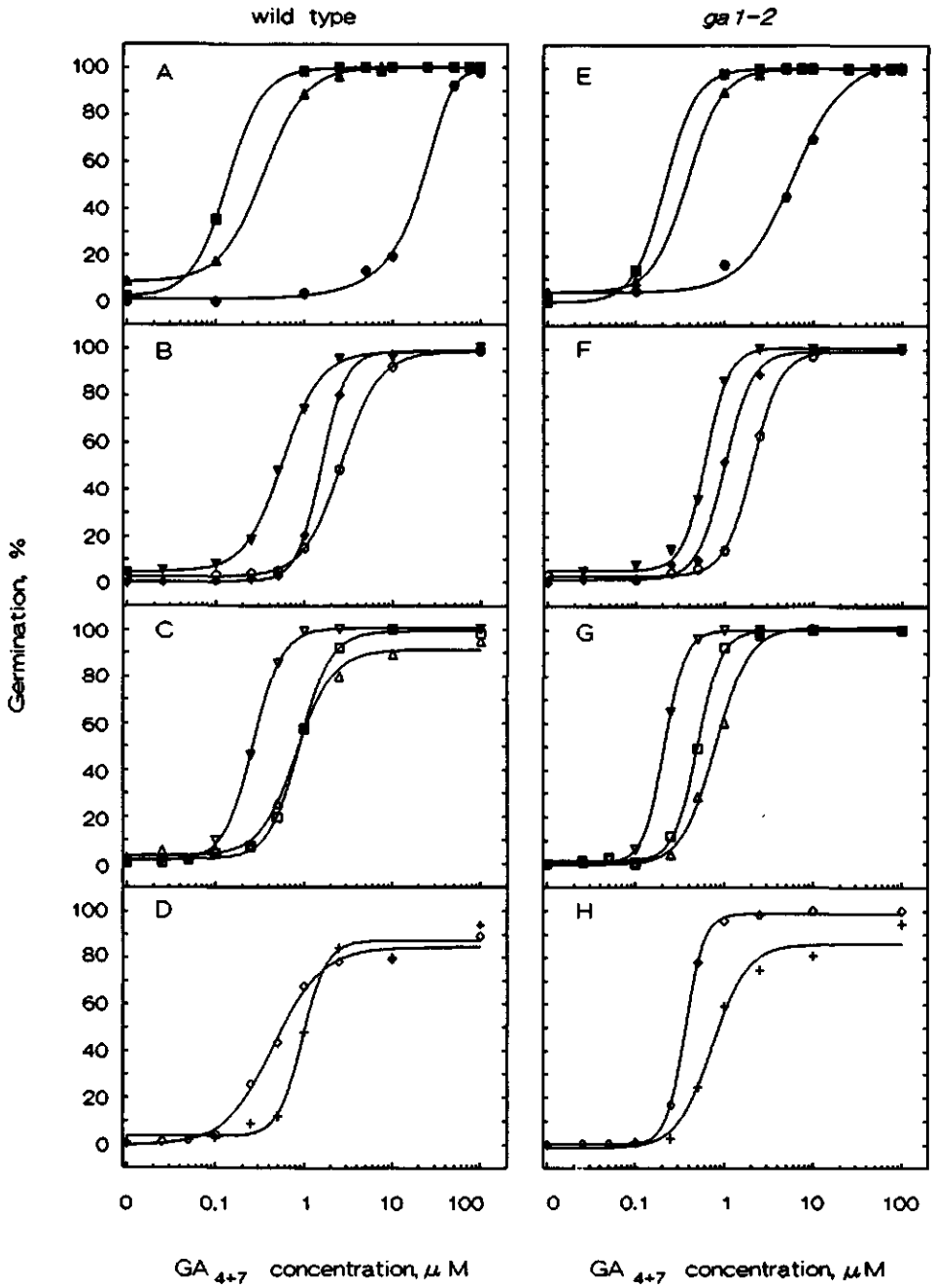

wild type
ga 1-2
A
B
C
D
E
F
G
H
Germination, %
0
20
40
60
80
100
0
0.1
1
10
100
GA 4+7 concentration, μ M
GA 4+7 concentration, μ M

Table 5.1 Calculated curve parameters and standard deviations of GA dose response curves of wild-type seeds of *A. thaliana*. At the indicated times seeds were transferred from outside temperatures to 24 °C. Germination was tested in GA_{4+7} in darkness. Some of these curves are shown in Figs. 5.2A-D. R_{min} = the minimum response in the absence of applied GA_{4+7}, R_{max} = the maximum response, $[GA]_{50}$ = the GA_{4+7} concentration required for half-maximum response, p = Hill-coefficient, slope = slope at half-maximum response. n.d = not determined.

Date	R_{min} (%)	R_{max} (%)	$[GA]_{50}$ (μM)	*p*	*slope*
03-08-89	1 ± 2	98 ± 2	16.57 ± 2.45	n.d	2.1 ± 0.1
01-09-89	4 ± 2	100 ± 2	1.29 ± 0.06	1.9 ± 0.2	2.0 ± 0.1
15-09-89	2 ± 2	100 ± 3	0.52 ± 0.05	1.8 ± 0.2	2.0 ± 0.1
29-09-89	9 ± 2	100 ± 2	0.34 ± 0.03	1.8 ± 0.1	2.0 ± 0.1
13-10-89	3 ± 2	101 ± 4	0.69 ± 0.07	1.3 ± 0.1	1.9 ± 0.1
03-11-89	2 ± 1	100 ± 1	0.14 ± 0.01	2.2 ± 0.3	2.1 ± 0.1
23-11-89	4 ± 1	99 ± 2	0.54 ± 0.02	2.3 ± 0.1	2.1 ± 0.1
15-12-89	5 ± 2	99 ± 4	0.57 ± 0.04	2.0 ± 0.2	2.0 ± 0.1
05-01-90	0 ± 0	99 ± 1	1.56 ± 0.03	3.1 ± 0.1	2.2 ± 0.1
05-02-90	-1 ± 3	100 ± 6	1.23 ± 0.13	1.3 ± 0.2	1.9 ± 0.1
23-02-90	0 ± 2	100 ± 3	0.72 ± 0.04	1.5 ± 0.1	2.0 ± 0.1
19-03-90	3 ± 1	99 ± 3	2.62 ± 0.14	2.0 ± 0.2	2.0 ± 0.1
10-04-90	1 ± 1	95 ± 2	1.60 ± 0.07	2.6 ± 0.2	2.1 ± 0.1
18-05-90	4 ± 2	91 ± 4	0.83 ± 0.06	2.1 ± 0.3	2.0 ± 0.1
22-06-90	1 ± 2	100 ± 3	0.62 ± 0.03	2.1 ± 0.2	2.1 ± 0.1
20-07-90	2 ± 2	97 ± 3	0.90 ± 0.05	2.5 ± 0.3	2.1 ± 0.1
17-08-90	5 ± 1	95 ± 3	0.57 ± 0.03	2.6 ± 0.3	2.1 ± 0.1
05-10-90	0 ± 2	100 ± 4	0.51 ± 0.02	2.5 ± 0.3	2.1 ± 0.1
16-11-90	1 ± 1	99 ± 1	0.27 ± 0.00	2.7 ± 0.1	2.2 ± 0.1
04-01-91	0 ± 2	84 ± 4	0.45 ± 0.04	1.6 ± 0.2	1.9 ± 0.1
08-02-91	4 ± 3	84 ± 6	0.97 ± 0.09	3.2 ± 0.2	2.2 ± 0.1
08-03-91	6 ± 4	90 ± 7	0.24 ± 0.02	2.9 ± 0.7	2.2 ± 0.1

Table 5.2 Calculated curve parameters and standard deviations of GA dose response curves of *ga1-2* seeds of *A. thaliana*. At the indicated times seeds were transferred from outside temperatures to 24 °C. Germination was tested in GA_{4+7} in darkness. Some of these curves are shown in Figs. 5.2E-H. Curve parameters as in Table 5.1.

Date	R_{min} (%)	R_{max} (%)	$[GA]_{50}$ (μM)	*p*	*slope*
03-08-89	4 ± 2	104 ± 4	5.98 ± 0.45	1.5 ± 0.2	1.9 ± 0.1
01-09-89	4 ± 2	101 ± 2	0.53 ± 0.04	1.7 ± 0.2	2.0 ± 0.1
15-09-89	11 ± 1	100 ± 2	0.20 ± 0.01	1.5 ± 0.1	1.9 ± 0.1
29-09-89	4 ± 1	100 ± 2	0.38 ± 0.04	2.2 ± 0.2	2.1 ± 0.1
13-10-89	2 ± 1	100 ± 2	0.50 ± 0.05	2.5 ± 0.4	2.2 ± 0.1
03-11-89	0 ± 1	100 ± 2	0.21 ± 0.02	2.4 ± 0.2	2.2 ± 0.1
23-11-89	2 ± 2	102 ± 3	0.26 ± 0.02	1.8 ± 0.2	2.0 ± 0.1
15-12-89	5 ± 1	101 ± 2	0.61 ± 0.02	3.3 ± 0.3	2.3 ± 0.1
05-01-90	2 ± 1	99 ± 2	1.01 ± 0.03	2.8 ± 0.2	2.2 ± 0.1
05-02-90	1 ± 1	99 ± 2	1.40 ± 0.04	3.5 ± 0.2	2.3 ± 0.1
23-02-90	0 ± 1	100 ± 3	1.09 ± 0.04	3.1 ± 0.4	2.2 ± 0.1
19-03-90	3 ± 1	99 ± 2	2.08 ± 0.07	2.7 ± 0.2	2.2 ± 0.1
10-04-90	0 ± 2	98 ± 3	1.02 ± 0.05	2.7 ± 0.3	2.2 ± 0.1
18-05-90	0 ± 1	100 ± 2	0.80 ± 0.03	2.3 ± 0.2	2.1 ± 0.1
22-06-90	2 ± 1	100 ± 3	0.53 ± 0.02	4.0 ± 0.6	2.4 ± 0.1
20-07-90	1 ± 1	100 ± 1	0.50 ± 0.01	3.3 ± 0.2	2.3 ± 0.1
17-08-90	1 ± 1	100 ± 1	0.54 ± 0.01	2.9 ± 0.1	2.2 ± 0.1
05-10-90	4 ± 2	101 ± 3	0.45 ± 0.01	3.6 ± 0.4	2.3 ± 0.1
16-11-90	1 ± 1	100 ± 1	0.21 ± 0.00	3.7 ± 0.2	2.3 ± 0.1
04-01-91	0 ± 1	99 ± 1	0.37 ± 0.01	4.1 ± 0.2	2.4 ± 0.1
08-02-91	-1 ± 3	86 ± 5	0.74 ± 0.06	2.2 ± 0.3	2.0 ± 0.1
08-03-91	1 ± 1	101 ± 3	0.45 ± 0.02	2.4 ± 0.2	2.1 ± 0.1

GA dose responses

To investigate whether changes in dormancy, as depicted in Fig. 5.1B, are accompanied by shifts in requirement for GAs, the germination capacity of wild-type and *ga1-2* seeds that had been incubated outdoors, was tested at a range of GA_{4+7} concentrations. To avoid interference with endogenous GAs in wild-type seeds as much as possible, germination tests were performed in darkness. However, effects of phytochrome in the farred-absorbing form (Pfr) originating from seed development ('pre-existing' Pfr) cannot be circumvented in this way. The curve parameters for all fitted GA dose response curves at 24 °C are listed in Tables 5.1 and 5.2 for wild-type and *ga1-2* seeds, respectively. Sets of illustrative curves are shown in Fig. 5.2. Both genotypes showed little variation in minimum (R_{min}) and maximum (R_{max}) responses, R_{min} was mostly close to 0 % and R_{max} close to 100 %, with the exception of the tests with wild-type seeds early 1991 (Table 5.1). Major changes occurred in the dose for half-maximum response $[GA]_{50}$ (Tables 5.1, 5.2). The most prominent shift was visible in the first month of incubation outdoors (Figs. 5.2A, E). The increased GA sensitivity (decreasing $[GA]_{50}$) during the first 3 months was reversed in the period November, 1989 - March, 1990. Thereafter GA sensitivity increased again gradually until November, 1990. In this month GA sensitivity, as expressed by $[GA]_{50}$, was ten times as high as in March, 1990 for both genotypes. From November, 1990 to February, 1991 GA sensitivity again declined somewhat. The shifts in requirement for GAs were parallel as can be deduced from the logarithms of the slopes (∂Response/∂log[GA]) of the dose response curves, that were not significantly different from each other (Tables 5.1, 5.2). The Hill-coefficient (p) fluctuated from 1.3 to 3.2 in wild-type seeds (Table 5.1) and from 1.5 to 4.1 in *ga1-2* seeds (Table 5.2).

To compare shifts in GA requirement for wild-type and *ga1-2* seeds and to see whether shifts in GA requirement occurred in similar periods of the year as shifts in requirement for a 15 min red light irradiation, inverse values of $[GA]_{50}$ are plotted for both genotypes against the time of incubation outdoors (Fig. 5.1C). Inverse values were chosen because a high inverse value of $[GA]_{50}$ corresponds with a high GA sensitivity. Seasonal shifts in requirement for GAs were remarkably similar for wild-type and *ga1-2* seeds. Periodicity of

shifts in GA sensitivity did only partly correspond to shifts in requirement for 15 min red light irradiation, especially in the first dormancy cycle.

The timing of changes in $[GA]_{50}$ determined at 10 °C was comparable to that observed at 24 °C, although changes were less pronounced (data not shown).

It can be questioned whether shifts in requirement for GAs as observed during imbibition of seeds under seasonal temperature conditions are not simply reflections from endogenous rhythms within the seeds themselves. Therefore, in a new set of experiments started in June, 1991 GA dose response curves were determined in a parallel way on seeds either imbibed under natural conditions or stored dry at 2 °C over equivalent periods (Figs. 5.3A-C). In analogy with the experiment started in August, 1989, alterations in response to applied GAs occurred during incubation outdoors, both in wild-type and *ga1-2* seeds (Figs. 5.3A, B). The curve parameters are listed in Table 5.3. At the start of the experiment in June, 1991 R_{max} of both genotypes at 24 °C did not exceed 65 % germination. R_{max} rose during the first month of imbibition at outside temperatures and was accompanied by a decrease in requirement for applied GAs (Figs. 5.3A, B, Table 5.3). This decrease went on during summer and autumn. The maximal increase in GA sensitivity was about 40-fold in wild-type seeds and about 100-fold in *ga1-2* seeds. After October, 1991 until the end of the experiment in February, 1992 the GA dose response curves of *ga1-2* seeds clearly returned to higher GA concentrations (Fig. 5.3B), whereas wild-type seeds showed only a small shift to the right from December to February (factor 2) (Fig. 5.3A). Shifts in requirement for GAs were more or less parallel as could be derived from the slopes of the dose response curves (Table 5.3). The Hill-coefficient showed fluctuations between 1.0 and 2.6 (Table 5.3). Wild-type seeds of this seed lot hardly germinated in the absence of exogenous GA, neither at 24 °C (Fig. 5.3A) nor at 10 °C (data not shown). R_{min} values were near to zero as was also the case in *ga1-2* seeds (Table 5.3).

Although a gradual increase in GA sensitivity was noticed at 24 °C in dry after-ripened wild-type seeds (Fig. 5.3C), this increase was by far not comparable to shifts observed in seeds imbibed under outside temperature conditions (Fig. 5.3A).

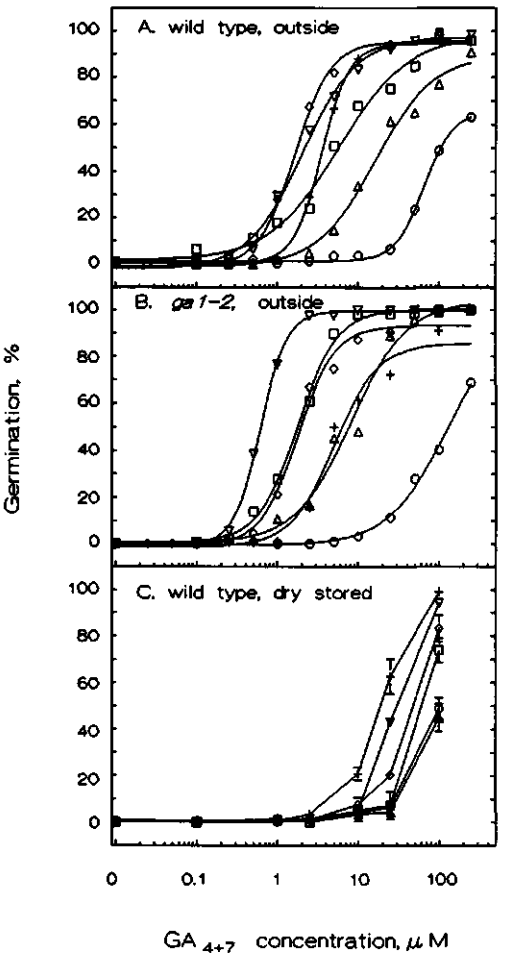


Figure 5.3A, B. Gibberellin dose response curves of *A. thaliana* wild-type (A) and *ga1-2* (B) seeds incubated under an outside temperature regime and transferred to laboratory conditions after several intervals of incubation outdoors. Incubation outside as described in Fig. 5.1 started in June, 1991 (○). Seeds were transferred to germination conditions in July, 1991 (△), August, 1991 (□), October, 1991 (▽), December, 1991 (◇) and February, 1992 (+).

C. Gibberellin dose response curves of wild-type seeds that were stored dry at 2 °C over equivalent periods as those incubated as described in Figs. 5.3A, B. Dry storage was 3 weeks (June, 1991, ○), 6 weeks (July, 1991, △), 12 weeks (August, 1991, □), 19 weeks (October, 1991, ▽), 27 weeks (December, 1991, ◇) and 38 weeks (February, 1992, +). Germination capacity was tested in darkness at a range of GA_{4+7} concentrations, buffered at pH 5. Germination temperature was 24 °C. Germination data of A and B were fitted as logistic dose response curves and best fitting curves were produced.

Table 5.3 Calculated curve parameters and standard deviations of GA dose response curves of wild-type and *ga1-2* seeds of *A. thaliana* in Figs. 5.3A, B. Curve parameters as in Table 5.1.

Month	R_{min} (%)	R_{max} (%)	$[GA]_{50}$ (μM)	*p*	*slope*
	wild type				
19-06-91	2 ± 1	66 ± 2	65.3 ± 2.8	2.4 ± 0.2	2.0 ± 0.1
11-07-91	-1 ± 2	89 ± 7	16.1 ± 2.6	1.2 ± 0.2	1.8 ± 0.2
23-08-91	2 ± 3	98 ± 8	5.7 ± 1.0	1.0 ± 0.2	1.8 ± 0.2
09-10-91	-2 ± 2	97 ± 4	2.0 ± 0.2	1.3 ± 0.1	1.9 ± 0.1
03-12-91	-1 ± 2	95 ± 3	1.7 ± 0.1	1.9 ± 0.2	2.0 ± 0.1
18-02-92	1 ± 1	95 ± 2	3.5 ± 0.1	2.5 ± 0.2	2.1 ± 0.1
	ga1-2 mutant				
19-06-91	-1 ± 2	69 ± 18	61.0 ± 4.7	1.2 ± 0.3	1.8 ± 0.2
11-07-91	0 ± 2	104 ± 4	8.1 ± 0.8	1.2 ± 0.1	1.9 ± 0.1
23-08-91	0 ± 1	101 ± 2	1.8 ± 0.1	1.7 ± 0.1	2.0 ± 0.1
09-10-91	-1 ± 1	100 ± 1	0.6 ± 0.0	2.6 ± 0.1	2.2 ± 0.1
03-12-91	-1 ± 1	93 ± 3	1.8 ± 0.1	1.9 ± 0.2	2.0 ± 0.1
18-02-92	-1 ± 2	86 ± 4	5.0 ± 0.5	1.6 ± 0.2	1.9 ± 0.1

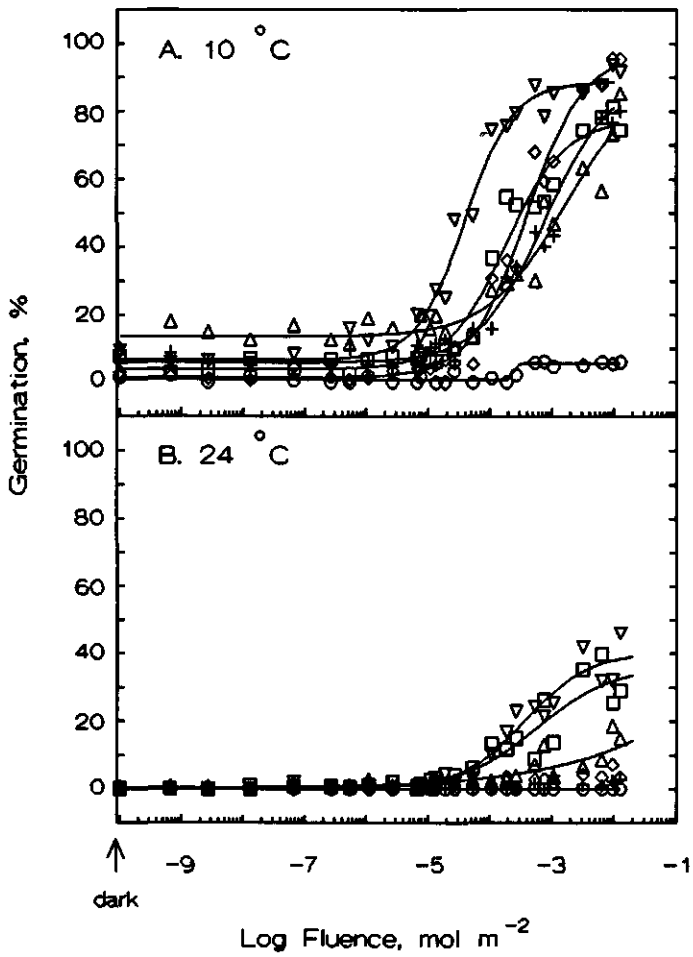


Figure 5.4 Fluence response curves of *A. thaliana* wild-type seeds incubated under outside temperature conditions and transferred to laboratory conditions at similar times as described in Fig. 5.3. Incubation outside occurred as described in Fig. 5.1. Germination was tested in a phosphate citrate buffer (see Materials and methods). Germination temperature was 10 °C (A) or 24 °C (B). The germination data were fitted as logistic dose response curves and best fitting curves were produced.

Table 5.4 Calculated curve parameters and standard deviations of fluence response curves of wild-type seeds of *A. thaliana* in Fig. 5.4. R_{min} = minimum response in the absence of light, R_{max} = maximum response, $[Fl]_{50}$ = fluence required for half-maximum response, p = Hill-coefficient, slope = slope at half-maximum response. n.d = not determined (see text).

Month	R_{min} (%)	R_{max} (%)	$FL_{50}*10^4$ (mol m^{-2})	*p*	*slope*
	wild type, germination at 10 °C in 0 µM GA_{4+7}				
19-06-91	1 ± 0	6 ± 0	n.d	n.d	n.d
11-07-91	14 ± 2	77 ± 17	10.0 ± 4.2	0.7 ± 0.2	1.5 ± 0.3
23-08-91	4 ± 1	78 ± 4	1.8 ± 0.3	0.9 ± 0.1	1.6 ± 0.2
09-10-91	7 ± 2	89 ± 3	0.4 ± 0.1	1.1 ± 0.1	1.7 ± 0.1
03-12-91	1 ± 1	98 ± 4	4.0 ± 0.5	0.9 ± 0.1	1.7 ± 0.1
18-02-92	6 ± 2	91 ± 10	7.9 ± 3.0	0.8 ± 0.2	1.6 ± 0.2
	wild type, germination at 24 °C in 0 µM GA_{4+7}				
19-06-91	n.d	n.d	n.d	n.d	n.d
11-07-91	n.d	n.d	n.d	n.d	n.d
23-08-91	0 ± 2	36 ± 9	5.8 ± 4.6	0.7 ± 0.3	1.2 ± 0.3
09-10-91	0 ± 5	41 ± 4	3.7 ± 1.2	0.8 ± 0.2	1.3 ± 0.2
03-12-91	n.d	n.d	n.d	n.d	n.d
18-02-92	n.d	n.d	n.d	n.d	n.d

Fluence responses

Changes in the response to a saturating red light irradiation were shown in Fig. 5.1 for wild-type seeds. In order to determine whether changes in phytochrome-receptor level can explain the observed periodicity, detailed fluence response experiments were performed.

For wild-type seeds fluence responses were determined in the absence of GA_{4+7}. The fitted curves are presented in Fig. 5.4 and the curve parameters of germination data of which the response range exceeded 20 % are listed in Table 5.4. Dark controls are represented by the first point of each series (R_{min} values in Table 5.4) and were close to zero except for wild-type seeds tested in July, 1991 at 10 °C. During the experimental period responses only occurred in the low-fluence-range at fluence values exceeding 10^{-5} mol m^{-2}. At 24 °C germination did not exceed 50 %. Nevertheless, it is clear that the response to light increased from June until October and was reversed thereafter (Fig. 5.4B). Due to low light sensitivity at 24 °C it is not possible to calculate curve parameters accurately. Therefore, data obtained at 10 °C are included (Fig. 5.4A, Table 5.4). At this temperature R_{max} increased dramatically during the first month. Up to October this increase was accompanied by a shift of the curves to lower fluence values, thus sensitivity to light increased (Fig. 5.4A). In the period October-February this reaction was reversed. R_{max} values, however, did not decline. All shifts were parallel as could be derived from the slopes of the fluence response curves. The Hill-coefficient varied between 0.7 and 1.1.

The study of shifts in requirement for light in *ga1-2* seeds is somewhat more complicated. Germination tests have to be conducted in the presence of exogenous GA. Changes in R_{min} (dark germination in the presence of GA) therefore represent shifts in GA sensitivity. Fluence response curves for *ga1-2* seeds are shown in Fig. 5.5 for tests at 24 °C and the concomitant curve parameters in Table 5.5. Like in wild-type seeds responses only occurred in the low-fluence-range. At the start of the experiment in June, 1991 *ga1-2* seeds were extremely insensitive to light. One month later, R_{max} was somewhat increased, as was also the response to 1 μM GA_{4+7} (R_{min}) (Fig. 5.5B). Both parameters further increased until October. Moreover, curves shifted to the left. Reversion of these responses was visible

from October onwards in 0.25 μM GA_{4+7} (Fig. 5.5A) and from December onwards in 1 μM GA_{4+7} (Fig. 5.5B).

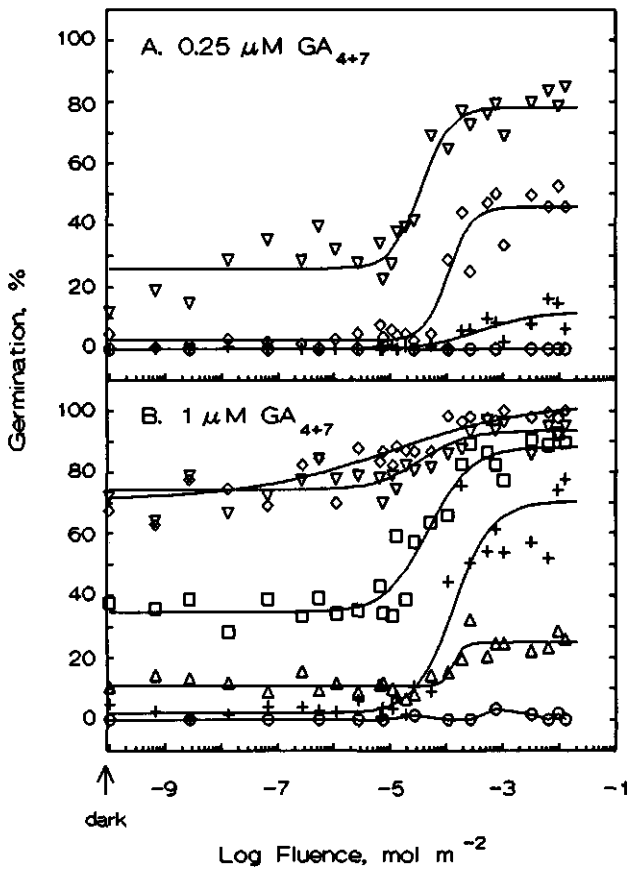


Figure 5.5 Fluence response curves of *A. thaliana ga1-2* seeds incubated under outside temperature conditions and transferred to laboratory conditions at similar times as described in Fig. 5.3. Incubation outside occurred as described in Fig. 5.1. Germination was tested in 0.25 (A) or 1 (B) μM GA_{4+7}, buffered at pH 5. Germination temperature was 24 °C. The germination data were fitted as logistic dose response curves and best fitting curves were produced.

Table 5.5 Calculated curve parameters and standard deviations of fluence response curves of *ga1-2* seeds of *A. thaliana* in Fig. 5.5. Curve parameters as in Table 5.4.

Month	R_{min} (%)	R_{max} (%)	$FL_{50}*10^4$ (mol m^{-2})	*p*	*slope*
	ga1-2 mutant, germination at 24 °C in 0.25 μM GA_{4+7}				
19-06-91	0 ± 0	0 ± 0	n.d	n.d	n.d
11-07-91	n.d	n.d	n.d	n.d	n.d
23-08-91	n.d	n.d	n.d	n.d	n.d
09-10-91	26 ± 2	78 ± 4	0.4 ± 0.1	1.7 ± 0.3	1.7 ± 0.2
03-12-91	3 ± 1	46 ± 3	1.1 ± 0.2	2.1 ± 0.3	1.5 ± 0.2
18-02-92	0 ± 1	12 ± 2	n.d	n.d	n.d
	ga1-2 mutant, germination at 24 °C in 1 μM GA_{4+7}				
19-06-91	0 ± 0	2 ± 0	n.d	n.d	n.d
11-07-91	11 ± 1	25 ± 2	n.d	n.d	n.d
23-08-91	35 ± 2	88 ± 4	0.5 ± 0.1	1.1 ± 0.2	1.6 ± 0.2
09-10-91	74 ± 2	93 ± 4	n.d	n.d	n.d
03-12-91	71 ± 2	103 ± 6	0.1 ± 0.1	0.3 ± 0.1	0.8 ± 0.4
18-02-92	2 ± 1	70 ± 3	1.4 ± 0.2	1.3 ± 0.2	1.7 ± 0.1

Nitrate dose responses

Responses to nitrate were minimal, both at 10 and 24 °C. Therefore, shifts in sensitivity to nitrate do not play any decisive role in the control of dormancy and germination of *A. thaliana* seeds (data not shown).

Discussion

Wild-type seeds of *A. thaliana* incubated at outside temperatures showed at a test temperature of 24 °C variability in response to a saturating dose of red light over the seasons (Fig. 5.1B). At 10 °C a seasonal pattern of changes in dormancy was seen in the absence of red light (Fig. 5.1A). The lack of absolute light requirement at 10 °C may be explained by the presence of 'pre-existing' phytochrome (Pfr) in the seeds originating from the mother plant (Cone and Kendrick, 1985). Because levels presumably decline during dark incubation, its contribution after some time of incubation is questionable. However, when seeds are very sensitive to light, very low amounts of Pfr may be sufficient to stimulate germination, as was also found after 2 months of burial of *Datura ferox* seeds (Scopel *et al.*, 1991). Changes in dark germination may result from fluctuations in sensitivity to 'pre-existing' Pfr, as was also concluded by VanderWoude and Toole (1980) for lettuce seeds. Additionally, the dim green light to which the seeds were exposed when transferred from outside conditions to germination conditions, may stimulate germination of extremely light-sensitive seeds, as was also demonstrated in Chapter 4. High germinability in late summer and autumn represents some winter-annual behaviour as was also described by Baskin and Baskin (1983). The small flush in late winter indicates that winter temperatures can also relieve dormancy, thus representing some summer-annual behaviour, as described by Laibach (1951). In this respect, our ecotype may be classified as non-strict winter annual, following the terminology by Baskin and Baskin (1985). As a matter of fact *ga1-2* seeds did not exhibit seasonal changes in dormancy in the absence of GAs (data not shown).

To get insight in the nature of the endogenous changes that were evoked by temperature as the main controlling environmental factor, shifts in sensitivity to GAs, light and nitrate

were determined. A quantitative assessment was made by fitting the dose response data as logistic dose response curves. Interpretation followed definitions by Firn (1986), further extended by Weyers *et al.* (1987) and Fitzsimons (1989). However, it should be emphasized that this interpretation is not definite because as long as the receptors concerned have not been isolated, assumptions required for justification cannot be verified. In addition, it should be mentioned that the interpretation is derived from a relatively simple molecular model, whereas the germination process is highly complex. For instance, little is known about hormone binding and about the transduction chain between the formation of hormone-receptor complex and the final germination response. Bearing this in mind, it can be concluded that the logistic dose response curves fitted the germination data very adequately.

Interpretation of curve parameters on basis of a simple model

The parameter R_{min} represents the base level of response in the absence of added ligand. R_{max} is a measure of the capacity to respond when ligand availability is not limiting. Changes in R_{max} may point to changes in the number of receptors but also to changes in the response chain initiated by the binding of ligand to its receptor (Weyers *et al.*, 1987). When the number of receptors rises above the number required for a maximal physiological response (100 % germination) dose response curves will shift to the left. Parallel shifts of dose response curves can also result from alterations in the affinity of the receptors, expressed by K_D values that are equivalent to $([ligand]_{50})^p$. The Hill-coefficient p is related to the abruptness of the dose response curve. It is dangerous to give a biological interpretation to p values, solely based on dose response curves (Weyers *et al.*, 1987). Values of p are presented because of their contribution in equation 1 but are not further discussed. The p values are also not used to calculate K_D values because several assumptions are required that cannot be tested at the moment. In addition slopes of dose response curves were calculated according to equation 2 to see whether shifts were parallel.

Interpretation of calculated curve parameters

GA dose responses

Both wild-type (Figs. 5.2A-D, 5.3A) and *ga1-2* (Figs. 5.2E-H, 5.3B) seeds did not germinate at 24 °C in the absence of applied GAs in darkness, as could also be derived from R_{min} values that were all the time close to zero (Tables 5.1, 5.2 and 5.3). This means that the endogenous concentration of GA is very low or even fully absent. Thus, interference of endogenous GA with sensitivity to GAs can be neglected under these circumstances. When R_{min} in wild-type seeds was not exactly zero, it could be reduced to this value by adding tetcyclasis, indicating that *de novo* synthesis of GAs was involved (data not shown).

Changes in $[GA]_{50}$ may therefore be regarded as true shifts in GA sensitivity. Although $[GA]_{50}$ values and shifts therein were quantitatively somewhat different in the 1989 and 1991 experiment, it is clear that incubation at outside temperatures influences the GA requirement of the seeds. The large decrease in $[GA]_{50}$ values in the first 4 months of both experiments was however only partially reversed in winter (Figs. 5.2A, B, E, F, 5.3A, B, Tables 5.1, 5.2, 5.3). In the 1989 experiment an increased response to a 15 min red light irradiation in summer and autumn, 1990 (Fig. 5.1B) was accompanied by a 10-fold decrease in $[GA]_{50}$ (shown as an inverse in Fig. 5.1C). However, when the responsiveness to red light had already started to decline, the responsiveness to GA_{4+7} still increased. Because changes in sensitivity to light do not necessarily correlate to changes in sensitivity to GAs, it is postulated that temperature exerts different effects on receptors for phytochrome and for GAs.

Shifts in requirement for GAs were parallel (Tables 5.1, 5.2, 5.3). These parallel shifts can be explained by changes in the affinity of the receptors for the hormone (Firn, 1986; Weyers *et al.*, 1987). However, also the total number of available receptors may be involved. Supplementary, some regulation may occur in the response chain initiated by the interaction of GA and its putative receptor.

Seeds that were stored dry at 2 °C showed a gradual increase in GA sensitivity in 1991-1992 (Fig. 5.3C). However, this increase was rather small in comparison to changes in GA sensitivity in seeds exposed to a seasonal temperature regime in the same period

(Fig. 5.3A). Therefore, regulation by an endogenous seasonal rhythm is unlikely. A truly endogenous rhythm was found in *Phaseolus vulgaris* seeds (Spruyt *et al.*, 1988), whereas it was absent in two *Datura* species (Reisman-Berman *et al.*, 1991).

Fluence responses

wild-type seeds Values of R_{min} close to zero ruled out a significant contribution of 'pre-existing' Pfr. The curve parameters R_{max} and Fl_{50} exhibited some seasonal periodicity. It is striking that R_{max}, being a measure of the capacity to respond when phytochrome availability is not limiting, does not exceed more than 50 % germination at 24 °C (Fig. 5.4B). An extremely low light sensitivity at the start of the experiment was also apparent at 10 °C (Fig. 5.4A). R_{max} did not exceed 6 %. However, after 3 weeks of exposure to temperatures of June and July, R_{max} had increased dramatically.

ga1-2 seeds In *ga1-2* seeds reversibility in several curve parameters was observed (Fig. 5.5, Table 5.5). Changes in R_{min} obviously reflect changes in sensitivity to the applied dose of GA_{4+7} and can thus not be regarded as shifts in sensitivity to light. The observed shifts were in good agreement with shifts observed in GA dose response curves (Fig. 5.3B). The seasonal periodicity in the response range (R_{max} - R_{min}) and in Fl_{50} can, however, be interpreted as changes in sensitivity to light.

The observed changes in curve parameters of fluence response curves in both genotypes indicate that the number of available receptors may fluctuate seasonally. Binding characteristics of phytochrome receptors or metabolic processes in the response chain, initiated by binding of phytochrome to its receptor may be involved as well (Weyers *et al.*, 1987). This control may be mediated by secondary messengers, such as calcium (Strickland and Loeb, 1981; Roux *et al.*, 1986).

Nitrate dose responses

Although changes in light sensitivity were strikingly similar to changes in sensitivity to nitrate in the related species *Sisymbrium officinale* (Derkx and Karssen, 1993a; Chapter 6), such similarity was not found in *A. thaliana* seeds. Actually, effects of nitrate were only minimal in this *A. thaliana* seed lot (data not shown). Nitrate obviously is not required for

the light-induced GA biosynthesis of *A. thaliana* seeds in contrast to *S. officinale* seeds (Hilhorst and Karssen, 1988). However, a small effect of nitrate on light-induced germination of wild-type seeds has been observed in another *A. thaliana* seed lot (Derkx and Karssen, 1993b; Chapter 2).

A dormancy model

Temperature conditions to which seeds are exposed in the imbibed state determine whether or not a seed has the potential to germinate at particular times, provided that environmental conditions are suitable for germination, as was also concluded by Bouwmeester (1990). Obviously, temperatures in summer and autumn increase sensitivity to light whereas winter temperatures reverse these reactions. These changes may occur at the level of receptors. Although circumstantial evidence is not available, it has been proposed that phytochrome receptors are located in a membrane (Taylorson, 1988). Temperature may affect the rate of receptor synthesis or the availability of receptors by altering physical properties of membranes (Raison *et al.*, 1980; Di Nola and Mayer, 1986). Also the affinity of the receptors may be influenced and the response chain initiated by the interaction of phytochrome with its receptor. As a result of an increased light sensitivity, the capacity to synthesize GAs is enhanced as well as the capacity to respond to GAs. GA receptors have also been proposed to be membrane-bound (Singh and Paleg, 1984; Hooley *et al.*, 1991). Moreover, GA sensitivity can also be directly controlled by temperature, thus without the interference of light. It is questionable whether sensitivity to GAs can be regarded as the limiting factor in germination of wild-type seeds. It was indeed low before the start of incubation at outside temperatures. However, sensitivity to light was also low at this time, additionally resulting in a low capacity to synthesize GAs. Sensitivity to GAs increased concomitantly with increased sensitivity to light but failed to expose dramatic shifts thereafter in contrast with clear changes in light sensitivity (Figs. 5.1B, C). Therefore, we propose that seasonal dormancy patterns of *A. thaliana* are mainly accompanied by changes in sensitivity to light, whereas sensitivity to GAs contributes to the observed pattern but is not the ultimate limiting factor for germination at any time of the year. GA biosynthesis is not required for

reversible changes in dormancy, as *ga1-2* seeds also exhibited a clear dormancy pattern. When the stimulus required for GA biosynthesis, i.e. light, is perceived in sufficient light-sensitive seeds the biosynthesis can start and germination follows. Thus, although the combination of GA biosynthesis and a sufficiently high GA sensitivity is indispensable for germination, both factors do not primarily regulate dormancy. The absence of control of dormancy by GA levels conforms conclusions by Metzger (1983), and Hepher and Roberts (1985) in *Avena fatua* and in *Trollius ledebouri*, respectively.

These conclusions are in good agreement with those based on changes in dormancy induced by incubating seeds under constant laboratory conditions (Chapter 4). This regulation is also in quite good agreement with that in *S. officinale* seeds, with the exception that nitrate and sensitivity to nitrate contributes significantly in the control of dormancy and germination in *S. officinale* seeds (Derkx and Karssen, 1993a; Chapter 6).

It is unlikely that primary and secondary dormancy have different mechanisms of action. Similarities in mechanisms governing primary and secondary dormancy have also been suggested for *Avena fatua* (Symons *et al.*, 1987), *Petunia hybrida* (Girard, 1990) and several other species (Khan and Samimy, 1982).

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Chapter 6

Changing sensitivity to light and nitrate but not to gibberellins regulates seasonal dormancy patterns in *Sisymbrium officinale* seeds

M.P.M. Derkx and C.M. Karssen

Summary

Seeds of *Sisymbrium officinale* (L.) Scop. that are buried under natural conditions in soil pass annually through a seasonal pattern of changes in dormancy. Dormancy is broken in autumn-winter and re-induced in summer. To elucidate dormancy regulation in this species under natural conditions, a detailed analysis of the changes in sensitivity to some relevant germination factors was carried out.

Germination data fitted as logistic dose response curves showed that sensitivity to light and nitrate, both indispensable stimuli for germination of this species, varied with the seasons. Patterns of shifts in requirement for light and nitrate were remarkably similar. Sensitivity increased when both primary and secondary dormancy were alleviated and it was reversed during induction of secondary dormancy. During alleviation of primary dormancy in spring, 1991, the fluence response curves exhibited a biphasic character with responses occurring both in the very-low-fluence-range and in the low-fluence-range. The nitrate dose response data could all be fitted as monophasic curves, although responses might have occurred in two distinct ranges as well. From interpretation of curve parameters, it is postulated that dormancy is regulated by changes in the number of phytochrome- and nitrate receptors, in shifts in the binding characteristics of the receptors and/or in shifts in the response chain initiated by the ligand-receptor interaction. Somewhere in this response chain, biosynthesis of gibberellins (GAs) is stimulated. By use of the GA-biosynthesis inhibitor tetcyclasis, it was indirectly proven that the capacity to synthesize GAs indeed varied with the seasons. Sensitivity to GAs gradually increased from burial onwards and was not particularly related to changes in dormancy. Thus, except for the first few months of burial,

GA sensitivity may not be regarded as a limiting factor in controlling dormancy in this species.

Introduction

Studies on the control of seed dormancy have indicated that the origin of dormancy is quite variable. So far, only a few mechanisms are known in some detail, for instance the role of water-impermeable seed coats in seed hardiness or embryo immaturity in dormancy of other species (Bewley and Black, 1982).

Most studies of seed dormancy have concentrated on breaking of dormancy only. However, for the majority of species seed dormancy is a reversible process. When environmental conditions are not suitable for germination, relief of dormancy is often followed by re-induction of dormancy. In these species, a satisfactory explanation of dormancy has to involve this reversible character.

In studies on germination periodicity it has been demonstrated that reversibility of seed dormancy naturally occurs in many species when seeds are buried in a soil seed bank (Karssen, 1982; Baskin and Baskin, 1985; Bouwmeester and Karssen, 1992). When germination is somehow inhibited, seeds may pass through a seasonal pattern of changes in dormancy for numbers of years. These patterns are studied by burial of seeds under field conditions. At regular intervals portions of seeds are exhumed to test germination capacity under controlled conditions in the laboratory.

So far, studies on seasonal dormancy patterns have been mainly descriptive. An essential observation was that temperature during burial plays a predominant role in the regulation of these dormancy patterns. However, a suitable temperature is also of critical importance for successful germination. These two roles of temperature are functionally connected because temperature conditions during burial determine the size of the temperature range over which germination can occur. In general, the range is widened during relief of dormancy, whereas the opposite occurs during dormancy induction (Vegis, 1964; Bouwmeester and Karssen 1992).

Whether or not a seed will germinate when the temperature requirements are fulfilled,

also depends on other environmental factors, such as light and nitrate. Light, acting through the photoreversible chromoprotein phytochrome as well as nitrate, stimulate germination in many species (Roberts and Smith, 1977; Taylorson, 1987). The stimulative actions of light and nitrate often interact in the germination of seeds (e.g. Vincent and Roberts, 1979; Probert *et al.*, 1987; Grubišić and Konjević, 1990; Singh and Amritphale, 1992) and in fern spores (Haas and Scheuerlein, 1990). It has been shown in several species that light requirement of seeds changes during alterations in dormancy (Taylorson, 1970, 1972). A few studies observed changes in the dependency on nitrate (Karssen *et al.*, 1988; Bouwmeester, 1990).

Seed germination of the cruciferous species *Sisymbrium officinale* (L.) Scop. depends on the simultaneous presence of light and nitrate, either exogenously applied or endogenously present in the seeds (Hilhorst and Karssen, 1989). During incubation at a constant temperature of 15 °C, sensitivity to both light and nitrate decreased (Hilhorst, 1990a, b). Detailed dose response analyses indicated that induction of secondary dormancy resulted from a decrease in the number of phytochrome- and nitrate receptors. Similarities in the changing responses to light and nitrate led to the hypothesis that phytochrome and nitrate share the same site of action.

The requirement for light and nitrate in *S. officinale* could be replaced by addition of gibberellins (GAs) (Hilhorst and Karssen, 1988). This combined action of light and nitrate could be reversed by tetcyclasis, an inhibitor of GA biosynthesis, indicating that the combination of both factors stimulated GA biosynthesis. Light also increased sensitivity to GAs. This second light effect was independent of nitrate. Whether dormancy induction is also accompanied by a decrease in sensitivity to GAs has not been studied in this species.

The annual dormancy cycle of *S. officinale* seeds has been described by Bouwmeester (1990). Dormancy of this species is relieved during periods of low temperature in autumn-winter, whereas high summer temperatures re-induce dormancy.

These previous studies indicate that seeds of *S. officinale* are an ideal model system for the study of the mechanisms of reversible changes in dormancy.

This paper presents the first detailed analysis of changes in the fluence responses and in the dose responses to nitrate and GAs of seeds buried under natural conditions for periods

up to 15 months. Thus, sensitivity changes during breaking of dormancy, both primary and secondary dormancy, as well as during dormancy induction, could be monitored.

Materials and methods

Seeds

Ripe seeds of *S. officinale* were collected in September, 1990 from plants growing at a roadside in Wageningen. After cleaning the seeds were stored dry in plastic containers in darkness at 2 °C.

Burial in the field

In November, 1990 portions of 1.5 $\pm$ 0.1 g seeds were buried in sandy loam outdoors. The seeds were packed in fine-mesh cotton gauze and each package was buried separately in a 10 cm plastic netpot filled with sandy loam. The soil completely surrounded the seeds to avoid any irradiation either during burial or during exhumation of the pots. After burial of the pots in the field, the seeds were at a depth of approximately 10 cm below the soil surface. Soil temperatures at this depth were recorded under bare soil at the Meteorological station in Wageningen. Decade means were used.

Germination conditions

At regular intervals, some pots were exhumed at random and the seeds from each package were divided into smaller portions of 40-70 seeds. The portions were transferred to 5 cm glass Petri dishes lined with one layer of filterpaper (Schleicher & Schüll, no 595, Dassel, Germany) and wetted with 1.5 ml of the test solution. Germination occurred at a range of GA_{4+7} concentrations (ICI, Yalding, U.K.), either in darkness or following a 15 min red light irradiation. GA_{4+7} was dissolved in a few drops of 1 N KOH and diluted with a phosphate citrate buffer containing 3.3 mM $K_2HPO_4.3H_2O$ and 1.7 mM citric acid. The pH

of the buffer was 5.0. By full-scan gas chromatography mass spectrometry it was demonstrated that the commercial GA_{4+7} mixture contained 54 % GA_4 and 46 % GA_7-isolactone, whereas GA_7 was not detected (Chapter 3). As a result of short exposure to KOH most GA_7-isolactone was converted to GA_7, whereas GA_4 was not affected (Chapter 3). Sometimes, 10 μM tetcyclasis (BASF, Limburgerhof, Germany) was added to the GA_{4+7} solutions. A stock solution of 100 μM tetcyclasis was made as described in Chapter 2. In addition, germination was tested at a range of KNO_3 concentrations (Suprapur, Merck, Darmstadt, Germany) after a saturating red light dose of 15 min and fluence response curves were performed on seeds incubated in a saturating KNO_3 concentration of 25 mM. KNO_3 was dissolved in milli-Q water.

A saturating red light (620-700 nm) irradiation was obtained from six red fluorescent tubes (Philips TL 20W/15, Eindhoven, The Netherlands) filtered through one layer of 3 mm plexiglass (Red 501, Röhm & Haas, Darmstadt, Germany), fluence at seed level being 11 μmol m^{-2} s^{-1}.

Fluence response curves were obtained by irradiating the seeds with light from a Leitz slide projector with a quartz-iodine lamp (Philips 250W, Eindhoven, The Netherlands) equipped with a narrow waveband interference filter (Balzers, Liechtenstein) of 667 nm (maximum transmission 47 %, bandwidth at 50 % of maximal transmission 10.9 nm). The fluence rate was varied by inserting neutral glass filters (NG, Schott u Gen., Mainz, Germany) behind the interference filter.

Irradiation time was 20 s but when fluence values higher than $1.06 * 10^{-3}$ mol m^{-2} were required, irradiation time was prolonged to maximally 4 min. Reciprocity was not affected at these longer times (Hilhorst, unpublished results). The fluence rate was calculated from the transmission characteristics of the neutral filters.

Germination was tested at 15 °C in a refrigerator (Bosch, KSR 2511/01, Van Rijn, Amsterdam, The Netherlands) converted into a cooled incubator. To determine germination percentages, both germinated and non-germinated seeds were counted 6 days after the start of the germination test. Radicle protrusion was taken as the criterion for germination. All manipulations with seeds after exhumation were performed in very dim green light, obtained by filtering one green fluorescent tube (Philips TL 40W/17, Eindhoven, The Netherlands)

with two layers of yellow no. 46 and two layers of blue no. 62 Cinemoid filters (Strand Electric, London, U.K.).

All treatments were in triplicates, except for the fluence response data, which were in duplicates.

Calculation of logistic dose response data

It is known that many dose response curves for plant hormones are sigmoidal in shape (Nissen, 1985, 1988), although many differ subtly from this model (Koshland *et al.*, 1982). Also nitrate- and fluence response curves in *S. officinale* were shown to be sigmoidal (Hilhorst, 1990a, b).

A quantitative description of the sensitivity of seeds to applied GA_{4+7}, to KNO_3 and to light was made by calculating logistic dose response curves from the individual data points, using a non-linear regression method (Tablecurve, Jandel Scientific, California, U.S.A.). The best fitting curves were produced.

The underlying interaction of a ligand (in our study: phytochrome, nitrate or GA) with its receptor can be summarized by:

$$H + Rec \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HRec \xrightarrow{k_r} response \qquad (1)$$

where: H = ligand

Rec = receptor

HRec = ligand-receptor complex

k_1 and k_{-1} are kinetic association and dissociation constants

k_r is a rate constant for the rate-limiting step in the series of events leading from the formation of HRec to response.

It is assumed that the receptors for one ligand are equivalent and independent, the response is proportional to the number of occupied receptors, the ligand can only exist in two states: free or bound to its receptor and the identity or velocity of the rate-limiting step does

not change during the response (Weyers *et al.*, 1987; Hilhorst, 1990a).

This relationship is similar to Michaelis-Menten kinetics. Modification by the Hill- or cooperativity coefficient p (Boeynaems and Dumont, 1980) has been proven to describe ligand-receptor interactions adequately (Weyers *et al.*, 1987).

The response (R) at a given dose concentration (H) is described by the following equation:

$$R = R_{min} + \frac{R_{max} - R_{min}}{1 + \left(\frac{[H]_{50}}{[H]}\right)^{p}} \qquad (2)$$

where: R = response
R_{min} = minimum response in the absence of exogenous [H]
R_{max} = maximum response
[H] = applied dose concentration
$[H]_{50}$ = dose concentration required for half-maximum response
p = Hill- or cooperativity coefficient

The slope at half-maximum response ([H] = $[H]_{50}$) is calculated by:

$$\log\left(\frac{\partial R}{\partial \log[H]}\right) - \log\left(\frac{(R_{max} - R_{min}) * p}{4} * \ln 10\right) \qquad (3)$$

Nitrate measurements

Immediately following exhumation of seeds, portions of about 50 mg were transferred to 1.5 ml Eppendorf tubes and stored at -20 °C until measurement of nitrate, as described by Hilhorst (1990b) and Derkx and Karssen (1993) (see also Chapter 2). Nitrate content was measured in 3 independent replications and expressed in nmol g^{-1} seed on a dry weight basis.

Results

A clear dormancy pattern was observed when germination of exhumed seeds was tested under saturating conditions of light and nitrate (Fig. 6.1). A germination temperature of 15 °C was chosen because dormancy patterns of this species, as described by Bouwmeester (1990), were best seen at this temperature. Release of primary dormancy was visible from the start of burial in November, 1990. Germination capacity remained high during winter and spring and started to decline in May. At high summer temperatures dormancy was maintained and when temperatures dropped in autumn, secondary dormancy was broken. The pattern coincided well with patterns earlier found by Bouwmeester (1990).

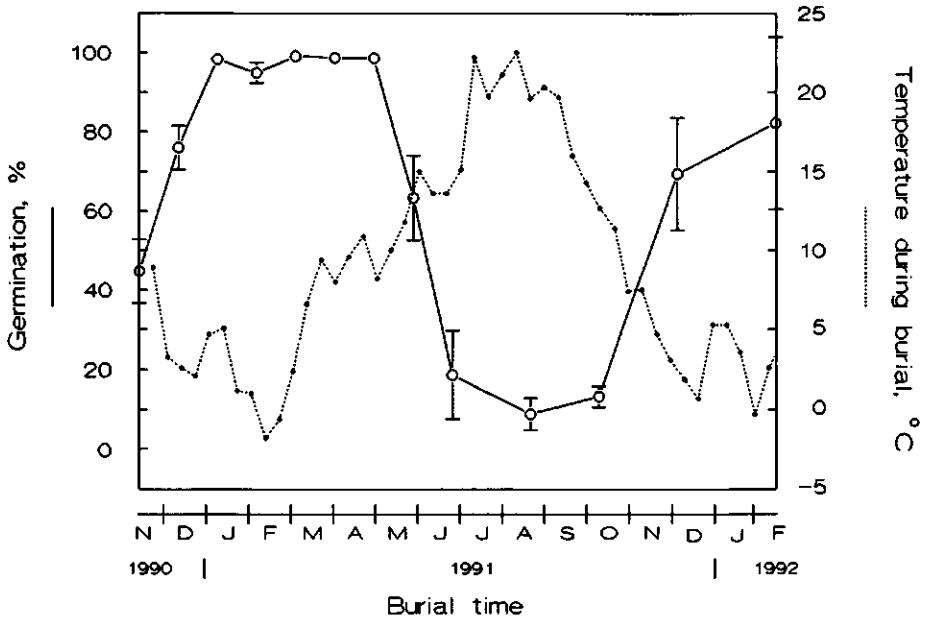

Figure 6.1 Seasonal variation in germination capacity of exhumed seeds of *S. officinale*. After exhumation at the indicated dates germination was tested at 15 °C in 25 mM KNO_3 after 15 min of red light irradiation. The dotted line indicates the soil temperature at 10 cm in bare soil.

As illustrated in Fig. 6.2, the range of temperatures over which germination took place, was much wider in spring than in summer. In summer germination only occurred at the low values of the range.

At all exhumation dates the responses of the seeds to red light, nitrate and GA_{4+7} were analysed. When the response range of germination did not exceed 20 %, not all curve parameters are presented because these values would be too inaccurate.

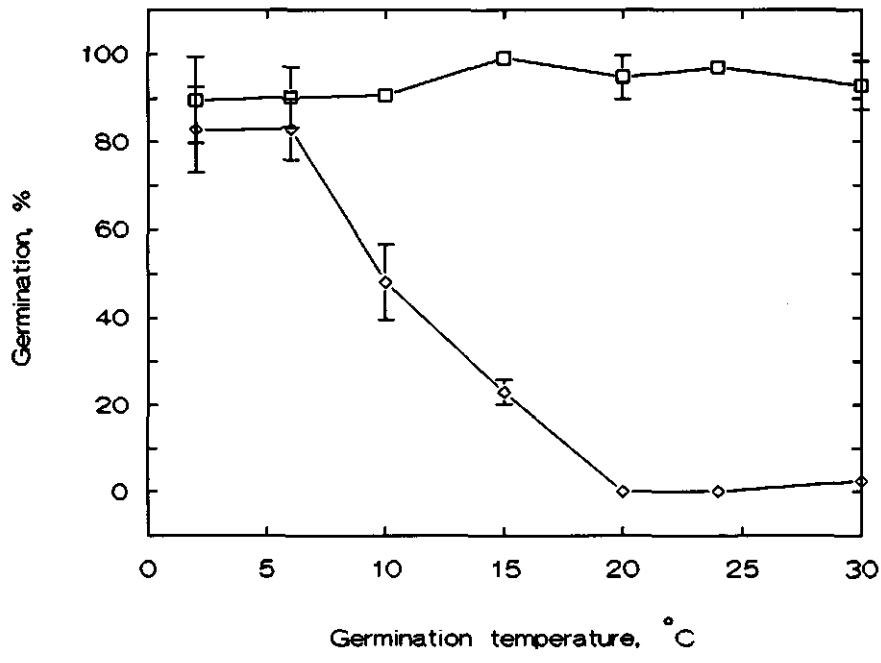

Figure 6.2 Germination of *S. officinale* seeds exhumed at 07-03-1991 (□) or 27-06-1991 (◇). Germination was tested at a range of temperatures in 25 mM KNO_3 after 15 min of red light irradiation.

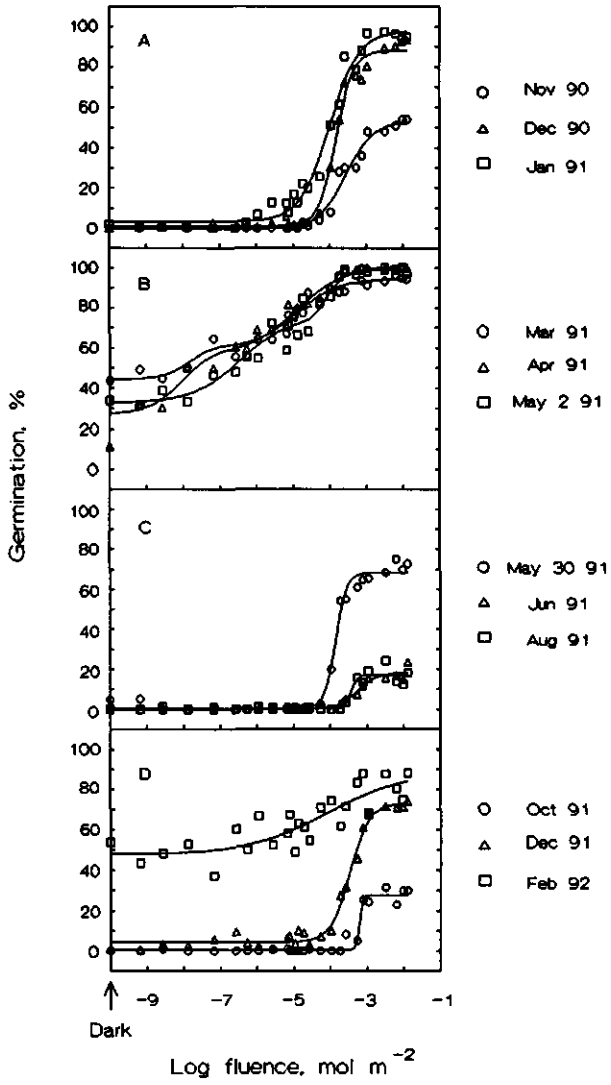

Figure 6.3 Fluence response curves of *S. officinale* seeds. After exhumation at the indicated dates germination capacity was determined at 15 °C in 25 mM KNO_3 at a range of fluence values. The germination data were fitted as logistic dose response curves. The best fitting curves were produced. The first point of each curve represents dark germination.

Table 6.1 Calculated curve parameters and standard deviations of fluence response curves in Figure 6.3. *R_{min} = minimum response, R_{max} = maximum response, Fl_{50} = dose required for half-maximum response, p = Hill-coefficient, slope = slope at half-maximum response, n.d = not determined (see text).*

Month	*R_{min} (%)*	*R_{max} (%)*	*$FL_{50}*10^4$ (mol m^{-2})*	*p*	*slope*
Nov 90	0 ± 1	53 ± 3	2.8 ± 0.0	1.1 ± 0.2	1.6 ± 0.2
Dec 90	1 ± 1	88 ± 2	1.5 ± 0.1	1.8 ± 0.2	2.0 ± 0.1
Jan 91	3 ± 1	98 ± 3	1.0 ± 0.0	1.1 ± 0.1	1.8 ± 0.1
March 91 LFR		95 ± 11	0.1 ± 0.1	0.8 ± 0.3	1.2 ± 0.3
March 91 VLFR	*44 ± 2*		*n.d*	*n.d*	*n.d*
April 91 LFR		102 ± 9	0.1 ± 0.1	0.6 ± 0.2	1.2 ± 0.3
April 91 VLFR	*27 ± 11*		*$1.0*10^{-4}$ ± $1.0*10^{-4}$*	*0.9 ± 0.9*	*1.2 ± 0.4*
May 2 91 LFR		99 ± 3	0.9 ± 0.0	1.8 ± 0.5	1.4 ± 0.2
May 2 91 VLFR	*33 ± 4*		*$36.0*10^{-4}$ ± $29.0*10^{-4}$*	*0.7 ± 0.4*	*1.2 ± 0.4*
May 30 91	1 ± 1	68 ± 2	1.4 ± 0.1	2.9 ± 0.4	2.1 ± 0.1
June 91	0 ± 0	18 ± 1	n.d	n.d	n.d
Aug 91	0 ± 1	17 ± 1	n.d	n.d	n.d
Oct 91	1 ± 1	28 ± 2	6.2 ± 0.5	11.2 ± 5.1	2.3 ± 0.1
Dec 91	4 ± 3	73 ± 2	3.4 ± 0.3	1.7 ± 0.2	1.8 ± 0.1
Feb 92	48 ± 5	89 ± 18	0.9 ± 1.8	0.4 ± 0.3	1.0 ± 0.6

Fluence response curves

To prevent limitation by other germination conditions, the fluence responses were tested in a saturating KNO_3 concentration of 25 mM (Hilhorst and Karssen, 1989). The fitted fluence response curves are shown in Fig. 6.3 and the curve parameters are listed in Table 6.1. The 'dark' (dim green light) controls are represented by the first point of each series.

During the burial period that was investigated two types of reactions to light could be distinguished. At the start of burial (November, 1990) and the two months thereafter light requirement only occurred in the low-fluence-range (LFR) at fluence values exceeding 10^{-5} mol m^{-2} (Fig. 6.3A). Within this range the maximum response (R_{max}) increased and the fluence required for half-maximum response (FL_{50}) decreased. The shifts were more or less parallel as can be derived from the slopes of the fluence response curves (Table 6.1). During spring, 1991 a second type of reaction appeared when germination became possible in the very-low-fluence-range (VLFR) at fluence values below 10^{-5} mol m^{-2} (Fig. 6.3B). The minimum response (R_{min}) increased significantly (Table 6.1). The curves obtained in March, April and the beginning of May clearly exhibited a biphasic character. The parameters for the VLFR and LFR were calculated separately (Table 6.1). Within the LFR the decrease in FL_{50}, that started already in November, 1990, continued until April. The slopes of the fluence response curves in the LFR were somewhat reduced in comparison with the previous months and were comparable to those in the VLFR (Table 6.1).

In May, 1990 the response in the VLFR disappeared (Fig. 6.3C). During summer and autumn changes in R_{max} and FL_{50} occurred in a way opposite to the breaking of dormancy in the previous winter (Figs. 6.3A, C).

The reactions during induction of secondary dormancy were reversed during winter, 1991-1992. Irrespective of a significant rise in R_{min}, the data points in February, 1992 could best be fitted by a monophasic curve (Fig. 6.3D).

The parameter p showed quite some variation, ranging from 0.4 to 2.9. In October, 1991 when R_{max}-R_{min} was less than 30 %, p was 11.2 (Table 6.1).

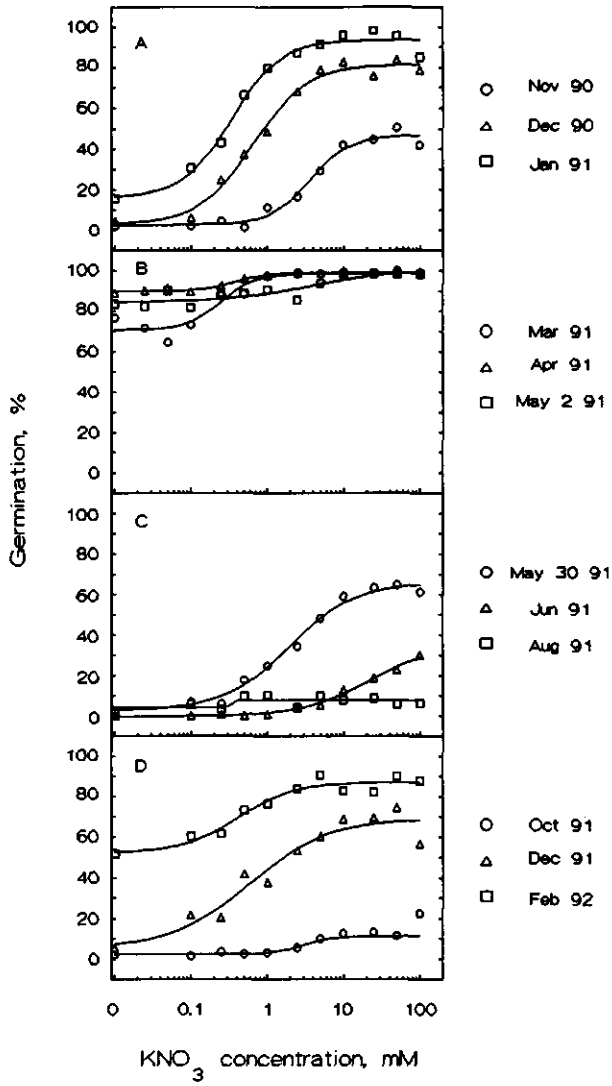

Figure 6.4 Nitrate dose response curves of *S. officinale* seeds. After exhumation at the indicated dates germination capacity was determined at 15 °C after 15 min of red light irradiation. The germination data were fitted as logistic dose response curves. The best fitting curves were produced.

Table 6.2 Calculated curve parameters and standard deviations of nitrate dose response curves in Figure 6.4. *R_{min} = minimum response, R_{max} = maximum response, $[KNO_3]_{50}$ = dose required for half-maximum response, p = Hill-coefficient, slope = slope at half-maximum response, n.d = not determined (see text).*

Month	*R_{min} (%)*	*R_{max} (%)*	*$[KNO_3]_{50}$ (mM)*	*p*	*slope*
Nov 90	3 ± 2	47 ± 4	3.6 ± 0.6	1.7 ± 0.4	1.6 ± 0.2
Dec 90	3 ± 3	82 ± 6	0.7 ± 0.1	1.2 ± 0.2	1.8 ± 0.2
Jan 91	16 ± 3	94 ± 5	0.4 ± 0.0	1.4 ± 0.2	1.8 ± 0.1
March 91	71 ± 2	99 ± 3	0.3 ± 0.1	2.0 ± 0.7	1.5 ± 0.2
April 91	90 ± 0	99 ± 1	n.d	n.d	n.d
May 2 91	85 ± 9	100 ± 5	n.d	n.d	n.d
May 30 91	3 ± 3	66 ± 7	2.0 ± 0.5	1.0 ± 0.2	1.6 ± 0.2
June 91	0 ± 2	37 ± 16	25.2 ± 24.5	0.9 ± 0.4	1.3 ± 0.3
Aug 91	4 ± 2	8 ± 3	n.d	n.d	n.d
Oct 91	2 ± 1	11 ± 1	n.d	n.d	n.d
Dec 91	5 ± 7	69 ± 11	0.6 ± 0.2	0.9 ± 0.3	1.5 ± 0.3
Feb 92	52 ± 7	87 ± 10	0.4 ± 0.2	1.2 ± 0.7	1.4 ± 0.3

Nitrate dose responses

In nitrate dose response experiments the seeds were irradiated with a saturating dose of red light after the start of imbibition in nitrate. The fitted curves are shown in Fig. 6.4 and the curve parameters in Table 6.2.

Shifts in nitrate requirement showed clear similarities with shifts in light requirement. During the alleviation of both primary and secondary dormancy in winter, 1990/91 and winter, 1991/92, respectively, R_{max} increased, followed by a rise in R_{min}. In the same period the requirement for exogenous nitrate ($[KNO_3]_{50}$) declined. During induction of secondary dormancy the opposite could be observed. Shifts in requirement for nitrate were parallel, as can be derived from the slopes of the dose response curves (Table 6.2). Variation in p was substantial.

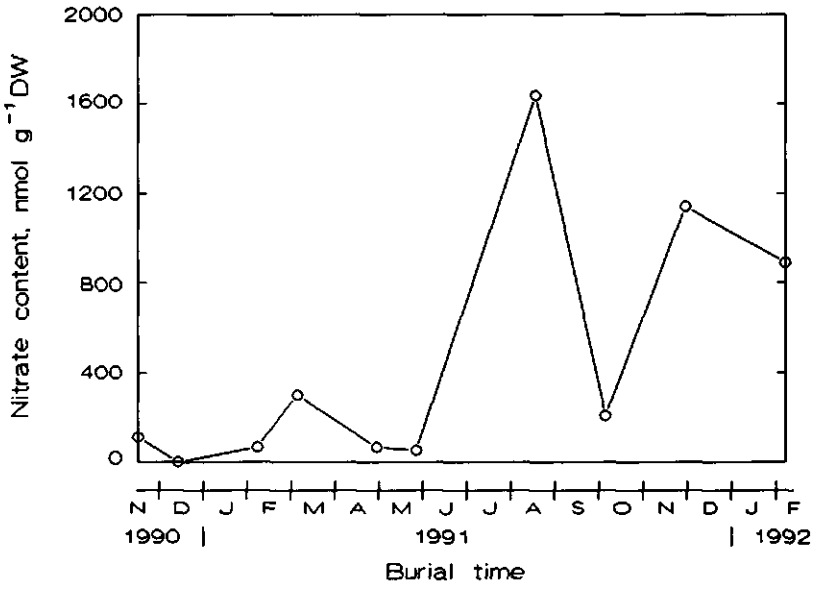

Figure 6.5 Variation in endogenous nitrate content of *S. officinale* seeds exhumed at the indicated dates.

All sets of data points could be fitted by monophasic curves although responses to nitrate occurred both in the very-low-nitrate-range (VLNR) (concentrations below 1 mM) and in the low-nitrate-range (LNR) (concentrations above 1 mM), as defined by Hilhorst (1990b). This author described biphasic nitrate dose responses.

The shifts in R_{min}, that were observed in Fig. 6.4 and Table 6.2 might reflect changes in the endogenous nitrate level of the seeds during burial. Therefore, endogenous nitrate content was determined of most exhumed samples. As shown in Fig. 6.5, the nitrate content was very high in August, and December, 1991, whereas R_{min} was very low (Fig. 6.4, Table 6.2). On the contrary, nitrate levels were low in most periods with high R_{min} values (late spring, 1991). February, 1992 is the only exception to this rule.

GA dose responses

The reaction of the exhumed seeds to GA_{4+7} was tested both in darkness and in combination with a 15 min red light pulse at the start of incubation in GA_{4+7}. The fitted GA dose response curves are depicted in Figs. 6.6A-D for the germination in darkness and in Figs. 6.6E-H for the light-induced germination. The parameters of the curves are shown in Tables 6.3 and 6.4, respectively.

Dark germination

At the start of burial and during the next two months the GA sensitivity of the seeds was very low (Fig. 6.6A). Even at the very high GA concentration of 1000 μM the dose response curves did not reach a plateau. Therefore curve parameters could not be calculated and are indicated with n.d in Table 6.3. From November, 1990 until October, 1991 values of $[GA]_{50}$ decreased gradually (Table 6.3, Figs. 6.6A-D). Thereafter $[GA]_{50}$ remained constant. Thus, reversion in requirement for GAs did not occur. The slopes of the curves hardly changed and R_{max} remained also at a constant value of about 100 %. The parameter p fluctuated between 1.2 and 2.3. Only R_{min} showed a certain seasonal periodicity: it was low in winter, 1990/91 (Fig. 6.6A), increased in late winter and spring (Fig. 6.6B), decreased in summer (Fig. 6.6C) and showed a second rise in February, 1992 (Fig. 6.6D).

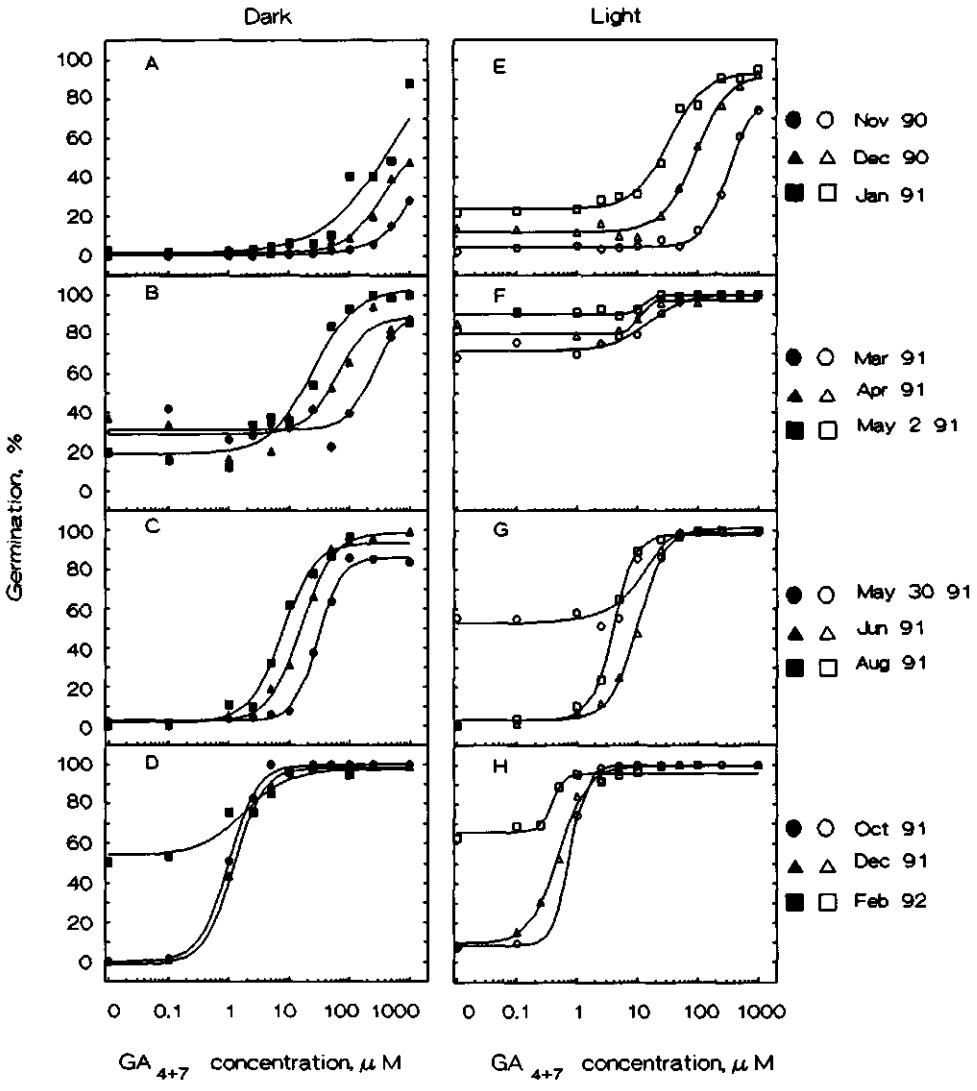

Figure 6.6 Gibberellin dose response curves of *S. officinale* seeds. After exhumation at the indicated dates germination capacity was determined at 15 °C in darkness (A-D) or after 15 min of red light irradiation (E-H) at a range of GA_{4+7} concentrations. The germination data were fitted as logistic dose response curves. The best fitting curves were produced.

Table 6.3 Calculated curve parameters and standard deviations of gibberellin dose response curves in Figure 6.6A-D (darkness). *R_{min} = minimum response, R_{max} = maximum response, $[GA]_{50}$ = dose required for half-maximum response, p = Hill-coefficient, slope = slope at half-maximum response, n.d = not determined (see text).*

Month	*R_{min} (%)*	*R_{max} (%)*	*$[GA]_{50}$ (µM)*	*p*	*slope*
Nov 90	1 ± 1	n.d	n.d	n.d	n.d
Dec 90	1 ± 1	n.d	n.d	n.d	n.d
Jan 91	1 ± 3	n.d	n.d	n.d	n.d
March 91	31 ± 2	92 ± 11	261.6 ± 100.2	1.9 ± 0.7	1.8 ± 0.2
April 91	29 ± 3	89 ± 6	61.5 ± 12.7	1.7 ± 0.5	1.8 ± 0.2
May 2 91	19 ± 3	103 ± 7	24.6 ± 4.4	1.2 ± 0.2	1.8 ± 0.2
May 30 91	3 ± 2	86 ± 4	29.7 ± 2.3	2.3 ± 0.4	2.0 ± 0.1
June 91	2 ± 2	99 ± 3	15.7 ± 1.0	1.6 ± 0.1	2.0 ± 0.1
Aug 91	2 ± 2	93 ± 5	7.6 ± 0.8	1.6 ± 0.3	1.9 ± 0.1
Oct 91	0 ± 1	100 ± 2	1.0 ± 0.1	1.8 ± 0.2	2.0 ± 0.1
Dec 91	-1 ± 1	99 ± 2	1.2 ± 0.1	1.8 ± 0.1	2.0 ± 0.1
Feb 92	54 ± 5	98 ± 10	1.8 ± 0.9	1.2 ± 0.7	1.5 ± 0.3
Dec 91 +tetc.	0 ± 2	97 ± 3	1.7 ± 0.1	1.8 ± 0.2	2.0 ± 0.1
Feb 92 +tetc.	8 ± 2	97 ± 5	2.7 ± 0.2	2.7 ± 0.6	2.1 ± 0.1

Table 6.4 Calculated curve parameters and standard deviations of gibberellin dose response curves in Figure 6.6E-H (15 min red light). Curve parameters as in Table 6.3. *n.d = not determined (see text).*

Month	R_{min} (%)	R_{max} (%)	$[GA]_{50}$ (μM)	*p*	*slope*
Nov 90	4 ± 1	81 ± 8	325.5 ± 45.5	2.1 ± 0.5	2.0 ± 0.1
Dec 90	12 ± 1	92 ± 4	93.4 ± 8.8	1.6 ± 0.2	1.9 ± 0.1
Jan 91	24 ± 2	93 ± 4	33.8 ± 4.2	1.5 ± 0.3	1.8 ± 0.1
March 91	72 ± 2	100 ± 3	n.d	n.d	n.d
April 91	80 ± 4	97 ± 2	n.d	n.d	n.d
May 2 91	90 ± 1	100 ± 1	n.d	n.d	n.d
May 30 91	53 ± 9	99 ± 1	11.3 ± 1.2	n.d	1.6 ± 0.2
June 91	3 ± 2	101 ± 3	10.1 ± 0.7	1.9 ± 0.2	2.0 ± 0.1
Aug 91	3 ± 2	98 ± 4	4.0 ± 0.2	2.5 ± 0.3	2.1 ± 0.1
Oct 91	8 ± 2	100 ± 2	0.8 ± 0.1	3.2 ± 0.7	2.2 ± 0.2
Dec 91	10 ± 3	100 ± 4	0.5 ± 0.0	1.8 ± 0.0	2.0 ± 0.1
Feb 92	66 ± 3	96 ± 5	0.4 ± 0.1	4.5 ± 2.6	1.9 ± 0.2
Dec 91 +tetc.	1 ± 3	96 ± 4	0.7 ± 0.1	2.4 ± 0.3	2.1 ± 0.1
Feb 92 +tetc.	2 ± 4	95 ± 7	0.8 ± 0.1	1.4 ± 0.3	1.9 ± 0.2

Light germination

Exhumed seeds that had been irradiated with a short red light pulse showed roughly the same pattern of change in $[GA]_{50}$ as observed in darkness. The values of $[GA]_{50}$ were, however, consistently lower than in darkness (Tables 6.3 and 6.4). As in dark germination shifts in response to GA_{4+7} were parallel in light-induced germination. The seasonal periodicity of R_{min} in light was even more pronounced than in darkness (Figs. 6.6E-H).

Essentially, the rise of R_{min}, as observed in both darkness and light, is an increased

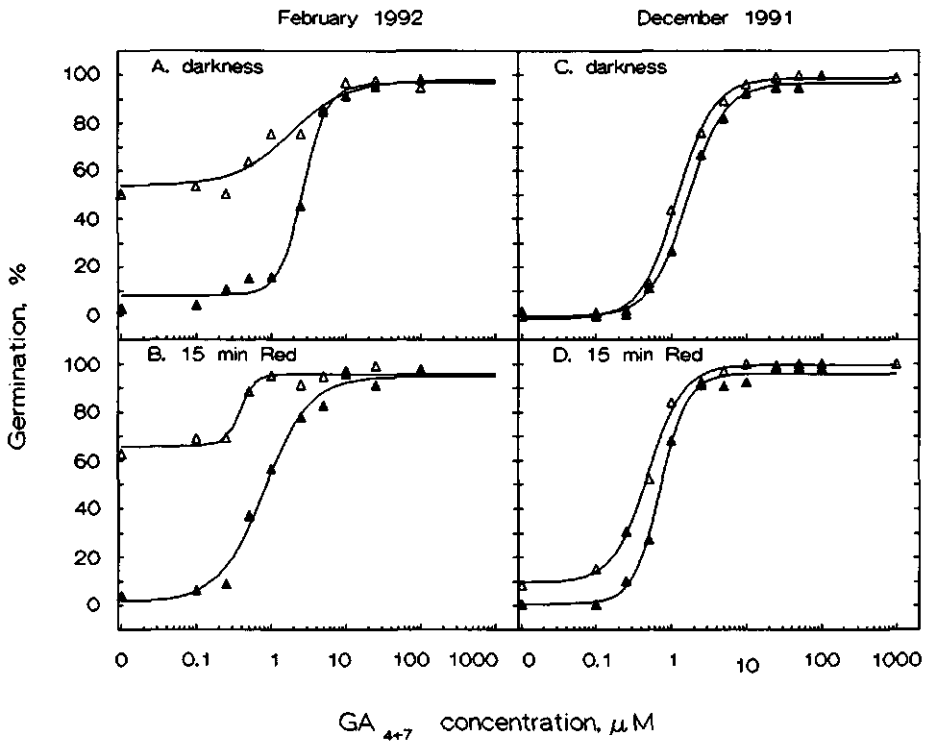

Figure 6.7 The effect of tetcyclasis on GA dose response curves of *S. officinale* seeds exhumed in February, 1992 (A,B) or December, 1991 (C,D). Germination was tested at 15 °C in darkness (A,C) or after 15 min of red light irradiation (B,D), with (▲) or without (△) 10 μM tetcyclasis.

capacity of the seeds to germinate in water. This independency on exogenous GA_{4+7} could be caused by an increased capacity to synthesize GAs. Indeed, the addition of 10 μM tetcyclasis, an inhibitor of GA biosynthesis, strongly reversed the rise in R_{min} both in darkness and in light of seeds exhumed in February, 1992 (Figs. 6.7A, B). Application of GAs in concentrations exceeding 5-10 μM could fully neutralize the inhibitory action of tetcyclasis. Sensitivity to GAs was not influenced by tetcyclasis. This could be seen in December, 1991 when R_{min} was negligibly low (Figs. 6.7C, D): values of $[GA]_{50}$ and the slopes of the curves were not influenced by tetcyclasis (Tables 6.3 and 6.4). Also when R_{min} reached high percentages in February, 1992, tetcyclasis did not influence $[GA]_{50}$. In darkness, tetcyclasis increased the slope of the curve somewhat (Table 6.3).

Discussion

From the present detailed dose response analysis, it can be concluded that changes in sensitivity to environmental stimuli like light and nitrate act as major regulatory factors in controlling reversible changes in dormancy of *S. officinale* seeds buried under natural conditions. Regulation by change in sensitivity to GAs is unlikely because a gradual increase in GA sensitivity is apparent from burial onwards, irrespective of dormancy breaking or -induction.

Because the term 'sensitivity' can have several meanings, we have attempted to quantify this term. By analogy with studies by Weyers *et al.* (1987) and Fitzsimons (1989), dose response data were fitted as logistic dose response curves that were adapted from Michaelis-Menten kinetics. The observed response is a function of several parameters: the concentration of ligand (in our study: GA, nitrate or phytochrome), the number of receptors, the affinity of the receptor for the ligand and the overall capacity of the responding system to respond to the number of occupied receptors. In addition, factors like ligand-uptake system and ligand-metabolizing enzymes may modify the response when the effects of applied ligands are studied (Firn, 1986). Although assumptions similar to those adopted in Michaelis-Menten kinetics can not be justified for plant-hormone responses, Weyers *et al.* (1987) stated that none of them appears unreasonable and sensitivity parameters were given some interpretive

value, that can only be tested when isolation of receptors concerned has been successful and binding properties studied. Germination, being an 'all-or-none' response at the end of a transduction chain initiated by some environmental factor(s), is a highly complex process. Consequently, care should be taken in the interpretation of sensitivity parameters derived from a 'simple' molecular model. In the absence of knowledge about the nature of ligand-receptor interaction, some physiological interpretation of sensitivity parameters will be presented, although tentative.

Interpretation of curve parameters on basis of a simple model

First, we will consider the interpretation of individual sensitivity parameters. The parameter R_{min} represents the base level of response in the absence of added ligand. The parameter R_{max} may be taken as a measure of the capacity to respond, when ligand availability is not limiting. R_{max} is a function of the total level of receptors according to: $R_{max} = k_r * [Rec]_T$ with $[Rec]_T = [Rec] + [HRec]$. Changes in R_{max} may reflect differences in the number of receptors. However, since $R_{max} = k_r * [Rec]_T$, changes in R_{max} may also reflect changes in the rate of metabolic processes initiated by the formation of ligand-receptor complex. When the number of receptors rises above the number required for a maximum physiological response (i.e 100 % germination), dose response curves will shift to the left, thus less ligand is required to achieve the same response. It is known from studies in seeds of lettuce (VanderWoude, 1985) and *S. officinale* (Hilhorst, 1990a) that the phytochrome-receptor level required to induce maximal germination may be 40 % of the level maximally present in the seeds. The parameter $[ligand]_{50}$ may be used to calculate K_D, the dissociation constant of the ligand-receptor complex, according to $K_D = ([ligand_{50}])^p$. The parameter p is a measure of the degree of cooperativity of ligand binding. It is unsafe to give a biological interpretation to p values, solely based on dose response curves (Weyers *et al.*, 1987). Similarly, calculation of K_D values would be dangerous. Therefore, values of p and K_D are not further discussed.

Interpretation of calculated curve parameters

The parameters R_{max}, Fl_{50} and $[KNO_3]_{50}$ in fluence- and nitrate dose response curves exhibited clear seasonal periodicities (Tables 6.1, 6.2, Figs. 6.3, 6.4). Shifts were parallel (Tables 6.1, 6.2). The observed shifts may represent seasonal changes in the number of available receptors, in the affinity of the receptors, and/or in the signal-transduction chain, initiated by the ligand-receptor interaction. The similarity in changing responses to light and nitrate may indicate that both share the same site of action. Reversible changes in R_{max} and $[GA]_{50}$ did however not occur in GA dose response curves. The parameter $[GA]_{50}$ decreased gradually from burial onwards, both in darkness (Table 6.3) and after 15 min of red light irradiation (Table 6.4). Light reduced the requirement for GAs. Therefore, it is concluded that dormancy is not regulated by changes in sensitivity to GAs.

The parameter R_{min} showed seasonal variation in fluence-, nitrate and GA dose response curves (Tables 6.1-6.4, Figs. 6.3, 6.4 and 6.6).

R_{min} in fluence response curves essentially represents light-independent germination. Shifts in R_{min} may arise in different ways. 'Pre-existing' Pfr may modify R_{min} (VanderWoude and Toole, 1980). Because levels presumably decline during burial, its contribution after some time of burial may be questioned. However, when seeds are extremely light sensitive, very low amounts of Pfr may be sufficient to initiate a VLFR, as was also found after 2 months of burial of *Datura ferox* seeds (Scopel *et al.*, 1991). It is also possible that Pfr action is not required anymore after some months of burial. Although we cannot fully exclude this possibility, it is not very likely from an ecological point of view. Moreover, in laboratory experiments seeds of *S. officinale* never germinated in darkness (Hilhorst and Karssen, 1988). Whether or not the dim green light to which the seeds were shortly exposed when transferred from burial conditions to germination conditions, contributes to R_{min} values in late winter and spring, cannot be established without real dark controls. However, it is known from short-term laboratory experiments that extremely light-sensitive seeds of *Arabidopsis thaliana* respond to low amounts of Pfr generated by low intensities of dim green light (Kendrick and Cone, 1985; Chapter 4). The absence of emergence in the field and hardly any seedlings in exhumed samples strengthen the hypothesis that the dim green light

contributed to R_{min} values. The absence of emergence in the field is of course also partly due to the depth of burial at 10 cm. An extremely high light sensitivity is apparent in spring. The fluence response curves clearly exhibited a biphasic character in spring, 1991 (Fig. 6.3B). One year later, R_{min} again increased, although the germination data could be fitted as a monophasic curve (Fig. 6.3D). A VLFR cannot be excluded. It is possible that the dim green light initiated a VLFR. Moreover, the response range was quite narrow. The germination data may equally well be fitted by a biphasic curve. VanderWoude (1985) used the dimeric model of the phytochrome molecule to explain biphasic fluence response curves. The response in the LFR results from the association of Pfr:Pfr with its receptor, while the response in the VLFR results from the binding of Pr:Pfr with its receptor. Pfr contents as low as 0.0001-0.01 % Pfr may induce a response in the VLFR, whereas 1-86 % Pfr induces a response in the LFR (Kronenberg and Kendrick, 1986). Biphasic fluence response curves were also observed in germination of several other species like lettuce (Blaauw-Jansen and Blaauw, 1975; Small *et al.*, 1979; VanderWoude and Toole, 1980), *Arabidopsis thaliana* (Cone *et al.*, 1985), *Rumex obtusifolius* (Kendrick and Cone, 1985), *Kalanchoë blossfeldiana* (Rethy *et al.*, 1987) and *Echinochloa crus-galli* seeds (Taylorson and Dinola, 1989). The ecological significance of a response in the VLFR is easily to interpret. Soil disturbance may expose seeds to light for short periods, but sufficiently long to induce a response in the VLFR. Fluences as low as those delivered by a few milliseconds of full sunlight may induce some germination, even when the seeds are covered with soil again (Hartmann and Nezadal, 1990).

Shifts in R_{min} in nitrate dose response curves did not simply reflect changes in the endogenous nitrate levels of the seeds, that may vary due to seasonal influences (Fig. 6.5), but most probably reflect changes in sensitivity to a saturating red light dose.

The shifts in R_{min} in GA dose response curves represent changes in the capacity to synthesize GAs as a result of Pfr action. By use of tetcyclasis it was proven that germination is preceded by *de novo* synthesis of GAs (Figs. 6.7A, B). Synthesis independent of Pfr action is not very likely because it would allow giant germination flushes of deeply buried seeds with little or no chance for the seedlings to reach the soil surface. In laboratory experiments it was convincingly demonstrated that GA biosynthesis absolutely depends on Pfr action

(Hilhorst and Karssen, 1988).

A dormancy model

From the present results it is concluded that temperature during burial determines the range of conditions under which germination can proceed. The dominant role of temperature in regulating seasonal dormancy patterns was extensively discussed by Bouwmeester and Karssen (1992). Temperature during burial results in reversible changes in sensitivity to light and nitrate. These changes occur at the level of receptors. Both phytochrome- (Taylorson, 1988) and GA (Hooley *et al.*, 1991) receptors are proposed to be membrane-bound. The observed changes may involve shifts in the number of available receptors, shifts in the binding characteristics or shifts in the response chain initiated by the ligand-receptor interaction. Whether or not these three parameters all play a direct role in dormancy control is debatable. Control via the availability of receptors seems very likely. It is difficult to estimate whether binding characteristics of receptors have a direct regulatory role in dormancy control, because shapes of dose response curves cannot, without many assumptions, be used to deduce the affinity of the ligand-receptor interaction (Hollenberg, 1985). Upon binding of phytochrome to its receptor and the unknown contribution of nitrate some response chain will be initiated. In this response chain the biosynthesis of GAs is stimulated, thus the capacity to synthesize GAs varies over the seasons, in analogy with shifts in light- and nitrate requirement. Light is also known to increase sensitivity to GAs (Hilhorst and Karssen, 1988). Shifts in requirement for GAs were, however, not comparable with those for light and nitrate. Thus, GA sensitivity was at least partly independent of light. Except for the first few months of burial, GA sensitivity may not be the limiting factor for germination. The presence of stimulatory factors like light and nitrate in combination with an appropriate temperature may stimulate the seeds to germinate, provided sensitivity to these factors is not limiting. The combination of GA biosynthesis and a sufficiently high GA sensitivity is essential for germination. Both factors do, however, not primarily regulate dormancy. It is not likely that post-receptor events play an important direct role in dormancy control. Soon after a germination-inducing treatment (i.e. light and nitrate provided to non-

dormant *S. officinale* seeds) the metabolic activity increases dramatically (Chapter 7). Regulation at this stage would not be preferable because seed reserves would be depleted within a relatively short time.

It should be pointed that changes in curve parameters alone should not be used to infer underlying dormancy mechanisms. Further analysis of sensitivity at the molecular level is required to verify our conclusions about dormancy control.

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Chapter 7

Respiration in seeds of *Sisymbrium officinale*. I. Temperature-induced changes in dormancy during prolonged incubation do not depend on respiratory activity

M.P.M. Derkx and C.M. Karssen

Summary

Effects of temperature-induced changes in dormancy on respiratory activity were measured in seeds of *Sisymbrium officinale* (L.) Scop. during prolonged incubation. Effects on dormancy could be studied in darkness without the interference of early germination events because germination of this species absolutely depends on the simultaneous action of light and nitrate. Germination and O_2 uptake were measured at 24 °C. Seeds were pre-incubated at 6, 10, 15 or 24 °C. At all temperature pretreatments a quick rise in O_2 uptake was followed by a gradual decrease. The initial rise in O_2 uptake correlated well with the rate of water uptake and with breaking of dormancy. However, the subsequent decline in O_2 uptake was not generally linked to induction of secondary dormancy. An increased O_2 uptake was not required during a following breaking of secondary dormancy, by a transfer of the seeds to 2 °C. Germination capacity increased in the course of 10 weeks but rates of O_2 uptake remained constant. Seeds kept for prolonged periods at 24 °C, showed neither a change in germination capacity nor in O_2 uptake. During re-induction of dormancy following the chilling pretreatment O_2 uptake did not further decline. It is concluded that changes in dormancy are not related to changes in respiratory activity. However, germination strongly depends on respiration. The increase in O_2 uptake started well before radicle protrusion. A farred irradiation only reversed this increase when it was given before germination escaped from its red light-antagonizing action.

Introduction

It is well established that seeds of many species can survive substantial periods of burial in soil (for references see Priestley, 1986). It is likely that for successful survival the metabolic activity of the seeds should be reduced to avoid rapid depletion of reserves. A low constant level of respiration during prolonged imbibition in water has been observed in lettuce seeds (Powell *et al.*, 1983). During burial seeds pass through an annual dormancy cycle (Karssen, 1982; Baskin and Baskin, 1985; Bouwmeester and Karssen, 1992). Vegis (1964) suggested that these cycles might be regulated by fluctuations in oxygen availability that result from fluctuations in temperature. However, in the two dimorphic seeds of *Xanthium pennsylvanicum* that differ in the degree of dormancy, no differences in oxygen availability were observed (Porter and Wareing, 1974). It has often been suggested that treatments leading to termination of dormancy, for instance chilling, give enhanced respiratory activity (Pollock and Olney, 1959; Alscher-Herman *et al.*, 1981), whereas Bogatek and Rychter (1984) concluded that respiratory processes do not play any decisive role in dormancy removal of apple seeds. Respiration rates of excised embryos from dormant and after-ripened wild oat seeds were similar, but the measurements were restricted to the first 10 h of imbibition (Simmonds and Simpson, 1971). On the contrary, Chen and Varner (1970) reported that dormant seeds of wild oat consume less oxygen than after-ripened seeds during the first 12 h of imbibition. Application of nitrate stimulated both O_2 uptake and germination of *Avena fatua* seeds (Adkins *et al.*, 1984). Because the increase in O_2 uptake occurred before radicle protrusion, they concluded that the rise was a possible cause of germination rather than a result. A similar conclusion was drawn by Hilton and Thomas (1986), who compared O_2 uptake and germination in several species differing in their response to potassium nitrate. However, the increased respiratory activity may still result from germination processes that prepare radicle protrusion, rather than being correlated to dormancy breaking. It is difficult to separate the metabolism associated with the breaking of dormancy from that of germination and growth (Bewley and Black, 1982; Ross, 1984). An increased O_2 uptake after a red light irradiation was reported in seeds of lettuce (Peckett and Al-Charchafchi, 1979; Powell *et al.*, 1984) and *Pinus sylvestris* (Nyman, 1961). The authors

attributed the increase to the germination process.

The objective of the present study was to investigate whether changes in dormancy of *Sisymbrium officinale* seeds are regulated by changes in metabolic activity. Seeds of *S. officinale* are an ideal model system to get a definite answer to this question because processes concerning breaking of dormancy can be easily distinguished from processes concerning germination. Germination of this species fully depends on the simultaneous presence of light and nitrate, either applied or endogenously present in the seeds (Hilhorst *et al.*, 1986; Hilhorst and Karssen, 1989; Derkx and Karssen, 1993; Chapter 6). Thus, germination is prevented in darkness in seeds with a low endogenous nitrate content. Temperature pretreatment in darkness causes variation in the intensity of dormancy. Such variation becomes visible when germination is tested in the presence of nitrate, following irradiation.

In order to establish whether changes in dormancy are correlated to changes in metabolic activity, seeds will be pre-incubated in darkness at different constant temperatures. After variable periods of time the rates of O_2 uptake will be measured in darkness and germination capacity will be determined in the presence of nitrate after irradiation. For comparison, O_2 uptake rates are also followed in non-dormant seeds that are stimulated to germinate as a result of the interaction of light and nitrate.

Materials and methods

Seeds

Ripe seeds of *Sisymbrium officinale* (L.) Scop. were collected in autumn, 1987 from wild plants growing in the laboratory garden in Wageningen. After harvest the seeds were cleaned and stored dry in plastic containers at 2 °C in darkness until use in experiments in 1989-1991. Under these conditions no changes in dormancy were observed in the dry seeds during the experimental period.

Pre-incubation and germination conditions

Triplicates of 50 seeds were sown in 5 cm Petri dishes on a single layer of filter paper (Schleicher & Schüll, no. 595, Dassel, Germany) and wetted with 1.5 ml of 0.5 or 25 mM potassium nitrate (Suprapur, Merck, Darmstadt, Germany). After various pre-incubation periods in darkness at different constant temperatures, ranging from 2 to 24 °C, germination capacity was tested by irradiating the seeds with red light during 15 min and thereafter transferring them to 24 °C in darkness. Germination was counted after three days. Radicle protrusion was taken as the criterion for germination. Both pre-incubation and germination occurred in refrigerators (Bosch, KSR 2511/01, Van Rijn, Amsterdam, The Netherlands) that were converted into cooled incubators. Red light (620-700 nm) was obtained from six red fluorescent tubes (Philips TL 20W/15, Eindhoven, The Netherlands) filtered through one layer of 3 mm plexiglass (Red 501, Röhm & Haas, Darmstadt, Germany). Photon fluence rate at seed level was 11 μmol m^{-2} s^{-1}. In some experiments the red light irradiation was followed by farred irradiation after several intervals. Farred (> 690 nm) was obtained from six incandescent tubes filtered by one layer Red 501 and two layers Blue 627 Plexiglass (Röhm & Haas, Darmstadt, Germany). Photon fluence rate at seed level was 12 μmol m^{-2} s^{-1} and irradiation time was 15 min.

All manipulations of incubated seeds were conducted in dim green light, obtained by filtering one green fluorescent tube (Philips TL 40W/17, Eindhoven, The Netherlands) with two layers of yellow no. 46 and two layers of blue no. 62 Cinemoid filters (Strand Electric, London, U.K.).

Oxygen uptake measurements

After several intervals of pre-incubation in darkness at different constant temperatures, O_2 uptake was determined using a Clark-type oxygen electrode. Except for one experiment, in which O_2 uptake was measured at temperatures ranging from 5 to 30 °C, O_2 uptake was determined at 24 $\pm$ 1 °C. All samples were run in at least three or four replicates. Unless otherwise mentioned, the 2 ml oxygen electrode chamber was filled with 1.5 ml 25 mM

KNO_3 to which 50 ± 0.2 mg (fresh weight) seeds were added and a small stir bar circulated these around the chamber to maintain a stable oxygen gradient across the membrane. The KNO_3 solution was not additionally aerated before filling the electrode because O_2 uptake from non-aerated solutions was similar to that from aerated solutions (data not shown). The O_2 uptake measurements were performed under dim green light. Whenever pre-incubation exceeded more than one week, the seeds were treated, at the start of pre-incubation, with 5 % hypochlorite during 1 min and five times thoroughly rinsed with distilled water. When the pre-incubation period exceeded more than 4 weeks the hypochlorite treatment was repeated before measuring O_2 uptake. The disinfection with hypochlorite did not affect O_2 uptake. Any contaminated or abnormal seeds were discarded.

Drying of seeds

In one experiment seeds were dehydrated at a relative humidity of 20 % after a pre-incubation period of 24 h in 25 mM KNO_3 at 24 °C. Drying occurred in a hygrostat above a saturated solution of LiCl, according to Weges and Karssen (1987). After several periods of dry storage, ranging from 1 to 10 days, the seeds were re-imbibed in 25 mM KNO_3 at 24 °C. O_2 uptake was monitored during the first 10 h of re-imbibition and germination capacity of re-imbibed seeds was determined.

Water uptake measurements

The rate of water uptake of seeds imbibing in water in darkness at different constant temperatures was determined by drying about 150 mg of seeds in an oven at 130 °C during 1.5 h after several intervals of imbibition. Before drying, the adjacent water was removed from the seeds on a Büchner funnel, immediately followed by weighing the seeds. The dry weights were determined after adjustment of the seeds to room temperature in an exsiccator above silicagel. All determinations were performed in triplicate.

All experiments were repeated at least once with similar results.

Results

O_2 uptake of dark-imbibed seeds

The seeds of *S. officinale* used in this study were not deeply dormant. Seeds that were irradiated after only 2 h of imbibition at temperatures of 6 (Fig. 7.1A), 10 (Fig. 7.1B), 15 (Fig. 7.1C) or 24 °C (Fig. 7.1D) showed 60-80 % germination in 25 mM KNO_3 at 24 °C. Germination capacity of seeds pre-incubated at 24 °C rose during the first 9 h and this increase was accompanied by an increase in the rate of O_2 uptake (inset in Fig. 7.1D). The increase in both parameters was followed by a decline. After 5 days of pre-incubation at 24 °C the seeds were no longer able to germinate under the chosen conditions and O_2 uptake was reduced more than twice in comparison with the level reached after 8 h of pre-incubation. The decline in O_2 uptake continued and was at a level of about 30 nmol min^{-1} g^{-1} seed after 336 h of pre-incubation.

The parallel patterns of changes in germination capacity and O_2 uptake observed during pre-incubation at 24 °C may be incidental rather than causally connected. Therefore, seeds were also pre-incubated at lower temperatures, at which changes in germination capacity occurred at different rates.

During pre-incubation at 15 °C maximum germination capacity at 24 °C was reached after 24 h and dormancy induction started after 72 h (Fig. 7.1C). Following an initial rise in O_2 uptake during 24 h, the rate started to decline after 30 h, thus preceding the decrease in germination capacity (Fig. 7.1C). After 336 h of imbibition the O_2 uptake was about 40 nmol min^{-1} g^{-1} seed.

At the lower pre-incubation temperatures of 6 and 10 °C it became even more evident that a decline in O_2 uptake was not directly connected with dormancy induction. Whereas the rate of O_2 uptake started to decline after about 30 h of pre-incubation at 6 (Fig. 7.1A) and 10 °C (Fig. 7.1B), the germination capacity remained high for much longer periods. It started to decrease slightly after 6 weeks of pre-incubation at 10 °C and was reduced to 10 % after 18 weeks of pre-incubation at 10 °C (Fig. 7.2). O_2 uptake reached a constant level of about 40-45 nmol min^{-1} g^{-1} seed after 4 weeks of pre-incubation, although fluctuating considerably.

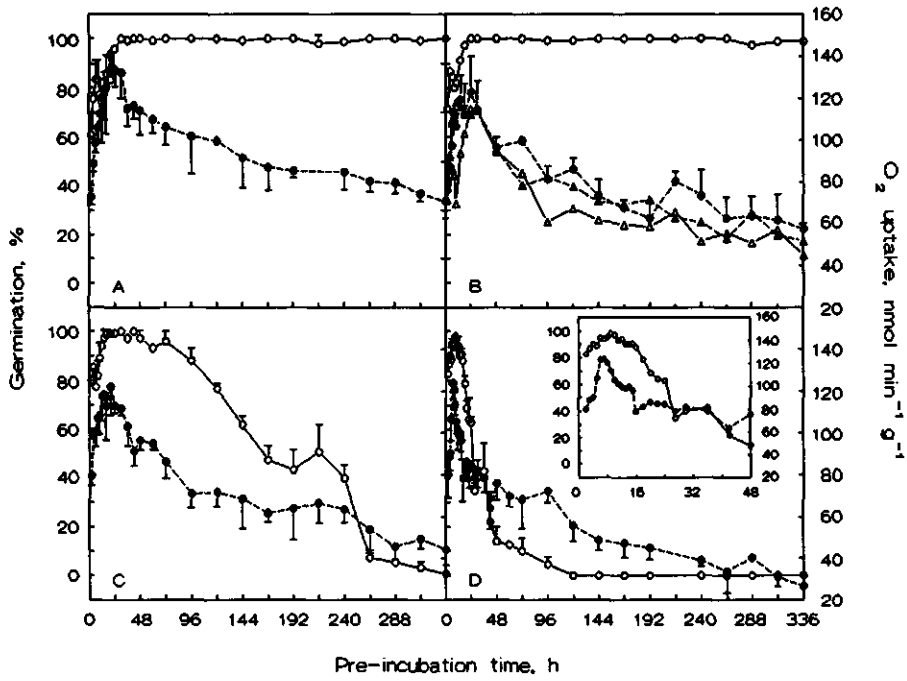

Figure 7.1 Changes in germination capacity (open symbols) and O_2 uptake rate (closed symbols) of *S. officinale* seeds during pre-incubation in 0.5 (triangles) or 25 mM KNO_3 (circles) in darkness at 6 (A), 10 (B), 15 (C) and 24 °C (D). Inset in Fig. 7.1D: Changes in germination capacity and O_2 uptake rate during the first 48 h of pre-incubation. Germination capacity and O_2 uptake rate were determined in the same KNO_3 concentration as during the pre-incubation treatment. Germination was tested at 24 °C after 15 min of red light irradiation. O_2 uptake rate was determined at 24 °C under dim green light. Vertical bars represent SD.

The decrease in germination capacity, starting as late as 6 weeks after the onset of imbibition at 10 °C in 25 mM KNO_3, may be accelerated when germination conditions are less optimal. If so, germination capacity and O_2 uptake may be correlated when the seeds are imbibed in a lower KNO_3 concentration. Therefore, we investigated whether both parameters showed similar shifts when pre-incubation and germination occurred in a KNO_3 concentration

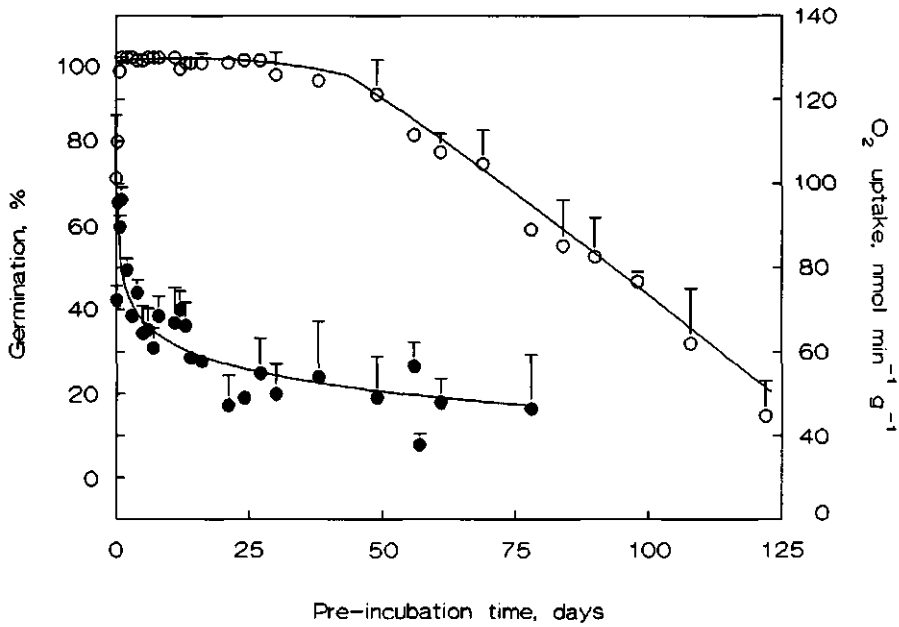

Figure 7.2 Changes in germination capacity (○) and O_2 uptake rate (●) of *S. officinale* seeds during prolonged pre-incubation at 10 °C in 25 mM KNO_3 in darkness. Germination capacity was tested in 25 mM KNO_3 at 24 °C after 15 min of red light irradiation. O_2 uptake rate was determined in 25 mM KNO_3 at 24 °C under dim green light. Vertical bars represent SD.

of 0.5 mM (triangles in Fig. 7.1B). The O_2 uptake pattern in 0.5 mM KNO_3 exactly resembled the pattern at a saturating concentration of 25 mM (Fig. 7.1B). The germination capacity in 0.5 mM KNO_3, however, declined after 30 h in contrast to the response in 25 mM KNO_3. Thus, in a non-saturating KNO_3 concentration the pattern of O_2 uptake during imbibition at 10 °C coincided well with the pattern of germination capacity, as was also found at 24 °C in a saturating KNO_3 concentration (Fig. 7.1D).

The initial rise in O_2 uptake after the start of imbibition (Fig. 7.1) is most probably the result of the water uptake itself. As shown in Fig. 7.3 the rate of water uptake was slightly temperature dependent. The increase in water content went on for quite long periods, varying

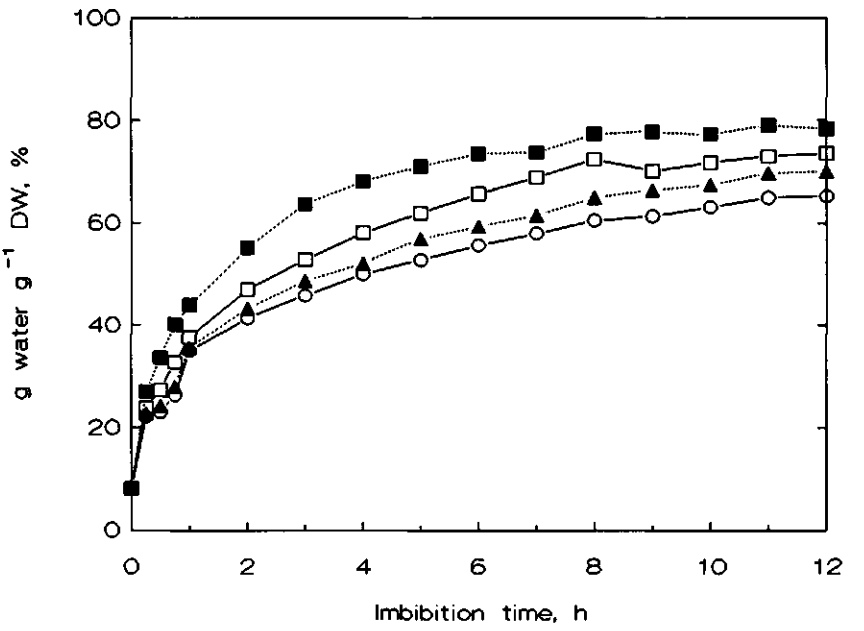

Figure 7.3 Increase in water content of *S. officinale* seeds during imbibition in darkness at 6 (○), 10 (▲), 15 (□) and 24 °C (■).

from about 14 h at 24 °C to about 30 h at 6 °C (data not shown). The water uptake correlated well with the rate of O_2 consumption at all four pre-incubation temperatures (Fig. 7.4).

A rise in O_2 uptake during early imbibition was however not observed in seeds that were re-wetted after a dehydration treatment. Germination capacity of dehydrated seeds was significantly elevated when compared with non-dehydrated seeds (data not shown).

The course of O_2 consumption was also followed during breaking of secondary dormancy, which has the advantage that early imbibition processes do not interfere with changes in germination capacity. Secondary dormancy was induced by pre-incubation at 24 °C during 7 days. Germination capacity was 0 % after 7 days and O_2 uptake about 45 nmol min^{-1} g^{-1} seed (Fig. 7.5A). Transferring the seeds to 2 °C resulted in breaking of secondary dormancy (Fig. 7.5B). Within 10 weeks germination capacity, determined at

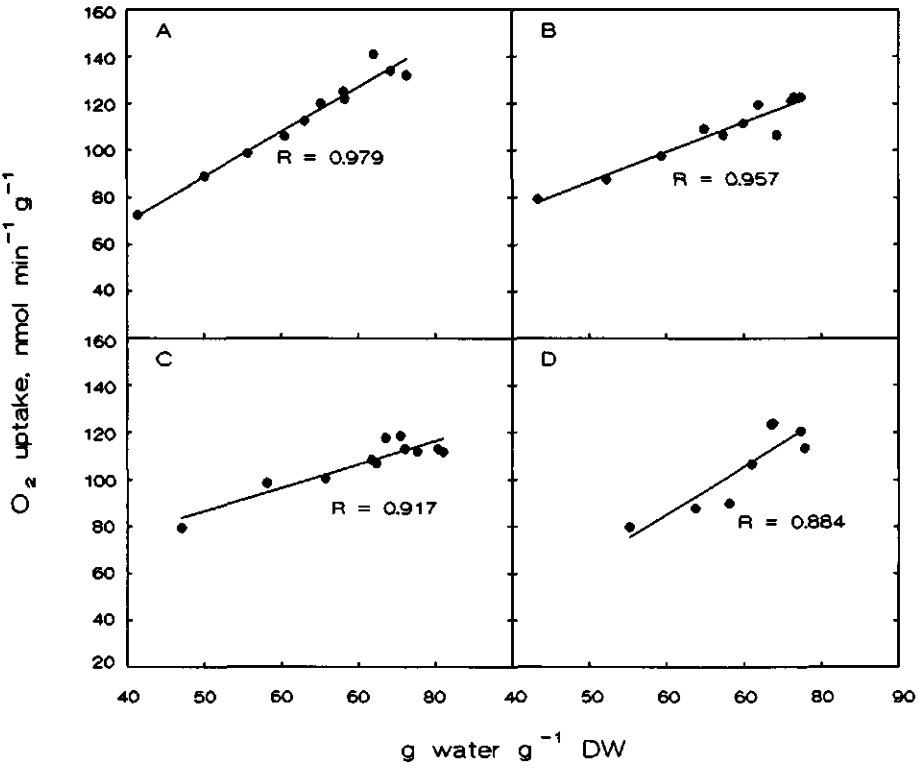

Figure 7.4 Correlation between water content of *S. officinale* seeds during imbibition at 6 (A), 10 (B), 15 (C) and 24 °C (D) in darkness and rate of O_2 uptake measured at 24 °C in 25 mM KNO_3 under dim green light.

24 °C, rose to 100 %. O_2 uptake, however, remained at a low constant level of 40-50 nmol min^{-1} g^{-1} seed. Similar results were obtained when the seeds were transferred to 6 °C (data not shown). When the seeds were not transferred to 2 or 6 °C, but kept at 24 °C, germination capacity remained low and O_2 uptake remained also at a low constant level, being somewhat lower than the O_2 uptake of seeds transferred to 2 °C (Fig. 7.5B). Following 76 days of pre-incubation at 2 °C dormancy was re-induced by transferring the seeds to 24 °C again. Germination capacity dropped to zero within one week but the rate of O_2 uptake was hardly

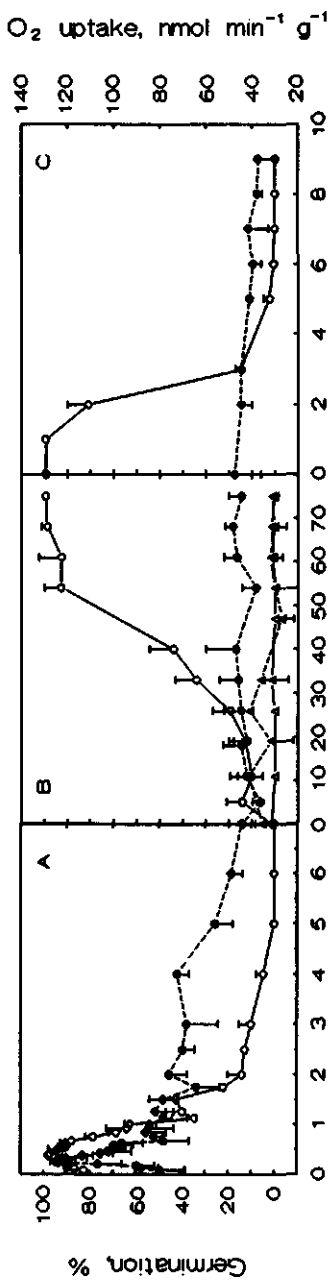

Figure 7.5 Changes in germination capacity (open symbols) and O_2 uptake rate (closed symbols) of *S. officinale* seeds during pre-incubation in 25 mM KNO_3 in darkness. Seeds were pre-incubated subsequently at 24 °C during 7 days (A), followed by 76 days at 2 °C (B) and finally by 9 days at 24 °C (C) (circles). Part of the seeds was not transferred to 2 °C but continuously imbibed at 24 °C (triangles). Germination capacity was tested in 25 mM KNO_3 at 24 °C after 15 min of red light irradiation. O_2 uptake rate was determined in 25 mM KNO_3 at 24 °C under dim green light. Vertical bars represent SD.

influenced (Fig. 7.5C). Thus, both breaking and induction of dormancy in *S. officinale* seeds are not ultimately linked to changes in O_2 uptake.

O_2 uptake measurements at different temperatures

In all previous experiments O_2 uptake was determined at 24 °C. At this temperature O_2 uptake stabilized at about 30-40 nmol min^{-1} g^{-1} seed after prolonged pre-incubation (Figs. 7.1, 7.2). Rates of O_2 uptake were also determined at a range of temperatures between 5 and 30 °C after pre-incubation at 24 °C over variable periods (Table 7.1). Temperature during measurement greatly affected the rate of O_2 uptake, irrespective of the duration of pre-incubation. O_2 uptake rate measured at 5 °C was about 7 times as low as that at 30 °C.

O_2 uptake after red light irradiation

When germination of non-dormant seeds was induced by 15 min red light 105 min after the start of imbibition, O_2 uptake started to rise immediately (Fig. 7.6 and inset in Fig. 7.7 for more detail). This rise clearly preceded the first visible signs of germination, i.e. cracking of the seed coat (indicated with B in Fig. 7.6), that started 14 h after the irradiation. The first radicle protrusion occurred 18 h after the irradiation and germination was complete within 48 h. Radicle protrusion was followed by a renewed and giant burst of respiratory activity (Fig. 7.6).

The relationship between the rise in O_2 uptake after the red irradiation and phytochrome control was further established in an escape experiment. In *S. officinale* seeds the escape from the antagonizing effect of farred irradiation started 1-2 h after the red light irradiation and was complete after 16 h (data not shown, Hilhorst *et al.*, 1986). When the farred irradiation was given 2 h after the red light pulse, the O_2 uptake pattern exactly resembled the dark control (Fig. 7.7). With a lag-time of 16 h between red and farred, the O_2 uptake pattern looked like the red light control (Fig. 7.7). A red light irradiation did not increase O_2 uptake when the seeds were not allowed to germinate as a result of pre-incubation at 24 °C during 8 days (data not shown).

Table 7.1 The effect of temperature on O_2 uptake of *S. officinale* seeds. After pre-incubation at 24 °C for 6, 24 or 336 h O_2 uptake was determined at different temperatures. Pre-incubation was in 25 mM KNO_3 in darkness and O_2 uptake rate was measured under dim green light in 25 mM KNO_3. **A.** Absolute values. **B.** O_2 uptake rates relative to the values measured at 30 °C.

A	O_2 uptake rate, nmol min^{-1} g^{-1} seed		
Temperature during O_2 uptake measurement, °C	Pre-incubation treatment		
	6 h 24 °C	24 h 24 °C	336 h 24 °C
5	21.9 ± 3.4	12.8 ± 0.2	8.2 ± 0.5
10	31.7 ± 3.3	22.0 ± 2.5	13.7 ± 2.8
16	60.5 ± 4.3	39.6 ± 1.6	19.8 ± 2.6
21	81.6 ± 2.5	55.6 ± 2.9	25.4 ± 2.1
25	113.4 ± 5.5	72.3 ± 7.6	33.1 ± 3.7
30	154.9 ± 3.5	104.1 ± 2.7	45.5 ± 5.6
B	Relative O_2 uptake rate		
Temperature during O_2 uptake measurement, °C	Pre-incubation treatment		
	6 h 24 °C	24 h 24 °C	336 h 24 °C
5	0.14	0.12	0.18
10	0.20	0.21	0.30
16	0.39	0.38	0.44
21	0.53	0.53	0.56
25	0.73	0.69	0.73
30	1	1	1

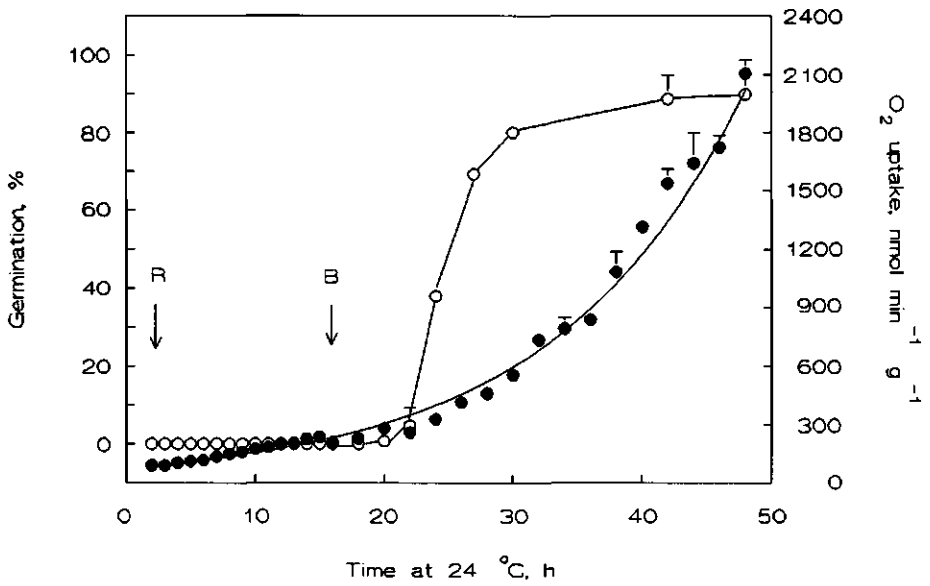

Figure 7.6 O_2 uptake rate of germinating seeds of *S. officinale*. Seeds were imbibed for 105 min in 25 mM KNO_3 at 24 °C in darkness, irradiated with red light during 15 min and thereafter returned to darkness. After several intervals radicle protrusion was scored (○) and O_2 uptake rate (●) was determined at 24 °C in 25 mM KNO_3 under dim green light. R indicates the red light pulse and B cracking of the seed coat.

Discussion

The present results unambiguously demonstrate that seeds of *S. officinale* allow a clear distinction between dormancy breaking and the actual germination process. An increased O_2 uptake is not ultimately required for dormancy breaking, whereas germination is accompanied by a large increase in O_2 uptake.

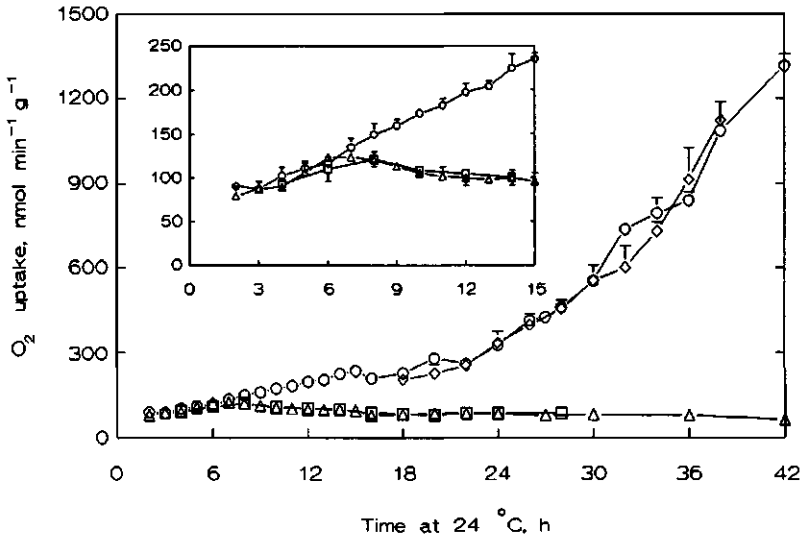

Figure 7.7 Rates of O_2 uptake of *S. officinale* seeds during imbibition at 24 °C. After 105 min of imbibition in 25 mM KNO_3 in darkness at 24 °C part of the seeds was irradiated with red light during 15 min and thereafter returned to continuous darkness (○), or to darkness interrupted with 15 min of farred irradiation after 2 h (□) or 16 h (◇). O_2 uptake of non-irradiated seeds is also included (△). O_2 uptake was determined in 25 mM KNO_3 at 24 °C under dim green light. Inset: Changes in O_2 uptake rate during the first 15 h of imbibition.

O_2 uptake of dark-imbibed seeds

Although the increased O_2 uptake upon dark imbibition nicely coincided with an increase in germination capacity (Figs. 7.1A-D), it can be attributed most probably to water uptake. O_2 uptake rate indeed correlated well with water uptake at four different temperatures (Fig. 7.4). It has been demonstrated for maize that the increased respiratory activity upon imbibition can result from an activation of the enzymes of respiratory pathways that were conserved in the embryo (Ehrenshaft and Brambl, 1990; Leprince *et al.*, 1992). In addition, activation of pre-existing mitochondria or *de novo* synthesis of mitochondrial proteins may

account for the increased respiratory activity (Morohashi, 1986). When seeds were re-imbibed following a dehydration treatment, O_2 uptake was not significantly enhanced (data not shown). This may indicate that repair- and activation processes that occur during the first imbibition period are not required again during re-imbibition. Increased germination capacity after a dehydration treatment (data not shown), as was also found by Karssen (1980/81), Karssen *et al.* (1988) and Bouwmeester (1990), in combination with a constant level of O_2 uptake, again presents evidence that both processes are not causally related.

This conclusion was strengthened by the experiments on breaking of secondary dormancy. Secondary dormancy (that was induced by 7 days pre-incubation at 24 °C) was alleviated by chilling. O_2 uptake, measured at 24 °C, did not increase but remained at a more or less constant level of 40-50 nmol min^{-1} g^{-1} seed (Fig. 7.5B). When the seeds were not transferred to 2 °C but stayed at 24 °C for another 76 days, germination capacity was not restored and O_2 uptake slightly further declined to about 30 nmol min^{-1} g^{-1} seed (triangles in Fig. 7.5B). This value was also found during prolonged imbibition of lettuce seeds at 25 °C (Evenari *et al.*, 1958; Powell *et al.*, 1983).

The reduction in O_2 uptake during pre-incubation at 24 °C started as early as 9 h after the start of imbibition and coincided with a decrease in germination capacity (Fig. 7.1D). At lower imbibition temperatures of 15 (Fig. 7.1C), 10 (Fig. 7.1B) and 6 °C (Fig. 7.1A) the decline in O_2 uptake clearly preceded induction of secondary dormancy (Figs. 7.1, 7.2). When imbibition at 10 °C occurred in a non-saturating KNO_3 concentration of 0.5 mM instead of the 25 mM that was used in all other experiments, the decline in O_2 uptake was accompanied by a decline in germination capacity (Fig. 7.1B). The rate of O_2 uptake was not affected by the concentration of KNO_3. Also in water O_2 uptake rates were similar to those in 25 mM KNO_3 (data not shown). During re-induction of dormancy at 24 °C, following a dormancy-breaking treatment of 76 days 2 °C, O_2 uptake remained at a constant level of 40 nmol min^{-1} g^{-1} seed (Fig. 7.5C). Thus, it can be concluded that, although the first induction of secondary dormancy can be accompanied by a decrease in O_2 uptake rate, the reduction in O_2 uptake rate is not generally required for dormancy induction.

A reduction in O_2 uptake during induction of secondary dormancy was also observed in lettuce seeds (Powell *et al.*, 1983). In a later study it was demonstrated that the lower levels

of O_2 uptake after imbibed storage were the consequence of changes within the embryo and could not be attributed to an increased restriction in O_2 uptake by the structures surrounding the seeds (Powell *et al.*, 1984). Although we did not measure O_2 uptake of isolated embryos, there are no reasons to assume that seed coat permeability in *S. officinale* seeds varies during prolonged imbibition and as a consequence would influence the rate of O_2 uptake.

O_2 uptake of germinating seeds

The decline in O_2 uptake during imbibition at 24 °C did not occur when the seeds received a 15 min red light irradiation 105 min after the start of imbibition. On the contrary, O_2 uptake rose considerably and seeds germinated for 90 % within 2 days (Fig. 7.6). This additional rise in O_2 uptake rate started at least 8 h before the first visible signs of germination, i.e. cracking of the seed coat, which means that O_2 uptake is a very sensitive parameter to determine whether or not radicle protrusion is to be expected (inset in Fig. 7.7). A second respiratory burst followed radicle protrusion (Fig. 7.6). An increase in O_2 uptake coinciding with red light-induced germination has also been reported by Nyman (1961), Woodstock and Toole (1976), Peckett and Al-Charchafchi (1979) and Powell *et al.* (1984).

A farred irradiation completely reversed the red light effect on O_2 uptake when it was given before the induction of germination escaped from the red light-antagonizing action of farred, whereas it could not reverse the red light effect after the escape was complete (Fig. 7.7). The close link between the germination process and O_2 uptake is in strong contrast to the absence of such a link in the control of dormancy. This conclusion does not correspond with findings in several other species (Bogatek and Lewak, 1978; Adkins *et al.*, 1984; Hilton and Owen, 1985; Hilton and Thomas, 1986). We conclude that in these other studies the metabolism associated with dormancy breaking could not be distinguished from that associated with germination. Our results clearly demonstrate that a rise in O_2 uptake before radicle protrusion cannot be used in conclusions about metabolic regulation of dormancy breaking.

Survival of seeds in soil

For survival in soil seeds have to maintain viability. Some of their storage reserves are constantly required to provide energy and substrates for maintenance of cellular integrity and repair and replacement of damaged subcellular components. We have demonstrated that after prolonged imbibition at 24 °C O_2 uptake rate at the same temperature was at a steady level of 30 nmol min^{-1} g^{-1} seed (Fig. 7.5). This rate can be used to calculate rates of depletion of substrates from the seeds with the objective to predict longevity. Provided that lipids are the main substrate, it can be calculated that about 0.9 mg lipids g^{-1} seed are depleted per day. Although we do not have any clue how much substrate can be used without exhausting the seed, it is clear that this rate of lipid depletion would not allow long survival. However, in soil much reduced levels of O_2 uptake can be expected than occurred at the artificial conditions of high temperature (24 °C) and moisture used in this study. Soil temperatures in temperate regions are generally much lower than 24 °C and temperature has been shown to affect O_2 uptake considerably (Table 7.1), as was also found by Ibrahim *et al.* (1983) and Laan *et al.* (1991). At 10 °C, for example, O_2 uptake rate was more than three times reduced in comparison with 25 °C (Table 7.1). Seeds in natural environments experience intermittent periods of hydration and drying. Under dry conditions O_2 uptake rates are dramatically reduced. Vertucci and Leopold (1984) could not detect O_2 consumption in soybean seeds below moisture contents of 8 % and a restricted metabolism was found between 8 and 25 % moisture. Because drying can mainly be expected in periods with high temperatures, much reduced levels of metabolism actually occur than maximally possible at these temperatures. This will lengthen the time over which storage reserves can be retained.

On purpose, we consistently used the term O_2 uptake in stead of respiration. It can be questioned whether O_2 uptake rates determined with oxygen electrodes accurately estimate respiration of seeds, rather than giving an overestimation. The determination of O_2 uptake rates by the use of oxygen electrodes has the implication that the seeds are immersed and constantly stirred. This situation is completely different from the seed's environment in soil. Therefore, we also determined rates of O_2 uptake with a gas chromatograph (GC), which has

the advantage that the seeds are fully imbibed without being immersed (Chapter 8). Fortunately, both methods gave comparable results, which means that the immersion and stirring of seeds during measurement with oxygen electrodes do not cause an extra increase in O_2 uptake rate. Moreover, GC measurements allow simultaneous determination of rates of O_2 uptake and CO_2 release, such that we can get insight in the type of substrate that is used. The measured rates of O_2 uptake may not only be attributable to respiration but also to other oxygen-consuming processes like for instance peroxidation or phenol oxidation. Calculation of respiratory quotients (CO_2 release/O_2 uptake) and the use of inhibitors of the cytochrome- and alternative pathway of respiration can give information on the possible involvement of other oxidation reactions, that also require substrates. These experiments are described in Chapter 8. In addition, we determined the contribution of different respiratory pathways in non-germinating seeds of *S. officinale* and investigated whether shifts occurred during changes in dormancy.

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Chapter 8

Respiration in seeds of *Sisymbrium officinale*. II. Changes in the contribution of different respiratory pathways during prolonged incubation in darkness

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Summary

The different pathways involved in respiratory activity in seeds of *Sisymbrium officinale* were followed during prolonged incubation at 24 °C in darkness. It was shown in Chapter 7 that during that incubation dormancy is broken and subsequently re-induced. Rates of O_2 uptake and CO_2 release rose during the first 8 h of imbibition and declined thereafter. After 3 weeks O_2 uptake at 24 °C was about 25-30 nmol min^{-1} g^{-1} seed and CO_2 release about 17 nmol min^{-1} g^{-1} seed. During prolonged imbibition up to 3 months, rates of O_2 uptake and CO_2 release remained at these constant levels. Respiratory quotients varied between 0.55 and 0.7. Rates of O_2 uptake measured with oxygen electrodes were similar to rates determined gas chromatographically, which means that immersion and stirring of seeds in oxygen electrodes does not give an overestimation of respiration rates. Effects of KCN and benzohydroxamic acid (BHAM) on germination and O_2 uptake rate were studied. It was remarkable that KCN concentrations that almost completely inhibited flow through the cytochrome pathway, did not dramatically reduce germination. BHAM already inhibited germination at concentrations that do not inhibit O_2 uptake. KCN at 1.5 mM fully inhibited the cytochrome pathway and BHAM at 30 mM the alternative pathway. These concentrations did not exert side-effects. The contribution of the respiratory pathways changed during imbibition at 24 °C. During the first 48 h of imbibition at 24 °C 70 % of O_2 uptake occurred via the cytochrome pathway, 10 % via the alternative pathway and 20 % was residual O_2 uptake, insensitive to both KCN and BHAM. Flow through the alternative path disappeared after 240 h. A role of the alternative pathway in the regulation of dormancy is

not very likely. The flow through the cytochrome path declined concomitantly with total O_2 uptake and residual O_2 uptake remained constant. Therefore, the contribution of the latter component became relatively more important with prolonged imbibition. After 2 weeks of imbibition at 24 °C 50 % of the electron flow occurred via the cytochrome pathway and 50 % was residual O_2 uptake. Irrespective of changes in the contribution of different respiratory pathways, respiratory quotients of CO_2 release and O_2 uptake did not change.

Introduction

In studies with seeds of *Sisymbrium officinale* breaking of dormancy can be clearly distinguished from the actual germination process because germination is absolutely dependent on the simultaneous presence of light and nitrate (Hilhorst, 1990; Derkx and Karssen, 1993; Chapter 6). When seeds are incubated in darkness, germination is prevented, but temperature can influence the degree of dormancy. The varying capacity to germinate can be determined subsequently in tests with addition of light and nitrate. It was demonstrated in Chapter 7 that although the rate of O_2 uptake increases dramatically during germination, such an increase is not required during breaking of dormancy.

During prolonged dark incubation at 24 °C O_2 uptake rate, also measured at 24 °C, stabilized at a level of 30 nmol min^{-1} g^{-1} seed. It was discussed in the previous chapter that this rate would not allow survival in soil for very long periods. Obviously, temperatures in soil are generally much lower than 24 °C, which considerably reduces O_2 uptake. Moreover, seeds in soil are not constantly fully imbibed, especially not in periods with high temperatures. O_2 uptake measurements with oxygen electrodes as used in the previous chapter involve full immersion of seeds that are constantly stirred in the electrode chamber. To check whether this artificial condition, that deviates considerably from a seed's natural environment, does not overestimate rates of O_2 uptake, the present study will compare measurements by oxygen electrodes and a gas chromatograph (GC), respectively. GC measurements do not require full immersion. Also rates of CO_2 release will be determined simultaneously.

The present study also aims at the characterization of the oxidative processes during

incubation at 24 °C. The existence of two pathways of mitochondrial electron transport has been demonstrated in seeds: the cyanide-sensitive but hydroxamic acid-insensitive cytochrome pathway and the cyanide-insensitive but hydroxamic acid-sensitive alternative pathway. Changes in the contribution of both pathways during imbibition have been reported for seeds of several species like soybean (Yentur and Leopold, 1976; Siedow and Girvin, 1980), mung bean (Siedow and Girvin, 1980), chick pea (Burguillo and Nicolás, 1977), tomato (Gui *et al.*, 1991) and maize (Leprince *et al.*, 1992). It has been proposed that the alternative pathway plays a regulatory role in germination (Yentur and Leopold, 1976) and breaking of dormancy (Esashi *et al.*, 1979, 1981a, b, 1982). Moreover, Esashi *et al.* (1979) argued that secondary dormancy arose from inactivation of the alternative pathway. However, several other authors suggested the presence of the alternative pathway, but not as a requirement for germination (Morohashi, 1986; Upadhyaya, 1986; Leprince *et al.*, 1992) or breaking of dormancy (Adkins *et al.*, 1984; Bogatek and Rychter, 1984). The activities of both pathways can be determined by specific inhibitors. The activity of the alternative pathway is determined as the O_2 uptake sensitive to substituted hydroxamic acids like salicylhydroxamic acid (SHAM) and benzohydroxamic acid (BHAM). The activity of the cytochrome pathway is determined by first blocking the alternative pathway by hydroxamates and then adding inhibitors of the cytochrome pathway, like KCN or azide. KCN is preferentially used since azide does not inhibit hydroxamate-dependent peroxidase activity. (Møller *et al.*, 1988). Part of the O_2 uptake may be insensitive to both KCN and hydroxamates. This residual respiration is only found in intact tissues and not in isolated mitochondria.

Except for the studies by Leprince *et al.* (1992) arbitrary concentrations of inhibitors of both the cytochrome- and alternative pathway have been used in all previous studies. However, side-effects of inhibitors of both pathways do exist (Van der Plas and Wagner, 1980; Lambers *et al.*, 1983; Van der Werf *et al.*, 1991). Hydroxamates at high concentrations may exert side-effects on the cytochrome pathway, whereas low concentrations may enhance peroxidase activity. These side-effects may easily cause errors in the interpretation of the effects of specific inhibitors. Therefore titration experiments will be performed as described by Møller *et al.* (1988). These experiments are required to assess concentrations of KCN and BHAM that fully inhibit the cytochrome- and alternative pathway

without exerting side-effects. After determining these concentrations, the respiratory shifts occurring during changes in dormancy are investigated.

Materials and methods

Seeds

In this study two different seed lots of *Sisymbrium officinale* (L.) Scop. were used. One seed lot was collected in autumn, 1987 from wild plants growing in the laboratory garden (experiment Fig. 8.1A) and the other seed lot was harvested in September, 1990 from plants growing at a roadside in Wageningen (other experiments). After harvest the seeds were cleaned and stored dry in plastic containers at 2 °C in darkness until use in experiments in 1990-1991. Under these conditions no changes in dormancy were observed in the dry seeds during the experimental period.

Pre-incubation and germination conditions

Portions of 50 or 200 mg seeds were sown in 5 cm Petri dishes on a single layer of filter paper (Schleicher & Schüll, no. 595, Dassel, Germany) and wetted with 1.5 ml 25 mM KNO_3 (Suprapur, Merck, Darmstadt, Germany). After various pre-incubation periods in darkness at 24 °C, rates of O_2 uptake and CO_2 release were determined.

Effects on germination of the respiratory inhibitors KCN (Merck, Darmstadt, Germany) and BHAM (Aldrich, Steinheim, Germany) were investigated on non-pretreated seeds. Germination was tested at 24 °C. Triplicates of 50 seeds were sown in 5 cm Petri dishes, to which 1.5 ml of the test solution was added. Both inhibitors were tested in the absence and in the presence of 25 mM KNO_3. KCN was buffered at pH 7 with a 0.01 M phosphate buffer and BHAM was not buffered. After 2 h of imbibition in darkness the seeds were irradiated with red light (Chapter 7) during 15 min and then returned to 24 °C in darkness. Petri dishes to which KCN was added were sealed for 24 h, whereas Petri dishes to which BHAM was added were not sealed. Sealing caused a small reduction in germination, probably due to lack

of oxygen. After 24 h of imbibition KCN and BHAM were replaced by water or 25 mM KNO_3. At this time radicle protrusion was already visible. For the remainder of the germination test at 24 °C Petri dishes were not sealed. Germination was counted after three days. Radicle protrusion was taken as the criterion for germination.

Pre-incubation and germination tests occurred in incubators as described in Chapter 7.

All manipulations of incubated seeds were conducted in dim green light (Chapter 7).

O_2 uptake and CO_2 release measurements

O_2 uptake was measured either with oxygen electrodes or with a gas chromatograph. Whenever pre-incubation exceeded more than one week, the seeds were treated, at the start of pre-incubation, with 5% hypochlorite during one min and five times thoroughly rinsed with distilled water. When the pre-incubation period exceeded more than four weeks the hypochlorite treatment was repeated before measuring O_2 uptake and CO_2 release. The disinfection with hypochlorite did not affect O_2 uptake or CO_2 release. Any contaminated or abnormal seeds were discarded.

oxygen electrodes

After several intervals of pre-incubation in darkness at different constant temperatures, O_2 uptake was determined using a Clark-type oxygen electrode, as described in Chapter 7. O_2 uptake was determined at 24 $\pm$ 1 °C in 25 mM KNO_3. All samples were run in at least three or four replicates. The O_2 uptake measurements were performed under dim green light.

When the different respiratory pathways were investigated, small volumes of stock solutions of KCN buffered at pH 7 with a 0.01 M phosphate buffer or BHAM dissolved in water, were injected in the electrode chamber after measuring the O_2 uptake in the absence of inhibitors.

The analysis of the distribution of electron flow along the respiratory pathways is based on the following equation (Theologis and Laties, 1978):

$$v_{tot} = \rho_{alt} V_{alt} + v_{cyt} + v_{res} \quad (1)$$

where

v_{tot} is the total rate of O_2 uptake in the absence of inhibitors

V_{alt} is the capacity of the alternative pathway

ρ_{alt} is the fraction (0-1) of the capacity of the alternative pathway that is engaged under the given conditions

$\rho_{alt} V_{alt}$ is the activity of the alternative pathway in the absence of inhibitors ($= v_{alt}$)

v_{cyt} is the activity of the cytochrome pathway in the absence of inhibitors of the cytochrome pathway ($= \rho_{cyt} V_{cyt}$, analogous to $\rho_{alt} V_{alt}$)

v_{res} is the residual O_2 uptake in the presence of inhibitors of both the alternative pathway and the cytochrome pathway

Titration experiments as described by Møller *et al.* (1988) were performed to determine concentrations of KCN and BHAM that fully inhibit the pathway concerned without exerting side-effects.

gas chromatograph

After pre-incubation at 24 °C in darkness over variable periods portions of 200 mg seeds were thoroughly rinsed with water on a Büchner funnel and then transferred to a fresh moist 5 cm filter paper. The filter paper was transferred to a 5 ml S/Cap Micro vial that was air-tight closed (Phase Separations B.V., Waddinxveen, The Netherlands). Gases were allowed to accumulate during 2 h in darkness at 24 °C and thereafter a sample of 100 μl was injected into a F & M Dual model 700-00 gas chromatograph at 105 °C, using a katharometer detector at 135 °C, as described by Hoekstra and Bruinsma (1975). One stainless steel column, 120 cm, 0.125 cm in diameter, was packed with silicagel 30-60 mesh, the other, 200 cm, 0.25 cm in diameter, with Becker Molecular Sieve 5A 45-60 mesh. The molecular sieve column was connected to the silicagel column with the detector in between. This enabled the measurement of CO_2, O_2 and N_2 with one injection. Retention times for CO_2 and

O_2 were 90 and 210 s, respectively. The sensitivity of the GC detection was enhanced by an additional amplifier, connected to an electronic integrator (Spectra Physics, SP 4100). Accuracy of the determination was enhanced by the fact that the sum of the injected gases CO_2, O_2, Argon and N_2, equalled 100 %. This allowed for a lesser precision of injected sample size. All treatments were in six replicates.

All experiments were repeated at least once with similar results.

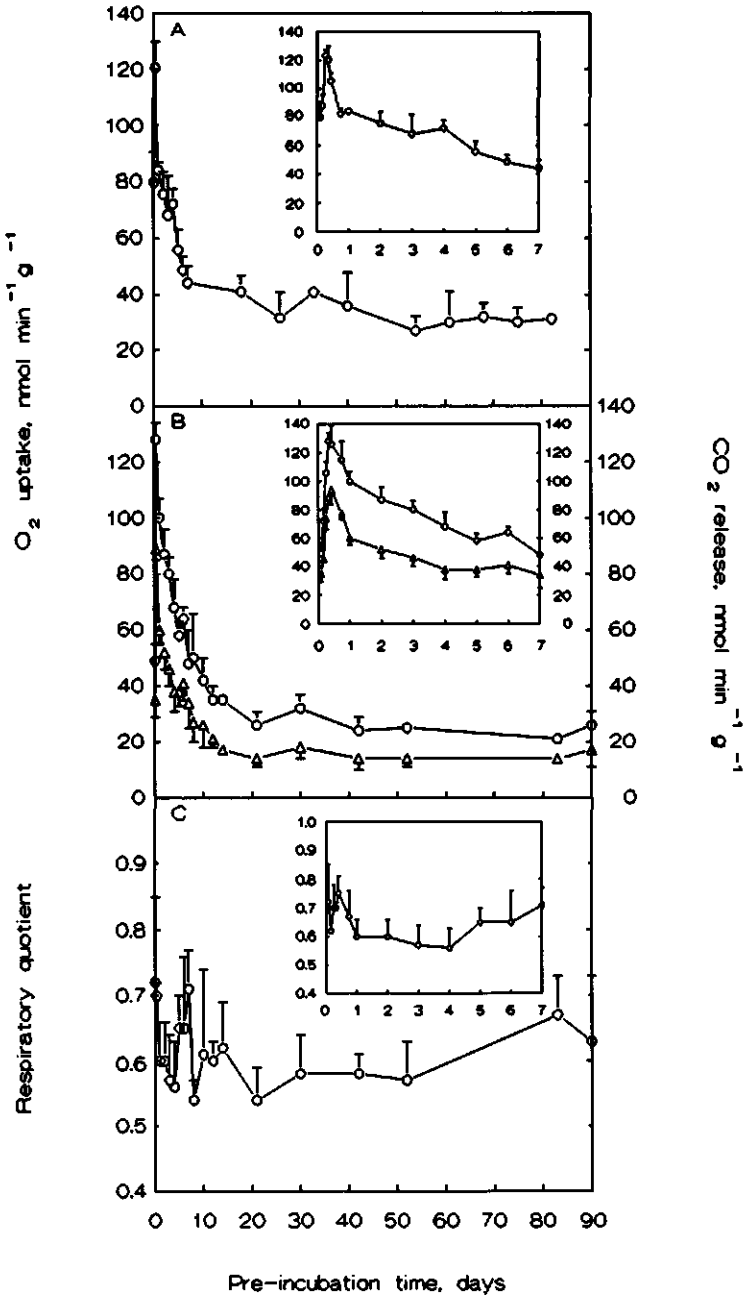
Results

O_2 uptake determined gas chromatographically and with oxygen electrodes

The O_2 uptake measured by GC followed during pre-incubation at 24 °C the same pattern as the data obtained with oxygen electrodes (Figs. 8.1A, B). An increase in O_2 uptake rate occurred during the first 8 h of imbibition, followed by a decline (inset in Figs. 8.1A, B). The pattern of CO_2 release was similar to that of O_2 uptake. CO_2 release was, however, considerably lower than O_2 uptake, resulting in RQ values between 0.55 and 0.7 (Fig. 8.1C). The rates of O_2 uptake and CO_2 release after 3 weeks of pre-incubation at 24 °C did not further decline when pre-incubation time was prolonged to 3 months. The rate of O_2 uptake remained at a stable level of about 25 nmol min^{-1} g^{-1} seed and CO_2 release at 17 nmol min^{-1} g^{-1} seed.

Effects of respiration inhibitors on germination

Red light-induced germination of *S. officinale* seeds was inhibited by BHAM (Fig. 8.2A), as well as by KCN (Fig. 8.2B). The difference in germination in the absence of inhibitor in Figs. 8.2A and 8.2B is most probably the result of sealing the Petri dishes during the first 24 h of the germination test in Fig. 8.2B, whereas those in Fig. 8.2A were not sealed. At BHAM concentrations of 1 mM seedlings looked abnormal, an increase of the concentration to 2.5 mM prevented germination in the presence of KNO_3 and strongly


A
B
C
O_2 uptake, nmol min^{-1} g^{-1}
CO_2 release, nmol min^{-1} g^{-1}
Respiratory quotient
Pre-incubation time, days

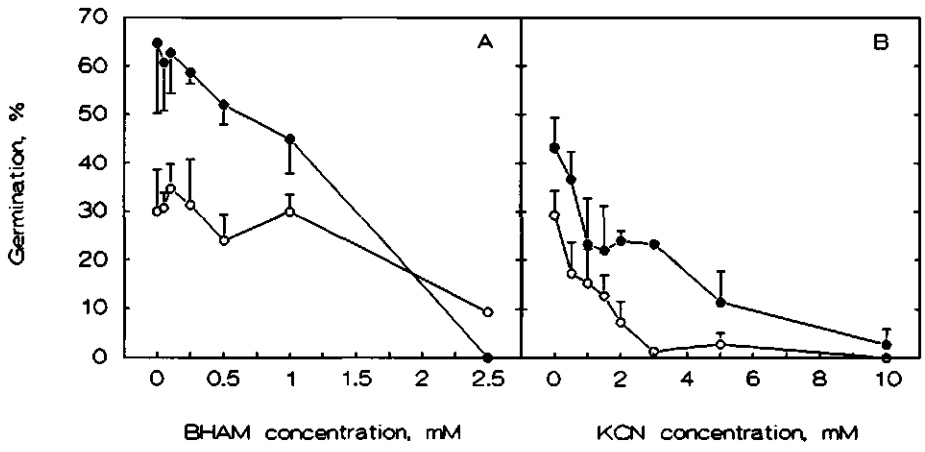


Figure 8.2 The effect of BHAM (A) and KCN (B) on the red light-induced germination of *S. officinale* seeds, in the absence (○) or presence (●) of 25 mM KNO_3. After 2 h of imbibition at 24 °C in darkness the seeds were irradiated during 15 min with red light and thereafter returned to darkness. When KCN was present, Petri dishes were sealed. After 24 h BHAM and KCN were replaced by water (○) or 25 mM KNO_3 (●). Vertical bars represent SD.

Figure 8.1 Changes in rates of O_2 uptake (○) (A, B), CO_2 release (▵) (B) and calculated respiratory quotients (C) (CO_2 release/O_2 uptake) of *S. officinale* seeds during pre-incubation at 24 °C in 25 mM KNO_3 in darkness. **A.** Rates of O_2 uptake determined by oxygen electrodes in 25 mM KNO_3 under dim green light. **B.** Rates of O_2 uptake and CO_2 release determined by gas chromatography. Seeds with filter papers were transferred to airtight glass vials 2 h before the end of pre-incubation and gases were allowed to accumulate at 24 °C in darkness. Gas samples of 100 µl were injected. **C.** Respiratory quotients calculated from B. Vertical bars represent SD. Inset: Changes in rates of O_2 uptake (A, B), CO_2 release (B) and RQ (C) during the first 7 days of pre-incubation at 24 °C.

inhibited germination in the absence of KNO_3. KCN concentrations of 1 mM reduced germination by about 50 % and 10 mM was fully inhibitory.

Titration experiments

Before determining the contribution of different respiratory pathways and possible shifts therein during changes in dormancy, titration experiments were performed to establish concentrations of KCN and BHAM that fully inhibited the pathway concerned without having side-effects. Titration experiments were performed with seeds that had been pre-incubated at 24 °C during 24 h.

The effect of a range of concentrations of BHAM on the O_2 uptake in the absence (circles) or presence (triangles) of 1.5 mM KCN is shown in Fig. 8.3A. It can be seen in Fig. 8.4A that this KCN concentration was sufficient to block the cytochrome pathway completely. Concentrations of BHAM below 1 mM stimulated O_2 uptake somewhat, both in the presence and absence of KCN. Increasing the BHAM concentration slightly inhibited O_2 uptake. Above a BHAM concentration of 20 mM no further inhibition was observed in the presence of KCN, whereas in the absence of KCN 30 mM BHAM was required to give maximal inhibition. The inhibition by BHAM in the absence of KCN indicates that the alternative pathway contributed for about 10 % in total O_2 uptake. The electrons predominantly flowed through the cytochrome pathway, contributing 70 % of total O_2 uptake (KCN sensitive). The engagement of the alternative pathway was determined by plotting the O_2 uptake rates in the presence of various concentrations of BHAM against the O_2 uptake rates in the presence of KCN and the same BHAM concentrations, after subtraction of residual O_2 uptake (v_{res}) from each value, a so-called ρ_{alt}-plot, according to Møller *et al.* (1988). A straight line was obtained with a slope of 1.35 (Fig. 8.3B). This appears to indicate that the activity of the alternative path (v_{alt}) exceeded its maximal capacity (V_{alt}), which is impossible ($\rho_{alt}=1$, full engagement of the alternative path). This discrepancy may be due to the small contribution of the alternative pathway in total O_2 uptake, being less than 10 %. As a consequence this excess amounts to only 3 % of the total respiration, well within the uncertainty limits of the O_2 uptake measurements (Fig. 8.3A). Residual O_2 uptake,

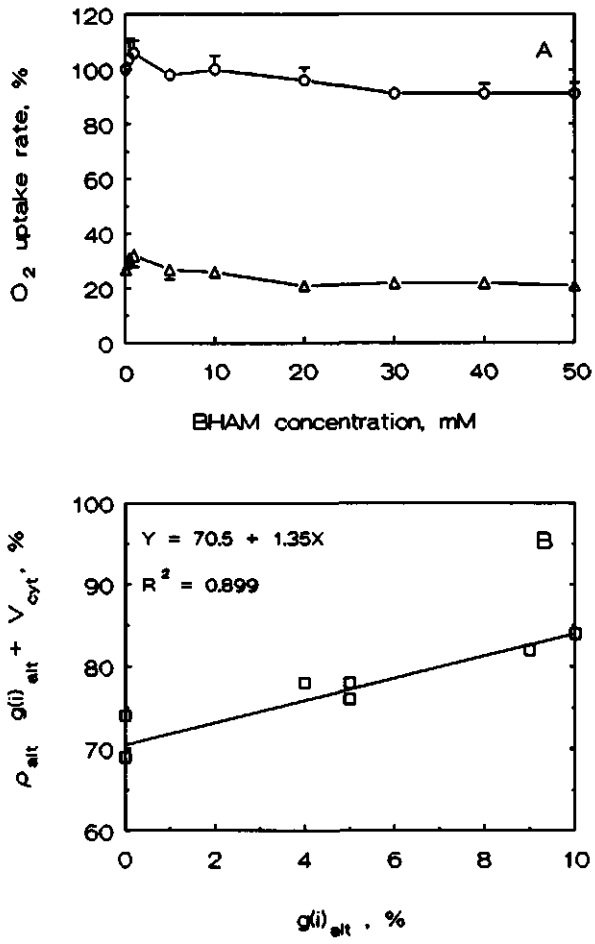


Figure 8.3 A. The effect of BHAM on the rate of O_2 uptake of *S. officinale* seeds in the absence (○) and presence (△) of 1.5 mM KCN. Seeds were pre-incubated during 24 h in 25 mM KNO_3 at 24 °C in darkness. Thereafter, O_2 uptake was measured in 25 mM KNO_3 at 24 °C under dim green light. 100 % O_2 uptake = 75 nmol min^{-1} g^{-1} seed. Vertical bars represent SD. **B.** The determination of the engagement of the alternative pathway using a ρ-plot. After correction for residual O_2 uptake, O_2 uptake rates in the absence of KCN (ρ_{alt}*$g(i)_{alt}$ + V_{cyt}) were plotted against O_2 uptake rates in the presence of KCN ($g(i)_{alt}$). Data are derived from A.

insensitive to both KCN and BHAM, accounted for about 20 % of total O_2 uptake (Fig. 8.3A).

Figure 8.4A shows the effect of a range of KCN concentrations on the O_2 uptake in the absence (circles) or presence (triangles) of 30 mM BHAM. At KCN concentrations above 1 μM the O_2 uptake rate started to decline (Fig. 8.4A, inset) and concentrations above 1 mM KCN gave no further inhibition, both in the absence and presence of 30 mM BHAM (Fig. 8.4A). A straight line with a slope of 1.0 was obtained, when after subtraction of v_{res} from each value, the O_2 uptake rates in the presence of various concentrations of KCN were plotted against O_2 uptake rates in the presence of both inhibitors (Fig. 8.4B). This means that side-effects do not occur when the BHAM concentration is 30 mM and the KCN concentration for instance 1.5 mM. These concentrations were chosen for further experiments.

Assessment of respiratory pathways during incubation at 24 °C

The contributions of cytochrome pathway, alternative pathway and residual respiration were evaluated during dark incubation at 24 °C in non-germinating seeds. After several intervals O_2 uptake was measured, first in the absence of inhibitors, then after adding BHAM and finally after adding KCN. To determine the capacity of the alternative path, the inhibitors were also applied in reverse sequence.

Figure 8.5A shows that during pre-incubation at 24 °C the activity of the cytochrome pathway (v_{cyt}) declined concomitantly with a decrease of total O_2 uptake. The contribution of the alternative pathway was small. At the start of imbibition the capacity of the alternative pathway of about 10 nmol min^{-1} g^{-1} seed was fully engaged. After 144 h of pre-incubation the activity of the alternative pathway had fully disappeared whereas the capacity was still about 2-3 nmol min^{-1} g^{-1} seed. Residual O_2 uptake was about 20 nmol min^{-1} g^{-1} seed and did not change significantly during 14 days of incubation at 24 °C. The decline in O_2 uptake via the cytochrome pathway and the constancy of residual O_2 uptake imply that the proportions of both components which were about 70 and 20 %, respectively, at the start of imbibition, change to about 50 % each after 336 h of pre-incubation at 24 °C (Fig. 8.5B).

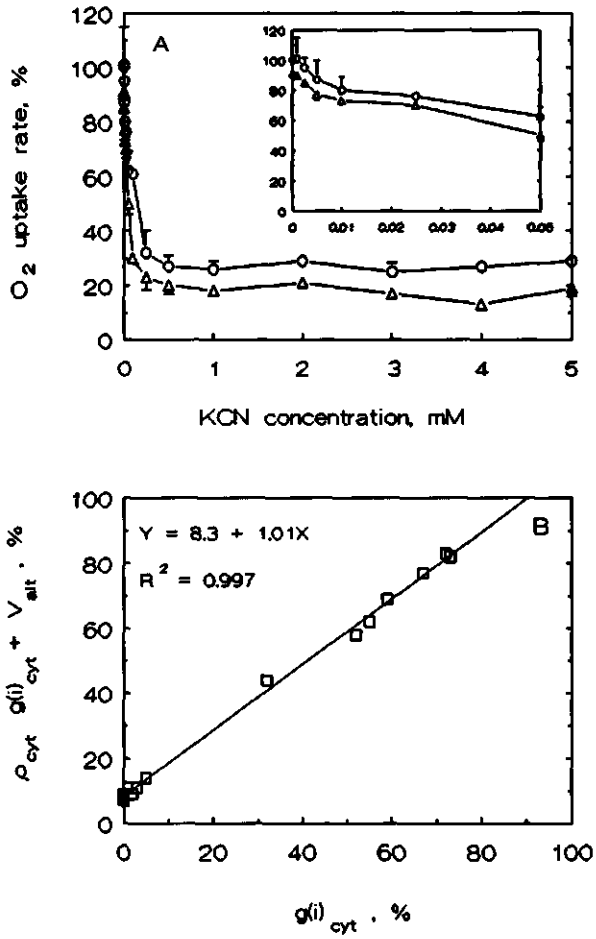


Figure 8.4 A. The effect of KCN on the rate of O_2 uptake of *S. officinale* seeds in the absence (○) and presence (△) of 30 mM BHAM. Pre-incubation and O_2 uptake measurements as in Fig. 8.3. 100 % O_2 uptake = 75 nmol min^{-1} g^{-1} seed. Inset: Effect of KCN over the low concentration range, between 0 and 0.05 mM. Vertical bars represent SD. **B.** The determination of ρ_{cyt}. After correction for residual O_2 uptake, O_2 uptake rates in the absence of BHAM (ρ_{cyt}*g(i)$_{cyt}$ + V_{alt}) were plotted against O_2 uptake rates in the presence of BHAM (g(i)$_{cyt}$). Data are derived from A.

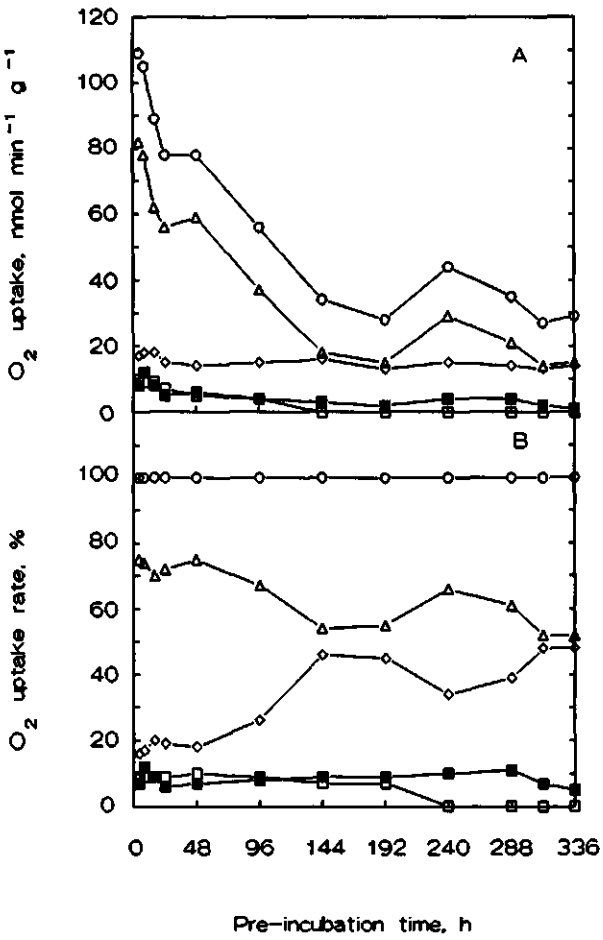


Figure 8.5 Changes in total O_2 uptake (○), the activity of the cytochrome pathway (▵), the activity of the alternative pathway (□), the capacity of the alternative pathway (■) and residual O_2 uptake (◇) during pre-incubation at 24 °C in darkness in 25 mM KNO_3. O_2 uptake was measured in 25 mM KNO_3 at 24 °C under dim green light, first in the absence of inhibitors, then after adding BHAM and finally after adding KCN. To determine the capacity of the alternative path, the inhibitors were also applied in reverse sequence. **A.** Absolute values. **B.** Relative contribution of different components of O_2 uptake.

Discussion

In the present study we examined several aspects of respiratory activity of non-germinating seeds of *S. officinale*. In a previous study (Chapter 7) it was demonstrated that after an initial rise at the start of imbibition, the rate of O_2 uptake dropped to a constant level of about 30 nmol min^{-1} g^{-1} seed at 24 °C. This level was not affected by dormancy-breaking and -inducing treatments. Only when irradiation of non-dormant seeds caused the actual start of germination, a giant increase in O_2 uptake rate was observed. It is not known whether the measured values of O_2 uptake are only due to respiration or also to other O_2 consuming processes like for instance peroxidation or phenol oxidation. Therefore, CO_2 production was measured together with O_2 uptake by GC. Fortunately, GC measurements closely resembled the data obtained with oxygen electrodes (Figs. 8.1A, B), in spite of the considerable differences between the methods. It is concluded that the full immersion of seeds during measurement with oxygen electrodes in combination with constantly stirring does not cause an extra increase in O_2 uptake rate. During imbibition at 24 °C rates of CO_2 production and O_2 uptake changed in a parallel way. In the previous study (Chapter 7) it was already demonstrated that changes in germination capacity during incubation at 24 °C followed the same pattern. RQ values were generally between 0.55 and 0.7 (Fig. 8.1C). A RQ value of 0.7 can be expected in the lipid-containing seeds of *S. officinale*. Values below 0.7 may point to other oxidation reactions in which less CO_2 is evoked, like oxidation of phenols originating in the seed coat.

The use of inhibitors of the respiratory pathways indicates that it is not likely that KCN-sensitive peroxidases contributed significantly to O_2 uptake. The plot of the O_2 uptake rates in the presence of KCN but absence of BHAM against the O_2 uptake rates in the presence of both inhibitors, shows a straight line with slope 1.0 (Fig. 8.4B), in correspondence with the theory of Bahr and Bonner (1973) that the cytochrome pathway always appears to work at the highest rate possible under the given experimental conditions. The slight increase in O_2 uptake rate by low BHAM concentrations (Fig. 8.3A) is most probably incidental. Because it was observed both in the absence and in the presence of KCN, it cannot be attributed to KCN-sensitive hydroxamate-activated peroxidases, which are reported to give

a significant contribution in O_2 uptake in roots of *Zea mays* (Spreen Brouwer *et al.*, 1986) and *Brachypodium pinnatum* (Van der Werf *et al.*, 1991). A slope of 1.0 of the ρ_{cyt} plot as shown in Fig. 8.4B also means that high concentrations of BHAM do not exert side-effects on the cytochrome pathway as reported by Van der Plas and Wagner (1980) for callus-forming potato tuber discs, by Bingham and Farrar (1987) for *Hordeum distichum* roots and by Lambers *et al.* (1983) for wheat- and maize roots.

From the titration experiments as shown in Figs. 8.3 and 8.4 concentrations of KCN and BHAM were chosen that fully inhibit the cytochrome- and alternative pathway, respectively, without having side-effects: 1.5 mM KCN (Fig. 8.4) and 30 mM BHAM (Fig. 8.3).

By using these concentrations it could be detected that during the first 48 h of imbibition at 24 °C 70 % of O_2 uptake was via the cytochrome pathway, 10 % via the alternative pathway and 20 % was residual O_2 uptake, insensitive to both KCN and BHAM. The alternative pathway was fully engaged (Fig. 8.5B). After 240 h of imbibition, flow through the alternative pathway had disappeared whereas its capacity was still 10 % (Fig. 8.5B). It is not likely that the alternative pathway plays a role in dormancy regulation of *S. officinale* seeds in contrast to a suggestion for cocklebur seeds (Esashi *et al.*, 1979, 1981a, b, 1982). The appearance of the alternative pathway in *S. officinale* seeds after the start of imbibition may be attributed to an excess of NADH that has to be oxidized, thus acting as an energy overflow (Lambers, 1980). Because the cytochrome pathway appears to work at the highest rate possible (Bahr and Bonner, 1973), the alternative pathway is only engaged when the cytochrome path is saturated. As the total flow of electrons declined with increasing imbibition time at 24 °C, the flow through the cytochrome pathway concomitantly declined (Fig. 8.5A). The excess of NADH that has to be oxidized is reduced and as a consequence flow through the alternative pathway is not required anymore.

It is striking that v_{res} did not decline with prolonged imbibition time but remained at a constant level of 20 nmol min^{-1} g^{-1} seed. Thus, its contribution in total O_2 uptake became relatively more important with prolonged imbibition (Fig. 8.5B). After 2 weeks of pre-incubation at 24 °C 50 % of total O_2 uptake could be attributed to the cytochrome pathway and 50 % to residual O_2 uptake. Therefore, it is evident that both components are regulated differently. Although the biochemical nature of the residual component is not known, it is

probably due to an extra-mitochondrial oxygen-consuming process like the α-oxidation of fatty acids in peroxisomes (Møller *et al.*, 1988), whereas the cytochrome pathway is located in the mitochondria. Although the contribution of the residual component of O_2 uptake increased with imbibition time at 24 °C, RQ values (Fig. 8.1C) were not affected, indicating that the state of oxidation of substrate oxidized via residual respiration, does not differ from that of the mitochondrial pathways.

Stimulatory effects on germination of KCN as reported for several other species (Hendricks and Taylorson, 1972; Esashi *et al.*, 1979; Zagórski and Lewak, 1983; Cohn and Hughes, 1986; Tilsner and Upadhyaya, 1987) and of hydroxamic acids (Brooks and Mitchell, 1988) have not been found in *S. officinale* seeds. It is striking that KCN at 0.25 mM reduced germination only slightly (Fig. 8.2B), while O_2 uptake via the cytochrome pathway was almost fully suppressed (Fig. 8.4A). Although the alternative pathway yields at the most only one third of the ATP produced via the cytochrome path, it can be concluded that the alternative path evidently provides sufficient energy for the start of the germination process (first 24 h of imbibition). The residual component of respiration generally is believed not to be coupled to ATP production. BHAM at 1-2.5 mM is a potent inhibitor of germination (Fig. 8.2A), whereas O_2 uptake is not inhibited (Fig. 8.3A). The BHAM inhibition of germination may be partly attributed to some action other than respiratory inhibition, perhaps involving chelation of transition metals (Schonbaum *et al.*, 1971).

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Chapter 9

General discussion

In the general discussion the results obtained in the previous chapters will be amalgamated into a coherent view on the regulation of dormancy and germination. The discussion will mainly concentrate on the physiological mechanisms of seasonal dormancy patterns. Although a period of developmental arrest, often coupled with deep dormancy, is common for seeds of many species, buds of many woody and herbaceous species, buds of underground organs, like tubers, rhizomes and bulbs and turions of water plants, cyclic changes of induction and release of dormancy are unique for seed dormancy. They can be repeated for numbers of years when seeds are buried in soil, deprived from light and other germination-stimulating factors. Because seeds in soil may survive for periods as long as decades to centuries (Priestley, 1986), it has to be supposed that seasonal dormancy cycles will be repeated over the whole length of such a period.

The ecological significance of reversible dormancy patterns is studied in detail (Karssen, 1982; Bouwmeester, 1990), but little is known about the physiological backgrounds. In this thesis the regulation of germination and reversible changes in dormancy was studied in the two related species *Arabidopsis thaliana* (L.) Heynh. and *Sisymbrium officinale* (L.) Scop. Figure 9.1 presents a scheme about the regulation of dormancy and germination. It will be explained in the following paragraphs. The different reactions are indicated with numbers. The text will refer to these reactions with the number placed between brackets.

Regulation of germination and dormancy

Requirement for stimulatory factors like light, nitrate and GAs

Germination of the two species under study is dependent on active phytochrome, that behaves as a dimer (Brockmann *et al.*, 1987) (Fig. 9.1). Upon irradiation with red light the

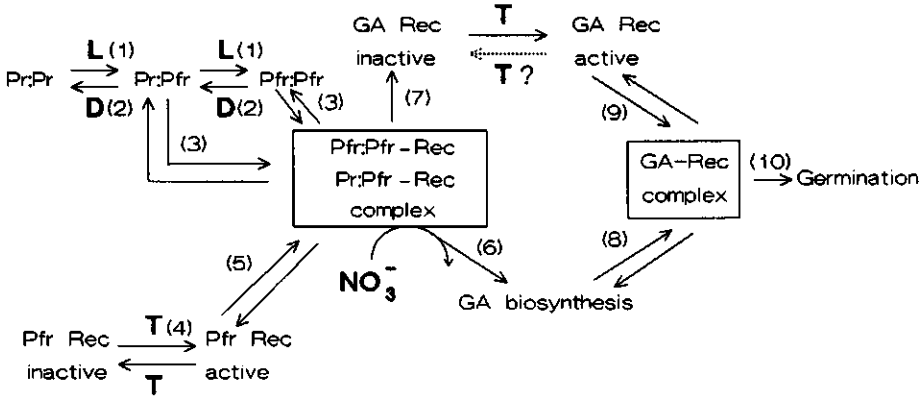

Figure 9.1 Schematic presentation for the regulation of seasonal changes in seed dormancy and germination by light, temperature and nitrate. L = light, D = darkness, T = temperature, Pr = inactive form of phytochrome, Pfr = active form of phytochrome, Rec = receptor, GA = gibberellin. Numbers are referred to in the text.

inactive form of phytochrome (Pr:Pr) will be converted to either Pr:Pfr or Pfr:Pfr (1). Pr:Pfr is induced by very low fluence values, resulting in a very-low-fluence-response (VLFR) and Pfr:Pfr results from irradiation by low fluence values, resulting in a low-fluence-response (LFR) (VanderWoude, 1985). In darkness these reactions are reversed (2). Both Pr:Pfr and Pfr:Pfr can bind to a phytochrome receptor, resulting in a ligand-receptor complex (3, 5). In seeds of *S. officinale* phytochrome action absolutely requires the co-action of nitrate (6) (Hilhorst, 1990b), in contrast to seeds of *A. thaliana* which only slightly responded to nitrate (Chapter 2). However, it can not be excluded that in the latter species endogenous nitrate is involved in the regulation of phytochrome action.

Application of GAs replaced the need for light (and nitrate). Most experiments in this thesis were performed with a commercially available mixture of GA_{4+7}. By RP HPLC and combined gas chromatography mass spectrometry (GC MS) it was demonstrated that the commercial mixture in fact contained GA_4 (54 %) and GA_7-isolactone (46 %), an isomer of GA_7, whereas GA_7 was not detected (Chapter 3). When the commercial mixture was

dissolved in a small volume of KOH before further diluting, a reduction in activity less than a factor 2 was observed in comparison with dissolving the mixture in ethanol. This reduction could be attributed to KOH-induced conversion of GA_7-isolactone to the 150 times less active GA_7. GA_4 was not affected by KOH. Because even low concentrations of ethanol can negatively influence germination, and solubility in KOH was better, we put up with the small reduction in activity of the KOH-dissolved mixture.

Because exogenous GAs replaced the requirement for light it was suggested that GAs are involved in the transduction pathway between the perception of light and the stimulation of germination. Indeed, in wild-type and GA-insensitive *gai* seeds of *A. thaliana* light-stimulated germination could be reversed by tetcyclasis, which inhibits early oxidations in the GA-biosynthetic pathway from *ent*-kaurene to *ent*-kaurenoic acid. This indicates that light-induced germination is preceded by *de novo* synthesis of GAs (6) (Chapter 4). Tetcyclasis also antagonized the action of phytochrome and nitrate in seeds of *S. officinale* (Hilhorst *et al.*, 1986; Chapter 6).

The conclusion that light stimulates GA biosynthesis was strengthened by preliminary GA determinations in wild-type and *gai* seeds of *A. thaliana*. Analyses of GAs by GC MS and quantification of GAs using deuterated standards demonstrated that irradiation of non-pre-incubated seeds gave elevated levels of GA_4 in wild-type and *gai* seeds and of GA_1 and GA_9 in *gai* seeds (Chapter 3). In light GA levels of *gai* seeds were 7 to 10 times as high as in wild-type seeds. Also vegetative parts of *gai* plants contained elevated levels of several GAs when compared to wild-type plants (Talon *et al.*, 1990). GA_3 was also detected in seeds of both genotypes, but was not quantified (Chapter 3). Of the biologically present GAs in wild-type seeds GA_4 was most active, as was also the case in *ga1* seeds. Sensitivity to GA_9 was about 50-100 times, and sensitivity to GA_1 and GA_3 10-25 times reduced when compared with GA_4, both in wild-type and *ga1* seeds (Chapter 3). Different receptors, differences in affinity of GA receptor(s), differences in uptake and transport to the site of action or differences in unspecific binding may account for the observed variability in activity.

The absolute GA requirement of germination processes in *A. thaliana* seeds has been proven convincingly in studies with the GA-deficient (*ga1*) mutant, and its recombinant with the GA-insensitive (*gai*) mutant (*gai/ga1*). Seeds of both genotypes did not germinate in the

absence of applied GAs, neither in darkness, nor in light (Karssen *et al.*, 1989; Chapter 4). Only ethylene can partially replace the need for GAs in *ga1* seeds. However, the seedling is seriously deformed. The mechanism of this ethylene action is not known (Karssen *et al.*, 1989).

Light also increased the sensitivity to GAs in seeds of both species (7) (Fig. 9.1) (Hilhorst *et al.*, 1986; Karssen *et al.*, 1989; Chapters 4, 6). Nitrate is not required for this second light action. Seeds can also respond to applied GAs in darkness, which indicates that an active GA receptor does not absolutely depend on Pfr action. However, it cannot be excluded that low levels of 'pre-existing' Pfr, which are present in darkness, participate in the response.

Temperature-induced shifts in requirement for light, nitrate and GAs

Temperature-induced changes in responsiveness to light and nitrate were extensively studied in both species. Moreover, changes in responsiveness to the commercial mixture of GA_{4+7} were followed.

Shifts in requirement for light and nitrate

Laboratory tests with exhumed seeds showed that the need for light and nitrate in buried seeds of *S. officinale* shifted in a seasonal pattern. The changes in requirement for both factors were remarkably parallel (Chapter 6). A loss of responsiveness to light and nitrate during pre-incubation at a constant temperature of 15 °C has been reported for *S. officinale* seeds by Hilhorst (1990a, b).

Shifts in requirement for a short red light pulse or continuous white fluorescent light were observed during pre-incubation of *A. thaliana* wild-type seeds at constant temperatures. Depending on temperature and duration, the requirement increased (e.g. 8 days 15 °C) or decreased (e.g. 7 days 2 °C) (Chapter 4). Chilling for periods up to 14 days lowered the fluence requirement for germination (Cone and Spruit, 1983; data not shown).

Reversible changes in sensitivity to light were also evident during imbibition of *A. thaliana* wild-type seeds under outside temperature conditions (Chapter 5).

For both species it is concluded that sensitivity to light increases during temperature-induced alleviation of dormancy and is reversed during dormancy induction. In *S. officinale* sensitivity to nitrate additionally increased during alleviation of dormancy and decreased during dormancy induction. The observed changes in curve parameters, that were derived from fitting the dose response data as logistic dose response curves, indicated that breaking of both primary and secondary dormancy are accompanied by an increase in the number of available phytochrome- (and nitrate) receptors, whereas the availability decreases during dormancy induction. In addition, receptor affinity or some processes in the signal-transduction chain initiated by the interaction of phytochrome, (nitrate) and their receptors, may vary seasonally.

Shifts in requirement for GAs

Changes in requirement for GAs had to be studied in the absence of GA biosynthesis. Therefore, GA dose responses in *S. officinale* seeds were studied in darkness and in *A. thaliana*, seeds of the *ga1* mutant were used. A GA-deficient situation can also be created in wild-type seeds, either by blocking GA biosynthesis by tetcyclasis or by depriving the seeds from light. However, action of eventually 'pre-existing' Pfr in the seeds can never fully exclude interference with endogenous GAs. When products of the GA-biosynthetic pathway subsequent to *ent*-kaurenoic acid are already present in the seeds, tetcyclasis cannot prevent further metabolism to some active GA. Such a mechanism might be the explanation for the incomplete reversal by tetcyclasis of dark germination of wild-type seeds in the absence of applied GAs (Fig. 4.8, Chapter 4).

In contrast to changes in requirement for light and nitrate, changes in requirement for GAs were not reversible in seeds of *S. officinale*. A gradual increase in GA sensitivity occurred from burial onwards (Chapter 6). Therefore, induction of secondary dormancy in summer can not be explained by a decreased GA sensitivity. During imbibition at constant temperatures in the laboratory changes in responsiveness to GAs were only minimal or fully absent (data not shown).

Whether or not changes in requirement for GAs were observed in wild-type and *ga1* seeds of *A. thaliana*, depended on several factors. It was for instance demonstrated that a

short chilling treatment of 7 days 2 °C increased responsiveness to GAs in freshly harvested seeds, whereas this chilling effect was absent in more GA-sensitive after-ripened seeds (Chapter 2). Chilling however still increased light responsiveness in these after-ripened seeds (data not shown). The temperature at which GA sensitivity was tested, also determined whether or not shifts in GA requirement were visible. An increased GA sensitivity, due to 7 days 2 °C, was best seen at 24 °C, whereas a decreased GA sensitivity due to 8 days 15 °C was best seen at 10 °C (Chapter 4). Again, shifts in requirement for light were visible at both temperatures (data not shown). This indicates that although changes in dormancy may be accompanied by changes in GA sensitivity, this correlation is not necessarily present. This was also seen during imbibition at outside temperatures. A very substantial enhancement of GA responsiveness was observed in the first few months of two experiments started in August, 1989 and June, 1991, respectively. Reversal of this reaction in winter was only partial, especially in the latter experiment. Changes in requirement for GAs further levelled off during prolonged imbibition (18 months) at outside temperatures, whereas changes in requirement for light were still very obvious. Therefore, it is concluded that changes in sensitivity to light rather than to GAs are of primary importance in determining whether or not seeds have the potential to germinate at particular times.

Germination- and dormancy-regulating principles

These principles will be summarized by means of the scheme visualized in Fig. 9.1. Seeds sense the time of the year mainly through changing temperatures in soil. Shifts in temperature regulate sensitivity to light (4) and eventually to other environmental factors, like for instance nitrate. The effect of temperature on the chain of events that regulates the responsiveness of the seeds to germination-stimulating factors, is far from clear. Although *in vivo* evidence can not be presented, strong indications suggest that the receptors for Pr:Pfr and Pfr:Pfr are located in membranes (Taylorson, 1988). Temperature may influence the synthesis of new receptors and/or the activation and affinity of receptors and thereby the availability of active receptors (4). Temperature-induced alterations in membrane structure or -composition (Raison *et al.*, 1980; Di Nola and Mayer, 1986) may be involved. Whether

or not nitrate binds to the same receptor as Pfr, is not clear. Parallel shifts in requirement for light and nitrate suggest that phytochrome and nitrate share the same site of action (6), as was also concluded by Hilhorst (1990b). The small nitrate ion may for instance alter the Pfr receptor, a phenomenon described by Hollenberg (1985) for several small ions. Because nitrate was only required for GA biosynthesis and not for the light-induced enhancement of GA sensitivity, it is also possible that nitrate exerts a specific effect in the signal-transduction chain to GA biosynthesis (6) and not in the chain that affects the GA receptor. In spores of *Dryopteris filix-mas* Haas and Scheuerlein (1990, 1991) also proposed a specific effect of nitrate on one of the early steps in the signal-transduction chain initiated by the formation of Pfr-receptor complex. Alterations in membrane structure or -composition may also influence permeability to germination-inhibiting substances like ABA and phenolic compounds or germination-stimulating substances. However, levels of ABA in mature *A. thaliana* seeds are normally low (Karssen *et al.*, 1983).

The increased responsiveness to light and nitrate remains unnoticed as long as the formation of a ligand- (in this case phytochrome or nitrate) receptor complex does not exceed the threshold that is required to initiate the chain(s) of events that ultimately lead(s) to germination. Upon irradiation a phytochrome-receptor complex is formed (3, 5), that can be affected by nitrate and possibly also by other environmental factors (Fig. 9.1). There is increasing evidence that calcium plays a role in signal transduction by its function as messenger (Roux *et al.*, 1986; Scheuerlein *et al.*, 1991; Trewavas and Gilroy, 1991). Somewhere in the signal-transduction chain GA biosynthesis is stimulated (6). Additionally, GA receptors are synthesized or activated (7). When either factor is limiting, formation of GA-receptor complex will not exceed the threshold required for germination. Upon formation of sufficient bio-active GA-receptor complex (8, 9) germination is initiated (10), provided other factors are not limiting. Receptors for GAs are also proposed to be membrane-bound (Hooley *et al.*, 1991). It is therefore not surprising that the GA-response system can also be directly altered by temperature, thus without the interference of light (Fig. 9.1). Effects of GAs on membranes have also been reported. Application of a mixture of GA_4/GA_7 was found to lower the phase-transition temperature in liposome model membranes (Pauls *et al.*, 1982). Although germination is controlled by the level of GAs and the availability of active GA

receptors, changes in dormancy are not necessarily accompanied by changes in both factors and therefore neither GA levels nor GA receptors regulate dormancy.

Discrimination between effects on GA biosynthesis and GA sensitivity is not always easy. An increased light-induced germination of wild-type and *gai* seeds of *A. thaliana* due to some temperature pretreatment may be the result of increased GA biosynthesis, a higher sensitivity to GAs or a combination of both. Technical improvements make determination of GA levels possible now (Chapter 3). It was shown that in chilled seeds of both genotypes GA levels in darkness and light were quite similar. Because germination did not occur in darkness but was very high in light, it is evident that GA levels can not be regarded as limiting for germination of these chilled seeds. Therefore, GA sensitivity may rather be limiting. As a result of irradiation GA sensitivity increases, and GA molecules find more action sites. However, this explanation is not fully satisfactory, especially not for wild-type seeds. In this genotype GA sensitivity can be directly increased by a chilling pretreatment, thus without the interference of light (Chapter 4). It is not clear why this increase does not cause dark germination as a result of the action of the endogenously present GA, because the increase in sensitivity to GAs by chilling is often comparable to the increase evoked by light (Chapter 4).

Enhanced GA sensitivity can also explain increased light germination of *gai* seeds after 7 days pre-incubation at 2 °C, although GA levels were reduced in comparison with irradiated non-chilled seeds. This reduction of GA levels again presents evidence that it is unlikely that GA biosynthesis plays a role in dormancy control. The increased level of bio-active GA_4 in darkness after chilling may be significant, but can on its own not stimulate germination. Light is additionally required to overcome the limitation at the receptor level. Although determination of GA levels provide information on the potential availability, nothing is known about its physicochemical nature and its subcellular distribution. Therefore, the preliminary results on determination of GA levels in Chapter 3 should be interpreted with great caution.

In conclusion, germination depends on the presence of the ligands active phytochrome, (nitrate) and bio-active GA. Without light seeds do often not germinate. The availability of

light is not limiting in any season and can therefore not explain the annual dormancy patterns. Whether light (and nitrate) induces germination depends primarily on the responsiveness of the seeds to these factors. It is concluded that the sensitivity to Pfr (and nitrate) is the primary oscillating factor in seeds during the seasons.

When the presence of active phytochrome and an active response system allows the formation of a ligand-receptor complex, GAs will be synthesized. Sensitivity to GAs is influenced by light and temperature, but does not fluctuate in a seasonal way. Thus, GAs and a GA-response system are absolutely required for germination, but are not the primary regulators of dormancy.

To verify our conclusions about changes in sensitivity, research should focus on binding experiments to identify specific binding sites for GAs, Pfr and nitrate. Because many proteins are capable of binding GAs, for example, it is very difficult to discover the few low-abundance proteins that possess genuine receptor characteristics. Therefore, progress in this search is rather slow. Newly emerging photo-affinity and protoplast techniques are promising in this respect. Membrane fractions have been isolated to which Pfr binds (Gallagher *et al.*, 1988). Whether or not this binding results in a biological response is not clear. It has also been hypothesized that Pfr directly influences gene transcription, which would imply a nuclear localization rather than a membrane localization. Insight in the distribution of Pfr at a cellular level can be obtained by several techniques, like spectrophotometry, immunocytochemistry and microbeam irradiation. However, all these techniques have limitations in that they detect Pfr regardless of its biological activity or they provide little resolution. Therefore, it still remains to be determined whether presence of Pfr is related to its mode of action.

Similarly, determination of GA levels in seeds following perception of environmental signals like light and temperature do not provide any information about an eventual relation between the endogenous level and some biological response. Moreover, nothing is known about its physicochemical nature near the primary action site on a subcellular level.

The present physiological studies certainly advance our knowledge of the regulation of seed dormancy and germination under natural conditions. Biochemical verification is highly

desirable, but as outlined above these studies cope with results that are rather difficult to interpret.

Ecological implications

Laboratory experiments have shown that processes involved in either dormancy breaking or dormancy induction may occur simultaneously in seeds. Rates of both processes depend on temperature. The kinetics of both processes may be different, as well as the optimum temperature. The net result of both actions determines whether a certain pre-incubation treatment can be classified as dormancy breaking or -induction (Cone and Spruit, 1983; Karssen *et al.*, 1988). In *A. thaliana* (Chapter 4) and *S. officinale* (Hilhorst *et al.*, 1986) the rate of dormancy breaking was increased at elevated temperatures. However, induction of secondary dormancy was also enhanced at these temperatures. A fast rate of dormancy breaking was therefore followed by a rapid induction of dormancy. At lower temperature both processes differ much more. In *A. thaliana* a chilling treatment as short as 7 days can induce maximal germination but induction of secondary dormancy became at first visible after months of chilling (Chapter 4). It is likely that also under field conditions alleviation- and induction of dormancy occur simultaneously. Consequently, the use of the terms dormancy breaking and -induction in this thesis involves the net result of both processes, rather than separate processes.

To assess the ecological importance of temperature-induced changes in sensitivity to light (Chapters 5, 6) and eventually to other environmental factors like nitrate (Chapter 6), consideration should be given to these conditions encountered by seeds in arable soil.

light

Exposure to light during seed maturation determines the ratio Pr/Pfr in mature seeds. Therefore, seeds that become buried in soil may have different levels of 'pre-existing' Pfr. Upon hydration of these seeds, the probability of germination depends on the interaction of

Pfr with its response system, as was discussed above. When light sensitivity is limiting, germination is prevented. This situation will occur when seeds of *A. thaliana* and *S. officinale* are shed in summer or in early autumn. A similar situation is likely for seeds of numerous species of summer- and winter annuals, when they are shed prior to the season with unfavourable conditions for plant growth. Burial of these seeds will reduce the level of Pfr, due to dark reversion to Pr, that depends on temperature and pH. Light is required for the opposite change. Thus, seeds that are not light-dependent upon shedding often develop a light requirement during burial (Pons, 1991). Because penetration of light in soils is extremely poor, seeds that are buried deeper than a few millimetres, are exposed to darkness (Tester and Morris, 1987). Although buried seeds show seasonal periodicity in sensitivity to light, germination is prevented as long as the amount of Pfr does not exceed the threshold required at a particular time. Mechanical disturbance of the upper soil layers by for instance ploughing, sowing or weeding will transport seeds to the soil surface or bring them to more elevated depths. In regularly cultured crop fields seed banks below the ploughing level of 15-40 cm are very common (Hartmann and Nezadal, 1990). Whether or not seeds transferred to the soil surface have a chance to germinate depends on a combination of both light sensitivity and light perception of the seeds. The latter factor is greatly affected by the presence of neighbouring crops or other vegetation (Smith *et al.*, 1990). Seeds with a low sensitivity to light, require more Pfr, and germination may be delayed. Competition may be avoided in this way. Seeds that during tillage do not end at or near the soil surface might have been exposed to repeated irradiations with daylight up to some seconds before they become re-buried. These short light exposures may cause emergence of weeds which can potentially occur at depths between 2 and 17.5 cm (Hartmann and Nezadal, 1990). Exposures to fluences equivalent to a few milliseconds of full sunlight were sufficient to promote germination of *Datura ferox* seeds after 2 months of burial (Scopel *et al.*, 1991). An extremely high light sensitivity such that seeds could respond to fluences in the VLFR, that requires only 0.0001-0.001 % Pfr (Kronenberg and Kendrick, 1986), was also found in exhumed seeds of *S. officinale* in spring, 1991. The response was reversed during dormancy induction in summer (Chapter 6). In such periods of extremely high light sensitivity short light exposures during tillage may greatly attribute to observed germination flushes. Tillage

operations at night may therefore reduce seedling emergence. Preliminary studies of Hartmann and Nezadal (1990) seem to indicate that mechanical cultivation at night reduced weed coverage to 2 % in comparison with 80 % when performed at noon.

Whether germination actually will occur when phytochrome and its response system are not limiting, depends on other environmental factors, like the availability of water and nitrate and the proper temperature conditions.

Water availability

The need for water is evident. However, intermittent periods of hydration and drying are quite common at or near the soil surface. Moreover, dehydration can affect dormancy (Karssen *et al.*, 1988; Bouwmeester, 1990).

Temperature

Secondly, the actual field temperatures have to overlap the temperature requirements for germination. Because the range of temperatures over which germination can proceed is widest when the response system is most sensitive (see e.g. Fig. 6.2 in Chapter 6), this requirement may be easily fulfilled. Temperatures at or near the soil surface often show considerable diurnal fluctuations, which are however less pronounced under vegetation. It is well known that there often is a need for a diurnal fluctuation in temperature (Probert, 1992). Moreover, short extreme temperature conditions can be effective in stimulating germination (Takaki *et al.*, 1981).

Nitrate

The presence of nitrate in soil may be an important co-factor for light-induced germination. In seeds of *S. officinale* germination is even absolutely nitrate dependent (Hilhorst, 1990b). Although nitrate levels in soil fluctuate within the range of 0-50 mmol l^{-1} (Young and Aldag, 1982), no general seasonal pattern was observed (Rice, 1984). Levels

increase as a result of mineralization and input via fertilizers and rain and decrease as a result of uptake by plants. In addition, factors like microbial activity, soil type, soil pH, moisture content, cultivation practices and depth in soil may influence nitrate levels. Seeds may take up nitrate from the soil. Both nitrate levels and water uptake influence the uptake (Goudey *et al.*, 1988). Moreover, nitrate may originate from seed development on the mother plant (Saini *et al.*, 1985; Bouwmeester, 1990). In *S. officinale* seeds it was demonstrated that part of the nitrate was tightly bound, whereas a larger pool was loosely bound and was easily extractable from the seeds (Bouwmeester, 1990; Hilhorst, 1990b). It has been shown that nitrate levels in soil might be in the range that is required to stimulate germination. However, the absence of a seasonal pattern in nitrate levels makes it unlikely that soil nitrate is part of the mechanism by which seeds sense the seasons (Karssen and Hilhorst, 1992). This conclusion was strengthened by nitrate determinations of exhumed seeds that were either dormant or non-dormant (Chapter 6). Whether or not seeds respond to the available nitrate, depends on the nitrate-response system. Seasonal fluctuations in sensitivity to nitrate in seeds of *S. officinale* were remarkably similar to shifts in sensitivity to light (Chapter 6). Such parallel shifts are undoubtedly of high ecological significance. They continuously allow the seeds to detect ever-changing levels of nitrate and phytochrome, such that germination is prevented when either of the two factors is limiting. It was suggested by Pons (1989) that nitrate may become limiting for seeds that are near to established plants.

An altered response capacity to nitrate may influence the effectiveness of the Pfr response or vice versa. Seeds that are too deeply buried to perceive light but have a high sensitivity to light and nitrate, may in nitrate-rich soils start to germinate in darkness as a result of 'pre-existing' Pfr and the amount of nitrate present. However, such dark germination may be fatal when storage reserves do not allow the seedling to reach the soil surface. However, at elevated depths small amplitudes of diurnal temperature fluctuations may be sensed by the seeds as a signal that germination conditions are not appropriate.

Other compounds in soil

Other compounds in soil may also inhibit or stimulate germination. Minerals, organic

substances and volatile compounds like ethylene are known in this respect (Karssen and Hilhorst, 1992). It was demonstrated by Schonbeck and Egley (1981) that sensitivity to ethylene increased in *Amaranthus retroflexus* seeds during burial. Changes in sensitivity to organic substances have been found in seeds of some root parasites (Visser, 1989).

Respiratory activity during changes in dormancy

The present study has clearly shown that changes in dormancy in seeds of *S. officinale* are not necessarily accompanied by changes in respiratory activity. After peaks in oxygen uptake and carbon dioxide release shortly after the start of imbibition, which correlated well with water uptake, the rates decreased to low levels. These levels were affected by the actual temperature but not by dormancy-breaking and -inducing temperature pretreatments. Such low levels of respiration during the seasonal pattern of dormancy is of great survival value. It prevents metabolic exhaustion of the seeds. The germination process required an increased oxygen uptake (Chapter 7).

The contribution of different respiratory pathways changed during incubation (Chapter 8). The alternative pathway gave a contribution of maximally 10 % during the first 48 h of incubation at 24 °C and disappeared thereafter. The relative contribution of the cytochrome pathway decreased during prolonged incubation at 24 °C. Residual respiration relatively increased, but its absolute level remained constant. Respiratory quotients of CO_2 release and O_2 uptake, that were 0.55-0.7, were not influenced by incubation time. It is unlikely that the alternative pathway plays a role in the regulation of dormancy in *S. officinale* seeds, in contrast to suggestions in *Xanthium pennsylvanicum* seeds (Esashi *et al.*, 1981). Its function at the start of incubation may involve oxidation of excessive NADH, thus acting as an energy overflow (Lambers, 1980).

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Summary

Seeds play an important role in the propagation and dispersal of many species. Upon ripening on the mother plant seeds often fall on the soil surface and may become buried. At the time of shedding, seeds of many wild species are often not able to germinate, even when environmental conditions like availability of water, temperature, light and soil composition are favourable. These seeds are dormant. Dormancy can be relieved over time by certain specific conditions, allowing seeds to germinate. However, when such non-dormant seeds are exposed to environmental conditions that inhibit germination, for instance because they are too deeply buried, dormancy is re-induced (secondary dormancy). Annual cycles of relief and induction of dormancy may be repeated for numbers of years until the seeds germinate or disappear by predation, rot or metabolic exhaustion of food reserves. Earlier studies have shown that in temperate regions temperature plays a dominant role in the regulation of annual dormancy cycles. Temperature is also important for the germination process. The temperature requirements for breaking of dormancy and for germination may be completely different, indicating that both processes are controlled by separate mechanisms. Stimulative actions on germination of non-dormant seeds by environmental factors like light and nitrate imply that seeds can detect their presence during some period of the year.

It was demonstrated before by use of tetcyclasis, an inhibitor of gibberellin (GA) biosynthesis, that light, eventually in co-action with nitrate, results in GA biosynthesis. The red component of visible light is perceived by the inactive form of phytochrome, that is then converted to the active form. Light also reduced the requirement for GAs, and thus enhanced GA sensitivity.

Hormone mutants of *Arabidopsis thaliana* that lack the capacity to synthesize GAs or have a strongly reduced GA sensitivity, are very powerful tools to study the involvement of GA biosynthesis and sensitivity to GAs in the control of dormancy and germination. Previous studies demonstrated that GAs are absolutely required for germination of this species. GA-deficient (*ga1*) seeds do not germinate in the absence of applied GAs, neither in darkness, nor in light. Germination of wild-type seeds also often depends on applied GAs in darkness,

but light may overcome the requirement for applied GAs.

Chapter 1 presents a general introduction about the regulation of dormancy and germination.

Freshly harvested seeds of *A. thaliana* grown in different seasons showed considerable variability in the requirement for light, GAs and nitrate (Chapter 2). This variability is regarded as an expression of differences in dormancy. Generally, sensitivity was high in seed lots harvested between September and March and significantly lower in those harvested between April and June. Conditions during seed development on the mother plant must be responsible for the observed differences in sensitivity. Differences in temperature and nitrate availability seemed the most likely candidates. Endogenous nitrate levels varied as much as maximum a factor 600 amongst seed lots, they were weakly correlated with light-induced germination.

The degree of dormancy rapidly declined upon dry storage of the seeds. It was concluded that studies on germination and dormancy of *A. thaliana* seeds are hindered by the large variability in seed material and the relatively high rate of after-ripening.

Until now, over 80 GAs have been described. In physiological studies, commercially available GA_3 and a mixture of GA_4 and GA_7 are generally used. The mixture was also used in the germination experiments in this thesis. However, full-scan gas chromatography mass spectrometry (GC MS) demonstrated that the commercial mixture contained GA_4 and GA_7-isolactone, whereas GA_7 was not detected (Chapter 3). The composition of the mixture was 54 % GA_4 and 46 % GA_7-isolactone. Because solubility of the commercial mixture in water was low, it was dissolved in a small volume of KOH before further diluting with a phosphate citrate buffer, pH 5. GA concentrations up to 1 mM could be realized in this way. As a result, most of the GA_7-isolactone was converted to GA_7, whereas GA_4 was not affected. Dissolving the mixture in a small volume of ethanol before further diluting, did not alter the composition. The maximum concentration of GA that could be dissolved in this way, was 0.2 mM. Because GA_7 had a much reduced activity in comparison with GA_7-isolactone, dissolving the commercial mixture in a small volume of KOH, gave somewhat reduced germination when compared with dissolving the mixture in ethanol. Dose response curves shifted less than a factor 2 to the right, thus about twice as much GAs were required of the

KOH-dissolved mixture. Because small amounts of ethanol may influence germination negatively and solubility in ethanol was reduced when compared to KOH, GAs were dissolved in KOH, with the exception of experiments in which the activity of pure GAs was tested.

Also the activities of pure GA_4, GA_1, GA_3 and GA_9 were tested on seed germination of wild-type and *ga1* seeds of *A. thaliana*. Sensitivity to GA_7 and GA_9 was about 50-150 times, and to GA_1 and GA_3 10-25 times reduced when compared with GA_4 and GA_7-isolactone (Chapter 3). A satisfactory explanation for the observed differences cannot be given. Incomplete metabolism, the existence of different GA receptors, differences in affinity of GA receptors, differences in uptake and transport to the site of action and differences in unspecific binding have to be considered as (part of) the explanation.

The influence of light and temperature on GA biosynthesis and sensitivity to the commercial mixture of GA_{4+7} was studied in seeds of *A. thaliana* (Chapter 4). When wild-type seeds were pre-incubated at different constant temperatures for different periods of time and thereafter irradiated with red light during 15 min, and transferred to 10 or 24 °C, a pattern of breaking and induction of dormancy was seen. The higher the pre-incubation temperature, the quicker the rate of dormancy breaking and the quicker the rate of re-induction of dormancy. Inhibition of light-stimulated germination by tetcyclasis presented indirect evidence that germination is preceded by *de novo* synthesis of GAs (Chapter 4). This conclusion was strengthened by preliminary GA determinations in wild-type and GA-insensitive *gai* seeds (Chapter 3).

Four GAs were identified in extracts from wild-type and *gai* seeds: GA_1, GA_3, GA_4 and GA_9. Effects of light and chilling on levels of GA_1, GA_4 and GA_9 by using deuterated standards were compared to effects on germination. In non-chilled seeds light both increased GA levels and germination in the absence of applied GAs. As a result of irradiation GA levels in *gai* seeds were 7-10 times as high as in wild-type seeds. Chilling (7 days 2 °C) increased the levels of GA_4 in darkness, but not in light, although light significantly enhanced germination. Therefore, it is concluded that a rise in the level of some GA(s) as a result of light and chilling might be important in the stimulation of germination, but definitely is not the only decisive factor.

Sensitivity to GAs also plays a decisive role in the control of germination (Chapter 4). It was enhanced by treatments like chilling (up to 13 weeks), dry after-ripening and light. Sensitivity declined when periods of chilling exceeded 13 weeks or when seeds were pre-incubated at higher temperatures, for instance 8 days at 15 °C. Apart from wild type shifts in GA requirement also occurred in *ga1* and *gai/ga1* seeds, that is in the absence of endogenous GAs, indicating that GA biosynthesis is not required for changes in dormancy. The recombinant only responded to light after dormancy was broken by either a chilling treatment of 1 week or a dry after-ripening period of some months. Germination temperature determined whether or not changes in GA sensitivity could be detected. Breaking of dormancy could best be studied at a temperature of relatively low GA sensitivity (24 °C) and dormancy induction at a temperature of relatively high GA sensitivity (10 °C).

It was concluded that the combination of GA biosynthesis and a sufficient high GA sensitivity is essential for germination, but both factors are not directly controlling dormancy. Both factors depend on the mechanisms that control the responsiveness to environmental factors. Temperature primarily regulates sensitivity to light and as a result of light perception GA biosynthesis as well as the responsiveness to GAs may be enhanced. GA sensitivity is also directly controlled by temperature, thus without the interference of light.

These conclusions were strengthened when regulation of dormancy and germination was studied under natural conditions (Chapter 5). Dark-incubated seeds of wild type and *ga1* mutant were exposed to a natural temperature regime for periods up to 18 months. At regular intervals the germination capacity of portions of seeds was tested at laboratory conditions. Germination temperature was 10 or 24 °C. When germination of wild-type seeds was tested in the absence of GAs after irradiation with red light a seasonal pattern of changes in dormancy was seen. Primary and secondary dormancy were broken in summer and seeds reentered dormancy in autumn and winter. A second small flush occurred in early spring. Seeds of *ga1* did not germinate at any time of the year when deprived from GAs, re-emphasizing the absolute GA requirement for germination of *A. thaliana* seeds.

Dose responses to GAs, light and nitrate were determined to study the role of sensitivity to these factors in regulating annual dormancy patterns and germination. Germination data were fitted as logistic dose response curves and curve parameters were calculated. Dose

responses to light were determined in wild-type seeds in the absence of exogenous GAs and in *ga1* seeds in the presence of 0.25 or 1 μM GA_{4+7}. Dose responses to light showed reversible parallel shifts in both genotypes. The responsiveness to GAs increased 50-100 fold during the first 3-4 months of imbibition at outside temperatures. Major changes occurred in the dose for half-maximum response. Minimum- and maximum responses showed little variation. Reversal of this reaction in winter was either negligible or only partial. Changes in sensitivity to GAs clearly levelled off during prolonged incubation at outside temperatures, whereas temperature-induced reversible changes in requirement for light remained. Nitrate or sensitivity to nitrate did not significantly contribute in the control of dormancy and germination of *A. thaliana* seeds.

A comparable study was performed on seeds of the related species *Sisymbrium officinale* (Chapter 6). Seeds of this species were buried under natural conditions in soil in November, 1990 and at regular intervals portions were exhumed to test dose responses to light, nitrate and GAs under laboratory conditions. Germination data were fitted as logistic dose response curves. Germination capacity was determined at 15 °C because previous studies had shown that dormancy patterns were best seen at this temperature. It was also demonstrated before that germination of this species invariably depends on the simultaneous presence of light and nitrate, either exogenous or endogenous. When germination capacity of exhumed seeds was tested in the presence of saturating doses of light and nitrate, a clear dormancy pattern was seen. Dormancy was alleviated in autumn-winter and re-induced in summer. To prevent limitation by other factors, dose responses to light were determined in the presence of a saturating concentration of KNO_3 and dose responses to nitrate were performed after a saturating dose of red light. Sensitivity to light and nitrate showed reversible changes over the seasons. Changes occurred in the maximum response, the minimum response and the dose required for half-maximum response. Shifts of dose response curves were parallel. Patterns of shifts in requirement for light and nitrate were remarkably similar. During breaking of primary dormancy in spring, 1991, the fluence response curves exhibited a biphasic character with responses occurring in the very-low-fluence-range and in the low-fluence-range. The nitrate dose response curves could all be fitted as monophasic curves. However, it cannot be excluded that in some of the curves responses occurred in two distinct

ranges as well, which was difficult to judge due to the narrow response range of germination. Sensitivity to GAs gradually increased from burial onwards and was not particularly related to changes in dormancy. Thus, GA sensitivity cannot be regarded as a limiting factor in regulating dormancy of this species.

From interpretation of curve parameters in both species, it is suggested that the observed shifts in requirement for light (and nitrate) involve changes in the level of available receptors, changes in the binding characteristics of the receptors or in the response chain initiated by the formation of ligand-receptor complex, that follows the perception of light, eventually in co-action with nitrate.

Seeds of *S. officinale* were also used to study a possible involvement of changes in metabolic activity in the regulation of dormancy (Chapter 7). The advantage of this species in this respect is that breaking of dormancy can be easily distinguished from early germination events. Depending on a combination of temperature and duration, pre-incubation of seeds in darkness results in alterations in the degree of dormancy. Germination of non-dormant seeds is prevented as long as they are deprived from light and nitrate. Oxygen uptake of dark-incubated seeds was measured with the use of oxygen electrodes at 24 °C and compared to germination capacity in 25 mM KNO_3 at the same temperature after 15 min of red light irradiation. Pre-incubation temperatures were 6, 10, 15 and 24 °C. At all pre-incubation temperatures a quick rise in oxygen uptake was followed by a gradual decline. This rise in oxygen uptake correlated well with the rate of water uptake as well as with breaking of dormancy. However, when seeds were re-imbibed following a dehydration treatment, rates of oxygen uptake did not increase, whereas germination capacity was enhanced in comparison with non-dehydrated seeds. During breaking of secondary dormancy by chilling an increase in oxygen uptake rate did also not occur. The decline in oxygen uptake that began 9-30 h after the start of imbibition, depending on the temperature, was not generally linked to induction of secondary dormancy. During re-induction of dormancy following a chilling pretreatment, oxygen uptake rate did not show a further decrease. When seeds were kept at 24 °C for prolonged periods germination capacity remained zero and oxygen uptake remained at a constant level as well. It is concluded that changes in respiratory activity are not required for changes in dormancy. A low level of oxygen uptake

during prolonged burial in soil, that is only affected by the actual temperature and the moisture content of the seed and not by dormancy breaking or -induction is of great survival value, because rapid depletion of food reserves is prevented. When non-dormant seeds were allowed to germinate by providing light and nitrate, rates of oxygen uptake increased dramatically. This increase started well before radicle protrusion, indicating that oxygen uptake is a very sensitive parameter to determine whether or not radicle protrusion is to be expected. A farred irradiation only reversed the increase in oxygen uptake when it was given before germination escaped from its red light-antagonizing action. Thus, germination depends on an increased respiratory activity.

In order to investigate whether full immersion and constantly stirring of seeds in oxygen electrodes do not overestimate respiration rates, rates of oxygen uptake were also determined gas chromatographically (Chapter 8). Fortunately, both methods gave comparable results. Rates of oxygen uptake and CO_2 release were followed during prolonged incubation in darkness at 24 °C. During the first 8 h of incubation both rates increased and declined thereafter. Constant levels of oxygen uptake (25-30 nmol min^{-1} g^{-1} seed) and CO_2 release (17 nmol min^{-1} g^{-1} seed) were reached after 3 weeks of incubation at 24 °C. During incubation for periods up to 3 months, rates of oxygen uptake and CO_2 release remained at these constant levels. Ratios of CO_2 release and oxygen uptake (respiratory quotients) varied between 0.55 and 0.7. A respiratory quotient of 0.7 can be expected in the lipid-containing seeds of *S. officinale*. Lower values may indicate that other oxygen consuming processes in which less CO_2 is evoked, like oxidation of phenols originating in the seed coat, contribute in oxygen uptake.

The existence of two pathways of mitochondrial electron transport has been demonstrated before in seeds of several species: the cytochrome pathway and the alternative pathway. Both pathways can be blocked by specific inhibitors: the cytochrome pathway by cyanide and the alternative pathway by hydroxamates. Effects of KCN and benzohydroxamic acid (BHAM) on germination and oxygen uptake rate were studied in seeds of *S. officinale* (Chapter 8). It was remarkable that KCN concentrations that almost completely inhibited flow through the cytochrome pathway, hardly reduced germination. BHAM already inhibited germination at concentrations that did not inhibit oxygen uptake. Titration experiments were performed to

establish concentrations of KCN and BHAM that fully inhibit the pathway concerned without having side-effects: 1.5 mM KCN and 30 mM BHAM were chosen. The contribution of respiratory pathways changed during imbibition at 24 °C. During the first 48 h of imbibition at 24 °C 70 % of oxygen uptake occurred via the cytochrome pathway, 10 % via the alternative pathway and 20 % was residual oxygen uptake, that is insensitive to KCN and BHAM. After 240 h of imbibition the flow through the alternative pathway fully disappeared. Its capacity was still 10 %. The flow through the cytochrome pathway declined and residual oxygen uptake remained constant. After 2 weeks of imbibition at 24 °C 50 % of oxygen uptake occurred via the cytochrome pathway and 50 % was residual oxygen uptake. Irrespective of changes in the contribution of different respiratory pathways, respiratory quotients of CO_2 release and oxygen uptake did not change. The appearance of the alternative pathway after the start of imbibition may be attributed to an excess of NADH that has to be oxidized, thus acting as an energy overflow.

The results from Chapters 2-8 are integrated in the general discussion in Chapter 9. A scheme is presented in which (soil) temperature is regarded as the most important factor in the mechanism by which seeds sense the time of the year. Soil temperature regulates sensitivity to light and eventually to other environmental factors, like for instance nitrate. Upon perception of light (and nitrate) some signal-transduction chain is initiated, ultimately leading to germination. In this chain GA biosynthesis is stimulated. Moreover, GA responsiveness is enhanced. GA sensitivity can also be directly increased by temperature. When either GA level or the GA-response system is limiting, germination is prevented. Although germination depends on the level of bio-active GAs and the available response system, changes in both factors do not necessarily accompany changes in dormancy and are therefore not primarily regulatory. The temperature-controlled response system to light (and to nitrate), and not the environmental factors themselves, determines in which period of the year germination is possible. Changes in dormancy do also not depend on changes in respiratory activity, whereas germination requires an increased respiratory activity.

Because germination of many species is phytochrome dependent, the regulation of dormancy and germination as described for seeds of *A. thaliana* and *S. officinale*, may be valid for many species.

Samenvatting

Zaden spelen een belangrijke rol bij de vermeerdering en verspreiding van vele soorten. Nadat zaden gerijpt zijn aan de moederplant, vallen ze vaak op de grond, maar kunnen ook in de grond terecht komen. Op dat moment zijn zaden van vele wilde soorten niet in staat om te kiemen, zelfs niet wanneer omgevingsfactoren, zoals de beschikbaarheid van water, temperatuur, licht en samenstelling van de bodem gunstig zijn. De zaden verkeren in kiemrust. Deze kiemrust kan gebroken worden wanneer de zaden gedurende een bepaalde tijd aan specifieke condities worden blootgesteld. De zaden zijn nu in principe kiembereid. Wanneer deze zaden echter vervolgens aan omgevingsfactoren worden blootgesteld, die de kieming remmen, - bijvoorbeeld omdat de zaden te diep begraven liggen -, kan opnieuw kiemrust geïnduceerd worden, secundaire kiemrust. Jaarritmes van uit- en in kiemrust gaan kunnen vele malen doorlopen worden totdat de zaden kiemen of verdwijnen door predatie, rotting of metabolische uitputting van voedselreserves. In eerder onderzoek is al aangetoond dat in gebieden met een gematigd klimaat temperatuur een dominante rol speelt in de regulatie van seizoengebonden wisselingen in kiemrust. Temperatuur is ook belangrijk voor de eigenlijke kieming. De temperatuurbehoeften voor breking van kiemrust en voor kieming kunnen totaal verschillend zijn, wat erop duidt dat beide processen verschillend gereguleerd zijn. Het feit dat omgevingsfactoren, zoals licht en nitraat, de kieming in bepaalde perioden van het jaar kunnen stimuleren, betekent dat zaden de aanwezigheid van deze factoren in deze perioden kunnen waarnemen.

Met behulp van tetcyclasis, een remstof die de synthese van de hormoongroep gibberellinen (GAs) remt, is eerder aangetoond dat licht, eventueel samen met nitraat, leidt tot synthese van GAs. De rode component uit zichtbaar licht wordt waargenomen door de inactieve vorm van fytochroom, die hierdoor in de actieve vorm omgezet wordt. Licht verlaagde daarnaast de behoefte aan GAs, en verhoogde dus de gevoeligheid voor GAs.

Hormoonmutanten van *Arabidopsis thaliana* (zandraket) die zelf geen GAs kunnen synthetiseren of een sterk verlaagde gevoeligheid voor GAs hebben, zijn waardevol om het belang van synthese van GAs en gevoeligheid voor GAs in de regulatie van kiemrust en

kieming te kunnen bepalen. Eerder is al aangetoond dat GAs absoluut noodzakelijk zijn voor de kieming van deze soort. Zowel in het donker, als in het licht kiemen GA deficiënte (*ga1*) zaden niet in afwezigheid van toegediende GAs. Kieming van wildtype zaden in het donker hangt meestal ook af van toegediende GAs, echter licht kan de behoefte aan toegediende GAs vervangen.

In Hoofdstuk 1 wordt een algemene inleiding gegeven over de regulatie van kiemrust en kieming.

Versgeoogste zaden van *A. thaliana* die verzameld waren van planten die in verschillende jaargetijden geteeld waren, lieten nogal wat variatie zien in hun behoefte aan licht, GAs en nitraat (Hoofdstuk 2). Deze variatie kan gezien worden als variatie in kiemrust. Over het algemeen was de gevoeligheid hoog in zaadpartijen die geoogst waren tussen september en maart en belangrijk lager in de periode april-juni. Deze variatie in gevoeligheid moet terug te voeren zijn naar de opkweekcondities tijdens zaadrijping aan de moederplant. Verschillen in temperatuur tijdens zaadrijping en nitraatbeschikbaarheid zijn de meest waarschijnlijke factoren. Verschillen in het endogene nitraatgehalte in de zaden waren aanzienlijk, tot zelfs een factor 600. Het endogene nitraatgehalte was zwak gecorreleerd met de door licht gestimuleerde kieming.

Wanneer het verzamelde zaad droog opgeslagen werd, nam de mate van kiemrust snel af. Geconcludeerd werd dat onderzoek naar de regulatie van kiemrust en kieming in *A. thaliana* zaad sterk gehinderd wordt door de grote variabiliteit van het zaadmateriaal en de relatief snelle narijping van het zaad wanneer het droog opgeslagen wordt.

Tot nu toe zijn meer dan 80 GAs beschreven. In fysiologisch onderzoek wordt vaak gebruik gemaakt van het commercieel verkrijgbare GA_3 en van een mengsel van GA_4 en GA_7. Het mengsel werd eveneens gebruikt in de meeste kiemingsexperimenten die in dit proefschrift beschreven staan. Met behulp van gas chromatografie en massa spectrometrie werd aangetoond dat het commerciële mengsel GA_4 en GA_7-isolacton bevatte, terwijl GA_7 niet aangetoond werd (Hoofdstuk 3). De samenstelling van het mengsel was 54 % GA_4 en 46 % GA_7-isolacton. Omdat de oplosbaarheid van het mengsel in water extreem laag was, werd het mengsel eerst opgelost in enkele druppels KOH en vervolgens uitverdund met een fosfaatcitraat buffer met een pH van 5. Op deze manier konden GA concentraties van

tenminste 1 mM bereikt worden. Als gevolg van deze oplosmethode was een groot deel van GA_7-isolacton omgezet in het isomere GA_7. GA_4 was niet omgezet. Oplossen van het mengsel in enkele druppels ethanol voordat het verder uitverdund werd, veranderde de samenstelling van het oorspronkelijke mengsel niet. De maximaal oplosbare concentratie bedroeg 0.2 mM. Omdat GA_7 aanzienlijk minder actief bleek te zijn dan GA_7-isolacton, was kieming bepaald in het commerciële mengsel opgelost in KOH wat lager dan wanneer het mengsel in ethanol was opgelost. Dosis-responscurven verschoven echter minder dan een factor 2 naar rechts, dus ongeveer 2 maal zoveel GAs waren nodig wanneer het mengsel opgelost was in KOH. Omdat een geringe hoeveelheid ethanol al een negatief effect kan hebben op de kieming en de oplosbaarheid in KOH hoger was, werden GAs opgelost in KOH, met uitzondering van experimenten waarin de activiteit van zuivere GAs getest werd.

De activiteit van zuiver GA_7, GA_7-isolacton, GA_4, GA_1, GA_3 en GA_9 werd getest in de stimulatie van kieming van wildtype en *ga1* zaad van *A. thaliana*. De gevoeligheid voor GA_7 en GA_9 was ongeveer 50-150 maal en voor GA_1 en GA_3 10-25 maal zo laag in vergelijking met de gevoeligheid voor GA_4 en GA_7-isolacton (Hoofdstuk 3). Een bevredigende verklaring voor de waargenomen verschillen kan niet worden gegeven. Onvolledige metabolisatie, het bestaan van verschillende receptoren, verschillen in affiniteit van GA receptoren, verschillen in opname en transport naar de plek waar ze aangrijpen en verschillen in a-specifieke binding kunnen deel uitmaken van de waargenomen verschillen in activiteit.

Effecten van licht en temperatuur op synthese van GAs en gevoeligheid voor het commerciële mengsel GA_{4+7} werden bestudeerd in zaden van *A. thaliana* (Hoofdstuk 4). Voorbehandeling van wildtype zaden bij verschillende constante temperaturen gedurende verschillende tijdsduren, gevolgd door 15 min belichting met rood licht en een incubatie bij 10 of 24 °C leidde tot een patroon van breking en inductie van kiemrust. Hoe hoger de voorbehandelingstemperatuur, des te sneller kiemrust gebroken werd en des te sneller kiemrust weer opnieuw geïnduceerd werd. Indirect bewijs dat kieming voorafgegaan wordt door *de novo* synthese van GAs, werd geleverd door de remming van de door licht gestimuleerde kieming door tetcyclasis. Deze conclusie werd verstevigd door pionier bepalingen van GAs in wildtype en GA ongevoelige *gai* zaden (Hoofdstuk 3).

Vier verschillende GAs werden geïdentificeerd in extracten van wildtype en *gai* zaden:

Vier verschillende GAs werden geïdentificeerd in extracten van wildtype en *gai* zaden: GA_1, GA_3, GA_4 en GA_9. Effecten van licht en een koudebehandeling op gehaltes van GA_1, GA_4 en GA_9, bepaald met behulp van gedeutereerde standaarden, werden vergeleken met effecten op de kieming. In niet-koudebehandelde zaden, verhoogde licht zowel GA gehaltes alsook de kieming in afwezigheid van toegediende GAs. Ten gevolge van belichting werden GA gehaltes in *gai* zaden 7-10 maal zo hoog als in wildtype zaden. Een koude behandeling van 7 dagen 2 °C verhoogde het gehalte aan GA_4 in het donker, maar niet in het licht, hoewel licht de kieming sterk stimuleerde. Daarom kan geconcludeerd worden dat een toename in het gehalte aan bepaalde GA(s) als gevolg van licht of koude belangrijk kan zijn voor de kieming, maar duidelijk niet de enig beslissende factor is.

De gevoeligheid voor GAs speelt ook een bepalende rol in de regulatie van kieming (Hoofdstuk 4). De gevoeligheid nam toe door een voorbehandeling bij 2 °C (tot 13 weken), droge narijping van het zaad of kieming in licht. De gevoeligheid nam daarentegen af wanneer de zaden aan een koudeperiode van meer dan 13 weken werden blootgesteld of aan hogere temperaturen, bijvoorbeeld 8 dagen aan 15 °C. Verschuivingen in GA gevoeligheid waren niet alleen te zien in wildtype zaad, maar ook in *ga1* en *gai/ga1* zaad, dus in afwezigheid van endogene GAs, wat erop duidt dat GA synthese niet nodig is voor wisselingen in kiemrust. Recombinant (*gai/ga1*) zaad reageerde pas op licht wanneer kiemrust gebroken was door een koudeperiode van 1 week of een droge narijpingsperiode van enkele maanden. De kiemtemperatuur bepaalde of verschuivingen in GA gevoeligheid wel of niet zichtbaar waren. Breking van kiemrust kon het beste bestudeerd worden bij een temperatuur waarbij de GA gevoeligheid relatief laag is (24 °C) en inductie van kiemrust bij een temperatuur van relatief hoge GA gevoeligheid (10 °C).

Geconcludeerd werd dat de combinatie van GA synthese en een voldoende hoge GA gevoeligheid essentieel is voor de kieming, maar dat beide factoren niet direct kiemrust reguleren. Beide factoren hangen af van de mechanismen die de responssystemen voor omgevingsfactoren sturen. In eerste instantie reguleert temperatuur de gevoeligheid voor licht. Na belichting kan zowel de synthese van GAs gestimuleerd worden, alsook de gevoeligheid voor GAs. GA gevoeligheid kan echter ook direct door temperatuur gereguleerd worden, zonder dat daar licht voor nodig is.

Deze conclusies werden versterkt wanneer de regulatie van kiemrust en kieming onder natuurlijke omstandigheden bestudeerd werd (Hoofdstuk 5). Zaden van wildtype en de *ga1* mutant werden buiten te imbiberen gelegd, zonder dat licht bij de zaden kon komen. De maximale imbibitieduur was 18 maanden. Regelmatig werd de kiembereidheid van porties zaden bepaald onder laboratoriumomstandigheden. De kieming werd bepaald bij 10 en 24 °C. Wildtype zaden lieten een duidelijk kiemrustpatroon zien, wanneer de kiembereidheid getest werd na roodbelichting in afwezigheid van GA. Primaire en secundaire kiemrust werden gebroken gedurende de zomer en kiemrust werd weer geïnduceerd in het najaar en in de winter. Een kleine kiemingspiek was zichtbaar in het vroege voorjaar. Zaden van de *ga1* mutant kiemden op geen enkel tijdstip zonder GAs, wat weer de absolute GA behoefte voor de kieming van *A. thaliana* bevestigt.

Om de rol van gevoeligheid voor GAs, licht en nitraat in de regulatie van seizoengebonden wisselingen in kiemrust en kieming te bepalen, werden dosis-respons experimenten uitgevoerd. Kiemingsgegevens werden gefit als logistische dosis-responscurven en de bijbehorende curve parameters werden berekend. Dosis-responscurven voor licht werden in wildtype zaden bepaald in afwezigheid van exogene GAs en in *ga1* zaden in aanwezigheid van 0.25 of 1 μM GA_{4+7}. In beide genotypen lieten de dosis-responscurven reversibele, parallelle verschuivingen zien. De gevoeligheid voor GAs nam 50-100 maal toe gedurende de eerste 3-4 maanden van imbibitie buiten. De grootste verschuiving vond plaats in de dosis die nodig was voor half-maximale respons. Minimum- en maximum respons varieerden maar weinig. De toename in GA gevoeligheid werd vrijwel niet of slechts gedeeltelijk gevolgd door een afname in de winter. Veranderingen in gevoeligheid voor GAs zwakten duidelijk af naarmate de zaden langer buiten te imbiberen lagen. Verschuivingen in gevoeligheid voor licht bleven daarentegen duidelijk bestaan. Nitraat of gevoeligheid voor nitraat speelde geen rol van belang in de regulatie van kiemrust en kieming van *A. thaliana* zaden.

Een vergelijkbaar onderzoek werd gedaan met zaden van de verwante soort *Sisymbrium officinale* (gewone raket) (Hoofdstuk 6). In november 1990 werden zaden van deze soort buiten onder natuurlijke omstandigheden begraven in de bodem. Geregeld werden porties zaden opgegraven om dosis-respons experimenten voor licht, nitraat en GAs in het

laboratorium uit te voeren. Kiemingsgegevens werden wederom gefit als logistische dosis-responscurven. De kiembereidheid werd bij 15 °C bepaald, omdat eerder onderzoek aangetoond had dat kiemrustpatronen het beste bij deze temperatuur te zien waren. Ook was eerder aangetoond dat kieming van deze soort absoluut afhankelijk is van de gelijktijdige beschikbaarheid van licht en nitraat, hetzij exogeen, hetzij endogeen. Wanneer de mate van kiembereidheid van opgegraven zaden getest werd in aanwezigheid van verzadigende hoeveelheden licht en nitraat, was een duidelijk seizoengebonden kiemrustpatroon te zien. Kiemrust werd gebroken in het najaar en in de winter en opnieuw geïnduceerd in de zomer. Om te voorkomen dat andere kiemingsfactoren beperkend zijn, werden dosis-respons experimenten voor licht gedaan in aanwezigheid van een verzadigende concentratie KNO_3 en dosis-respons experimenten voor nitraat na het geven van een verzadigende dosis rood licht. Reversibele, seizoengebonden wisselingen in gevoeligheid voor licht en nitraat waren duidelijk te zien. Er traden verschuivingen op in de maximum respons, de minimum respons en de dosis die nodig was voor half-maximale respons. De verschuivingen in dosis-responscurven verliepen parallel. Veranderingen in gevoeligheid voor licht en voor nitraat lieten opvallende overeenkomsten zien. Tijdens het breken van primaire kiemrust in het voorjaar van 1991, vertoonden de fluence responscurven een bi-fasisch verloop. De nitraat dosis-responscurven konden allemaal gefit worden als mono-fasische curven. Het kan echter niet uitgesloten worden dat sommige curven een bi-fasisch karakter hadden, hetgeen moeilijk te zien was omdat het verschil tussen minimale en maximale kieming soms gering was. De gevoeligheid voor GAs nam geleidelijk toe vanaf het moment dat de zaden in de grond begraven werden en was niet gecorreleerd aan wisselingen in kiemrust. Daarom kan gevoeligheid voor GAs niet beschouwd worden als een beperkende factor in de regulatie van kiemrust van deze soort.

De waargenomen verschuivingen in curve parameters van fluence- (en nitraat dosis-) responscurven die in beide soorten waargenomen zijn, kunnen geïnterpreteerd worden als veranderingen in de hoeveelheid beschikbare receptoren, veranderingen in de bindingseigenschappen van de receptoren en/of in de signaal-transductie keten die gestart wordt na de vorming van het complex ligand-receptor, dat gevormd wordt na belichting, eventueel in combinatie met nitraat.

Zaden van *S. officinale* werden ook gebruikt om te bepalen of metabolische veranderingen in de zaden een rol spelen bij de regulatie van kiemrust (Hoofdstuk 7). Deze soort heeft als voordeel dat een duidelijk onderscheid mogelijk is tussen rustbreking en vroege kiemingsprocessen. Wanneer zaden in het donker worden voorbehandeld, vinden, afhankelijk van temperatuur en tijdsduur van voorbehandeling, wisselingen in de mate van kiemrust plaats. Zolang de zaden geen licht en nitraat krijgen, kunnen in principe kiembereide zaden niet kiemen. De snelheid van zuurstofopname van zaden die in het donker voorbehandeld waren, werd bij 24 °C gemeten met behulp van zuurstofelectroden. De snelheid van zuurstofopname werd vergeleken met de mate van kiembereidheid in 25 mM KNO_3 bij dezelfde temperatuur, nadat de zaden 15 min waren belicht met rood licht. Voorbehandeling vond plaats bij 6, 10, 15 of 24 °C. Tijdens voorbehandeling bij alle temperaturen werd een snelle toename in de snelheid van zuurstofopname gevolgd door een geleidelijke afname. De toegenomen snelheid van zuurstofopname correleerde met de snelheid van wateropname, maar ook met breking van kiemrust. Echter wanneer zaden te imbiberen werden gelegd nadat ze teruggedroogd waren na een eerdere periode van imbibitie, nam de zuurstofopname niet toe, terwijl de kiembereidheid wel toegenomen was in vergelijking met niet-teruggedroogde zaden. Tijdens breking van secundaire kiemrust door een koudebehandeling vond ook geen toename in de snelheid van zuurstofopname plaats. De afname in de snelheid van zuurstofopname, die afhankelijk van de temperatuur, 9-30 uur na de start van imbibitie begon, was niet algemeen gecorreleerd met inductie van secundaire kiemrust. Wanneer kiemrust opnieuw geïnduceerd werd na een koudebehandeling, nam de snelheid van zuurstofopname niet verder af. Wanneer zaden lange tijd bij 24 °C te imbiberen werden gelegd, bleef de kiembereidheid 0 % en de snelheid van zuurstofopname bleef op een laag constant niveau. Geconcludeerd werd dat veranderingen in ademhalingsactiviteit niet noodzakelijk zijn voor wisselingen in kiemrust. Een laag niveau van zuurstofopname tijdens lange perioden van begraving in de bodem, dat alleen beïnvloed wordt door de temperatuur op dat moment en het vochtgehalte van de zaden, is van groot belang voor de overleving van het zaad in de bodem, omdat snelle uitputting van voedselreserves voorkomen wordt. Wanneer zaden die niet in kiemrust zijn licht en nitraat aangeboden werd, waardoor ze konden gaan kiemen, nam de snelheid van zuurstofopname enorm toe. De eerste toename

vond duidelijk plaats voordat het kiemworteltje door de zaadhuid heenbrak, erop duidend dat meting van de zuurstofopname een zeer gevoelige parameter is om vast te stellen of doorbreken van de zaadhuid verwacht mag worden. Een verrood bestraling voorkwam de toename in de snelheid van zuurstofopname wanneer ze gegeven werd voordat kieming ontsnapte aan de verrood antagonerende werking van rood licht. De kieming zelf hangt dus duidelijk af van een verhoogde ademhalingsactiviteit.

Om te onderzoeken of door het volledig onderdompelen en constant roeren van zaden in zuurstofelectroden de ademhalingssnelheid niet overschat werd, werd de snelheid van zuurstofopname ter vergelijking met behulp van een gaschromatograaf bepaald (Hoofdstuk 8). Gelukkig gaven beide methodes vergelijkbare resultaten. De snelheid van zuurstofopname en afgifte van CO_2 werd gevolgd tijdens langdurige imbibitie in het donker bij 24 °C. Tijdens de eerste 8 uur imbibitie namen beide snelheden toe en namen daarna weer af. Constante niveaus van zuurstofopname (25-30 nmol min^{-1} g^{-1} zaad) en CO_2 afgifte (17 nmol min^{-1} g^{-1} zaad) werden na 3 weken imbibitie bij 24 °C bereikt. Deze niveaus bleven verder constant gedurende minstens 3 maanden. De verhouding van CO_2 afgifte en zuurstofopname (respiratoir quotiënt) varieerde tussen 0.55 en 0.7. Een respiratoir quotiënt van 0.7 kan worden verwacht in de vethoudende zaden van *S. officinale*. Lagere waarden duiden erop dat ook andere zuurstof consumerende reacties plaatsvinden, waarbij minder CO_2 vrijkomt, bijvoorbeeld oxydatie van fenolen in de zaadhuid.

In zaden van diverse soorten is het voorkomen van twee routes van mitochondrieel electronentransport aangetoond: de cytochroomroute en de alternatieve route. Beide routes kunnen door specifieke remstoffen geblokkeerd worden: de cytochroomroute door cyanide en de alternatieve route door hydroxamaten. Effecten van KCN en benzohydroxyzuur (BHAM) werden bestudeerd op de kieming en op de zuurstofopname van zaden van *S. officinale* (Hoofdstuk 8). Het was opmerkelijk dat KCN concentraties die vrijwel volledig electronentransport via de cytochroomroute remden, de kieming nauwelijks deden afnemen. BHAM remde de kieming al bij concentraties die de zuurstofopname niet remden. Om concentraties van KCN en BHAM vast te stellen die de betreffende route volledig remmen zonder nevenwerkingen te laten zien, werden titratie curven gemaakt. Een KCN concentratie van 1,5 mM remde de cytochroomroute volledig en een BHAM concentratie van 30 mM de

alternatieve route. Deze concentraties oefenden geen neveneffecten uit. De relatieve bijdrage van de ademhalingsroutes veranderde tijdens imbibitie bij 24 °C. Tijdens de eerste 48 uur imbibitie was 70 % van de zuurstofopname toe te schrijven aan de cytochroomroute, 10 % aan de alternatieve route en 20 % was restademhaling, welke ongevoelig is voor KCN en BHAM. Na 240 uur imbibitie bij 24 °C verdween de electronenstroom door de alternatieve route volledig. De capaciteit van de alternatieve route bedroeg nog steeds 10 %. De bijdrage van de cytochroomroute nam af en de restademhaling bleef constant. Na 2 weken imbibitie bij 24 °C vond 50 % van de zuurstofopname plaats via de cytochroomroute en 50 % was restademhaling. Ondanks veranderingen in de bijdrage van verschillende ademhalingsroutes, veranderde het respiratoir quotiënt van CO_2 afgifte en zuurstofopname niet. Het voorkomen van de alternatieve route direct na de start van imbibitie kan wellicht toegeschreven worden aan een overmaat NADH die geoxydeerd moet worden. De alternatieve route fungeert dan als een soort overloop mechanisme.

De resultaten werden geïntegreerd in de algemene discussie in Hoofdstuk 9. Een schema wordt getoond waarin de (bodem)temperatuur beschouwd wordt als de belangrijkste factor in het mechanisme waarmee zaden de tijd in het jaar kunnen 'waarnemen'. De bodemtemperatuur reguleert de gevoeligheid voor licht en eventueel voor andere omgevingsfactoren, zoals bijvoorbeeld nitraat. Na perceptie van licht (en nitraat) wordt een signaal-transductie keten gestart, die uiteindelijk tot kieming leidt. In deze keten wordt de synthese van GAs gestimuleerd. Ook de gevoeligheid voor GAs wordt verhoogd. De GA gevoeligheid kan ook rechtstreeks door de temperatuur verhoogd worden. Wanneer hetzij het gehalte aan GAs, hetzij het responssysteem voor GAs limiterend is, wordt kieming voorkomen. Hoewel kieming zonder meer afhangt van het gehalte aan bio-actieve GAs en het aanwezige responssysteem, gaan veranderingen in kiemrust niet noodzakelijkerwijze gepaard met veranderingen in beide factoren en daarom zijn beide niet primair regulerend in kiemrustwisselingen. Het responssysteem voor licht (en nitraat), en niet de omgevingsfactoren zelf, bepaalt in welke periode van het jaar kieming mogelijk is. Wisselingen in kiemrust hangen ook niet af van veranderingen in ademhalingsactiviteit, terwijl de kieming een verhoogde ademhalingsactiviteit duidelijk nodig heeft.

Omdat de kieming van vele soorten afhankelijk is van fytochroom, kan de regulatie van

kieming en kiemrust, zoals die beschreven is voor zaden van *A. thaliana* en *S. officinale*, geldig zijn voor vele soorten.

Curriculum vitae

Maria Petronella Matthea Derkx werd geboren op 8 december 1962 te Venlo. Van augustus 1975 tot mei 1981 bezocht zij het Collegium Marianum te Venlo. Na het behalen van het diploma Gymnasium-ß begon zij in september 1981 met een studie tuinbouwplantenteelt aan de Landbouwuniversiteit te Wageningen. Het kandidaatsexamen werd in januari 1985 met lof behaald. Tijdens de doctoraalfase werden de volgende afstudeervakken gedaan: plantenfysiologie (verzwaard hoofdvak), tuinbouwplantenteelt (hoofdvak) en onkruidkunde (bijvak). Het doctoraalexamen (oude stijl) werd in november 1987 met lof behaald. Van 1 januari 1988 tot 1 januari 1992 heeft zij promotie-onderzoek verricht bij de vakgroep Plantenfysiologie van de Landbouwuniversiteit in Wageningen. Het projectvoorstel 'Regulatie van seizoengebonden wisselingen in kiemrust van zaden' had zij zelf bij de Vaste Commissie voor de Wetenschapsbeoefening (VCW) ingediend. Het promotie-onderzoek heeft geleid tot dit proefschrift.