HORMONAL REGULATION OF SEED DEVELOPMENT AND GERMINATION IN TOMATO. STUDIES ON ABSCISIC ACID- AND GIBBERELLIN-DEFICIENT MUTANTS

HORMONALE REGULATIE VAN DE ZAADONTWIKKELING EN KIEMING BIJ TOMAAT. ONDERZOEK AAN ABSCISINEZUUR- EN GIBBERELLINE-DEFICIËNTE MUTANTEN

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HORMONAL REGULATION OF SEED DEVELOPMENT AND GERMINATION IN TOMATO

STUDIES ON ABSCISIC ACID- AND GIBBERELLIN-DEFICIENT MUTANTS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. C. C. OOSTERLEE,
IN HET OPENBAAR TE VERDEDIGEN
OP WOENSDAG 20 MEI 1987
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWUNIVERSITEIT TE WAGENINGEN

ISN 259700

The investigations were supported by the Foundation for Fundamental Biological Research (BION), financed by the Netherlands Organization for the Advancement of Pure Research (ZWO) and performed at the Department of Plant Physiology, Agricultural University Wageningen, The Netherlands.

NN08201, 1140

STELLINGEN

I Hormoon-deficiënte mutanten zijn ideale blanco's bij het vaststellen van de regulerende rol van hormonen.

C.M. Karssen and E. Łaçka, 1986. A revision of the hormone balance theory of seed dormancy: studies on gibberellin and/or abscisic acid deficient mutants of *Arabidopsis thaliana*. In: Plant growth substances 1985 (M. Bopp, ed.), pp. 315-323. Springer-Verlag, Berlin-Heidelberg.
Dit proefschrift.

II De door endogene gibberellinen geïnduceerde hydrolyse van de endospermcelwanden is essentieel voor de kieming van tomatezaden.

Dit proefschrift.

III De door ontwikkelende zaden geproduceerde gibberelline(n) stimuleren bij tomaat de vruchtgroei en vertragen de vruchtrijping.

Dit proefschrift.

IV De op grond van experimenten met geïsoleerde embryo's gepostuleerde hypothese dat abscisinezuur in ontwikkelende zaden de synthese van reserve-eiwitten stimuleert en de voortijdige kieming remt, wordt tegengesproken door het ongewijzigd verloop van deze processen in ontwikkelende abscisinezuurdeficiënte zaden.

R.C. Ackerson, 1984. Regulation of soybean embryogenesis by abscisic acid. J. Exp. Bot. 35: 403-413.
R.C. Ackerson, 1984. Abscisic acid and precocious germination in soybeans. J. Exp. Bot. 35: 414-421.
Dit proefschrift.

V Bij het interpreteren van splitsingsverhoudingen in een koppelingsanalyse dient rekening gehouden te worden met mogelijke pleiotrope effecten op de pollen en/of zaadkieming.

Dit proefschrift.

VI De argumenten die De Lacaze-Duthiers en Kükenthal gebruiken ter afscheiding van de koraalsoort *coralloides* van het genus *Alcyonium* zijn onterecht.

H. De Lacaze Duthiers, 1900. Les coralliaires de Golf de Lion, Alcyonaires.
W. Kükenthal, 1916. Die gorgonarien Westindiens. Kap. 1 Die Scleraxonier.
S.P.C. Groot and S. Weinberg, 1982. Biogeography, taxonomical status and ecologie of *Alcyonium (Parerythropodium) coralloides* (Pallas, 1766). Marine ecology 3: 293-312.

VII Verbetering van het wortelstelsel door middel van veredeling kan een bijdrage leveren aan een grotere stresstolerantie van het bovengrondse deel van het gewas.

S. Zijlstra and A.P.M. den Nijs, 1987. Effects of rootsystems of tomato genotypes on growth and earliness, studied in grafting experiments at low temperature. Euphytica 36: in druk.

VIII Aantasting van klassiek genetisch onderzoek is een vorm van genetische erosie.

IX Kruisbestuiving tussen klassiek-genetisch en plantenfysiologisch onderzoek levert ongedachte 'hybrid vigour'.

X Afschaffing van het zevenvoudig brevettensysteem en de daaraan gekoppelde hiërarchie in de Nederlandse Onderwatersportbond (NOB) verbetert zowel de motivatie als de veiligheid van de beginnende sportduik(st)er.

XI Bij handhaving van het huidige regeringsbeleid ten aanzien van verlaging van de budgetten voor wetenschappelijke onderzoeksinstituten en verhoging van de subsidies aan het bedrijfsleven, lijkt het voortbestaan van wetenschappelijk onderzoek gebaat te zijn bij een onmiddellijke privatisering.

XII Roken in een restaurant getuigt van smakeloosheid.

Stellingen behorende bij het proefschrift: "Hormonal regulation of seed and fruit development and germination in tomato. Studies on abscisic acid- and gibberellin-deficient mutants." door Steven P.C. Groot.

Wageningen, 20 mei 1987

LANDBOUWUNIVERSITEIT

in herinnering aan mijn
grootvader, B. Boeve

VOORWOORD

Het voor u liggende proefschrift beschrijft het grootste deel van de resultaten van een driejarig onderzoeksproject dat is uitgevoerd op de vakgroep Plantenfysiologie van de Landbouwuniversiteit. Een groot aantal mensen heeft direct of indirect een bijdrage geleverd aan het project en aan de totstandkoming van dit proefschrift. Het is ondoenlijk om op deze plaats iedereen persoonlijk te bedanken, toch wil ik een aantal mensen met name noemen.

Allereerst Professor Dr. C.M. Karssen, begeleider bij het onderzoek en een van mijn twee promotoren. Beste Cees, jouw voorbereiding van het project maakte het welhaast onmogelijk om geen resultaten te verkrijgen, het bespaarde mij in ieder geval de frustraties van menige promovendus. Dat je jezelf weleens 'vader' van de zaadclub noemt, tekent je belangstelling voor ons, binnen en buiten het Botanisch Laboratorium.

Ingrid van Yperen verrichtte een groot deel van de biochemische analyses. Ingrid, dankzij jouw grote inzet en nauwkeurige wijze van werken was het mogelijk om uit de lastige problematiek van de reserve-eiwitten te komen.

Het vaak intensief meedenken van de overige leden van de zaadclub leverde waardevolle suggesties. Met Roelf Weges bracht ik 3 jaar op dezelfde kamer door. De knetterende discussies die ik met hem en Cees voerde over godsdienst en politiek heb ook ik zeer gewaardeerd. De praktische ervaring van Henk Hilhorst en zijn kennis van de Engelse taal waren een welkome steun; aan de zwempartijen met Harro Boumeester bewaar ik meer dan één prettige herinnering.

De resultaten kwamen mede tot stand door de grote inzet en betrokkenheid van de studenten en HBO-stagiaires (in chronologische volgorde): Henriëtte Schmiermann, Mien van der Ven, Joska Buitendijk, Robert Dijkman, Arjan Stolte en Theo Heimgartner. Het aanstekelijke enthousiasme van Robert ging zelfs zover dat hij zowel zijn stage als zijn afstudeeropdracht bij mij uitgevoerd heeft.

The enzymological studies were carried out in cooperation with the Polish scientist Barbera Kieliszewska-Rokicka, whom I would like to join once on a trip in the Tatra mountains.

Zonder de mutanten zou het onderzoek niet mogelijk geweest zijn. Zij werden beschikbaar gesteld door Professor Dr. J. van der Veen en Maarten Koornneef van de vakgroep Erfelijkheidsleer.

Professor Dr. J.A.D. Zeevaart, Michigan State University USA, demonstrated the gibberellin deficiency of the ga-1 and the ga-2 mutants.

De opkweek van planten voor de proeven en de productie van de vele zaden die nodig waren in de kiemingsexperimenten vond plaats dankzij de inzet van Gerrit van Geerenstein, Aart van Ommeren, Jan Verburg, Wilbert Alkemade, Willem van Blijderveen, Tia Bosma, Henk Kuipers en verscheidene vakantiehulpen.

Professor Dr. J. Bruinsma, mijn tweede promotor, dank ik voor de gastvrijheid die ik bij de vakgroep Plantenfysiologie genoten heb en tevens voor de waardevolle opmerkingen bij de jaarlijkse onderzoeksbesprekingen en bij het doornemen van de manuscripten.

Dat er uiteindelijk toch nog een bruikbare bepalingsmethode voor uiterst geringe hoeveelheden abscisinezuur tot stand kwam was te danken aan het koppige doorzettingsvermogen van Evert Vermeer en zijn grote vakkennis op het gebied van de gaschromatografie.

Toch kom je zonder goed functionerende apparatuur ook in de plantenfysiologie niet ver. Gelukkig waren Ruth van der Laan en Ben van der Swaluw altijd bereid te helpen.

Alle andere leden van de vakgroep Plantenfysiologie dank ik voor hun collegialiteit, de prettige sfeer en de medewerking op allerlei terreinen.

De endospermweerstandsmetingen zijn uitgevoerd bij het TNO Instituut voor Graan Meel en Brood. Met name Herman van der Haar, Jerry van Maanen en John Wokke van de afdeling Physica wil ik bedanken voor de gastvrijheid en voor de interesse die ze me bijbrachten voor het gebruik van de PC.

Allex Haasdijk en Paul van Snippenburg verzorgden de tekeningen, Sybout Massalt het fotowerk en Koos Keizer maakte de SEM-opname voor de omslag van het proefschrift. Frank Dumoulin hielp, met groot enthousiasme en vingervlugheid op de PC, bij de lay-out en het camera-gereed maken van de tekst.

Ten slotte wil ik vooral mijn ouders bedanken voor de mogelijkheden en de stimulans die ze me gaven om verder te leren en Marelle voor de steun bij het schrijven van het proefschrift.

CONTENTS

ABSTRACT

The role of endogenous gibberellins (GAs) and abscisic acid (ABA) in seed development and germination of tomato, was studied with the use of GA- and/or ABA-deficient mutants.

GAs are indispensable for the development of fertile flowers. Fertility of GA-deficient flowers is restored by application of exogenous GAs. Fruits and seeds develop without GA, with a possible exception for the initial stage of seed growth. However, seed-produced GA delays maturation of the seeds and ripening of the fruit by one week and increases final seed and fruit weights.

ABA levels in developing ABA-deficient mutant seeds are strongly reduced compared to wild-type seeds. Despite the strong reduction of endogenous ABA levels neither dry matter accumulation nor storage protein synthesis are affected.

In wild-type seeds embryo-produced ABA is responsible for the development of dormancy during seed development. ABA-deficient seeds germinate viviparously in over-ripe fruits. Germination of wild-type seeds is also inhibited after harvest. Dormancy is relieved during a short period of dry storage. Stored wild-type seeds are much more sensitive to osmotic inhibition of radicle growth and germination than ABA-deficient seeds. Both types of seeds are equally sensitive to inhibition by exogenous ABA.

GA is indispensable for tomato seed germination. GA produced by the embryo and excreted to the endosperm, induces hydrolysis of the galactomannan-rich endosperm cell walls. The hydrolysis causes weakening of the mechanical restraint of the endosperm layers that oppose the radicle tip, thereby permitting the radicle to protrude. The separation of ABA- and GA-action in time and in site is a strong argument against the existence of a hormone balance for the regulation of seed dormancy.

Abbreviations:

ABA	abscisic acid
dap	days after pollination
GA	gibberellin
GAs	gibberellins
GA_{4+7}	mixture of the gibberellins GA_4 and GA_7
PAGE	polyacrylamide gel electrophoresis
SD	standard deviation
SDS	sodium dodecyl sulphate

Chapter 1

General introduction

Seeds

Seeds have an essential function in the reproduction of higher plants and often facilitates dispersal of the species. They are also a main food source and are therefore indispensable for the survival of mankind. Seeds are the origin and produce of many agricultural crops and supply raw material for several technologies. Therefore, considerable efforts have been made to improve seed quality by breeding programs. Studies of seed development and germination may also contribute to the improvement of seed quality. In particular plant hormones are suggested to play an important role in the regulation of seed quality. A regulatory role in food storage and mobilization and in dormancy and germination has often been suggested (See Khan 1982, King 1982, Van Staden et al. 1982, Pharis and King 1985, for recent reviews).

The role of hormones in seed development

Cytokinins, auxins, gibberellins (GAs) and abscisic acid (ABA) have been chemically identified in extracts of immature seeds of a variety of species. In developing seeds they reach the highest levels known in plant organs. Nevertheless, definite proof for a regulatory role of endogenous hormones during seed development is still scanty (Karssen 1982a).

In the majority of studies on hormonal control of seed development conclusions are based on the simultaneous occurrence of peak values in hormone content and certain morphological and biochemical changes. It is obvious that such correlations do not prove that hormones are causally involved in specific aspects of seed growth and development. Therefore, attempts have been made to test the causality of correlative evidence by environmental, chemical, or genetic manipulation of endogenous hormone levels.

Environmental manipulation. In other plant organs endogenous hormone levels are often sensitive to subtle changes in the physical environment. Such induced changes are hardly known from studies of seed development, however. The levels of cytokinins and ABA in developing seeds can be manipulated by influencing their synthesis in roots and leaves, respectively. For instance,

exposure of roots to water-logging conditions increased the cytokinin content of barley grains (Michael and Seiler-Kelbitsch 1972), while application of water stress to leaves increased the transport of ABA to seeds (Goldbach and Goldbach 1977, Dewdney and McWha 1979). In developing seeds of tomato cytokinin levels also increased when partial removal of leaves augmented the competitive force of the seeds (Varga and Bruinsma 1974, Monselise et al. 1978).

Chemical manipulation. The synthesis of GAs in developing seeds has been successfully inhibited by such growth retardants as ACPC and chlormequat (Baldev et al. 1965, Zeevaart 1966).

Genetic manipulation. The use of isogenic lines differing in endogenous hormone content, or hormone sensitivity, offers a decisive means of elucidating the regulatory functions of plant growth substances. The method has been successfully applied in studies on the role of endogenous GAs in elongation growth and of ABA in stomatal closure (Phinney 1985, Tal and Nevo 1973). ABA-deficient mutants of Arabidopsis thaliana proved to be useful tools in studies on the role of this hormone in dormancy induction (Karssen and Łącka 1986).

Cytokinins. A role of cytokinins in seed development has been suggested in a few studies. In tomato partial defoliation increased cytokinin levels in seeds, which was accompanied by an enhanced rate of seed development (Monselise et al. 1978). A comparison of near-isogenic lines of barley with different grain weights showed that large-grain lines contained a significantly higher cytokinin content during early seed growth than small-grain lines (Seiler-Kelbitsch et al. 1975).

Auxins. In the same barley mutants high grain weight correlated also with a significant higher auxin level (Mounla et al. 1980).

Gibberellins. Spraying of plants during seed development with inhibitors of GA-biosynthesis, such as ACPC and chlormequat strongly reduced GA levels in seeds but hardly reduced seed fresh weight in Pharbitis nil (Zeevaart 1966) and pea (Baldev et al. 1965). Barendse et al. (1986) studied the effect of GA on seed development in Arabidopsis thaliana by comparing seeds of wild-type and the GA-deficient ga-1 line. The lack of endogenous GA in developing seeds did not affect any aspect of seed growth, but fruit growth was clearly inhibited.

Abscisic acid. Studies with ABA-deficient mutants of Arabidopsis thaliana unequivocally proved that in this species endogenous ABA is responsible for

the induction of dormancy during seed development (Karssen et al. 1983). A stimulatory role of endogenous ABA in the synthesis of storage proteins was suggested in studies on the cultivation of immature seeds or seed parts in vitro in the presence or absence of exogenous ABA (Triplett and Quatrano 1982, Schroeder 1984, Bray and Beachy 1985). Crouch et al. (1985) showed that a similar stimulatory effect could be obtained, even more rapidly, by cultivation in an osmoticum. This stimulatory effect was not mediated by an increase in the endogenous ABA level (Finkelstein and Chrouch 1986).

The role of hormones in the maintenance and relief of dormancy.

After development and maturation, most seeds enter a period of developmental arrest that is characterized by dehydration and cessation of growth and results in either quiescent or dormant seeds. Quiescent seeds only require rehydration for germination, whereas dormant seeds do not germinate at any set of environmental conditions (true dormancy) or at a more or less limited range only (relative dormancy). Breaking of dormancy results in a widening of the range of suitable conditions for germination, making seeds more sensitive to stimulatory factors such as light, nitrate and growth regulators (Karssen 1982b). Under natural conditions dormancy prevents germination under circumstances that are unfavourable for the survival of the seedling, such as burial in soil, shade or extreme temperatures. Modern agriculture demands full germination at broad range of environmental conditions. Therefore, studies on the mechanisms and regulation of dormancy and germination form an essential basis for agricultural practice.

It has often been suggested that dormancy and germination depend on the interaction between the growth-inhibiting hormone ABA and the growth-promotive hormones GA and, possibly, cytokinins (Khan 1977). The effects of environmental factors such as light and temperature on the maintenance and relief of dormancy are thought to be mediated by changes in the balance between growth-promotive and -inhibitory hormones. The hormone-balance theory of seed dormancy is mainly based on the effects of applied growth regulators. Doubts on the validity of the hypothesis have been expressed (Black 1980/81, Bewley and Black 1982, Wareing 1982).

Mutants which lack the capacity to synthesize specific hormone(s), or are insensitive to hormone(s), provide unique tools to test the theory. The germination of GA-deficient seeds of Arabidopsis thaliana is absolutely dependent on the application of exogenous GA (Koornneef and Van der Veen 1980). The requirement for GA is influenced by the action of ABA during seed development; seeds that are deficient for both GA and ABA require much lower GA levels for germination than GA-deficient seeds (Karssen and Łaçka 1986). Based on these

observations Karssen and Łaçka (1986) formulated a revision of the hormone--balance theory of seed dormancy. In their opinion endogenous ABA and GA never act simultaneously on dormancy in Arabidopsis thaliana. The role of ABA is restricted to the induction of dormancy during seed development, whereas GA is only active in the stimulation of germination. The intermediate state between the action of both (classes of) hormones is that of dormancy: low ABA-levels mean a low level of dormancy that requires low GA-levels during germination, high ABA-levels induce deep-dormant seeds that require relatively high GA--levels to germinate.

Present study

In the present thesis the role of endogenous GA and ABA in the regulation of seed development, dormancy and germination in tomato is studied by means of GA- and/or ABA-deficient mutants. Compared to the tiny seeds of Arabidopsis thaliana the large size of tomato seeds enables a study of the localization of hormone action in the different seed parts during development and germination.

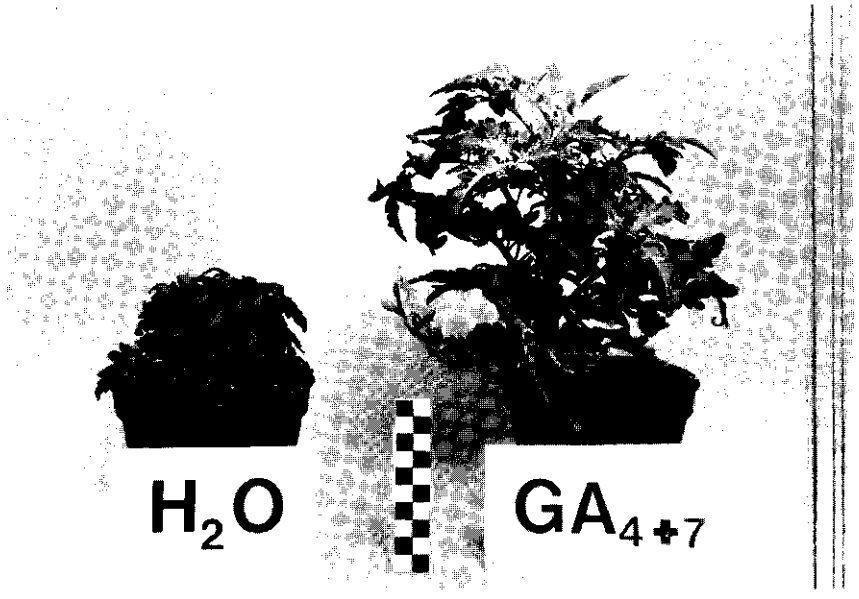

Fig. 1. GA-deficient tomato plants after 4 month cultivation. Plants were raised from ga-1 seeds from which endosperm and testa were removed in the area opposing the radicle. Plants grew without any application of exogenous GAs (H_2O) or with one single spray with 10 µM GA_{4+7} one week before the picture was taken. Bar represents 10 cm.

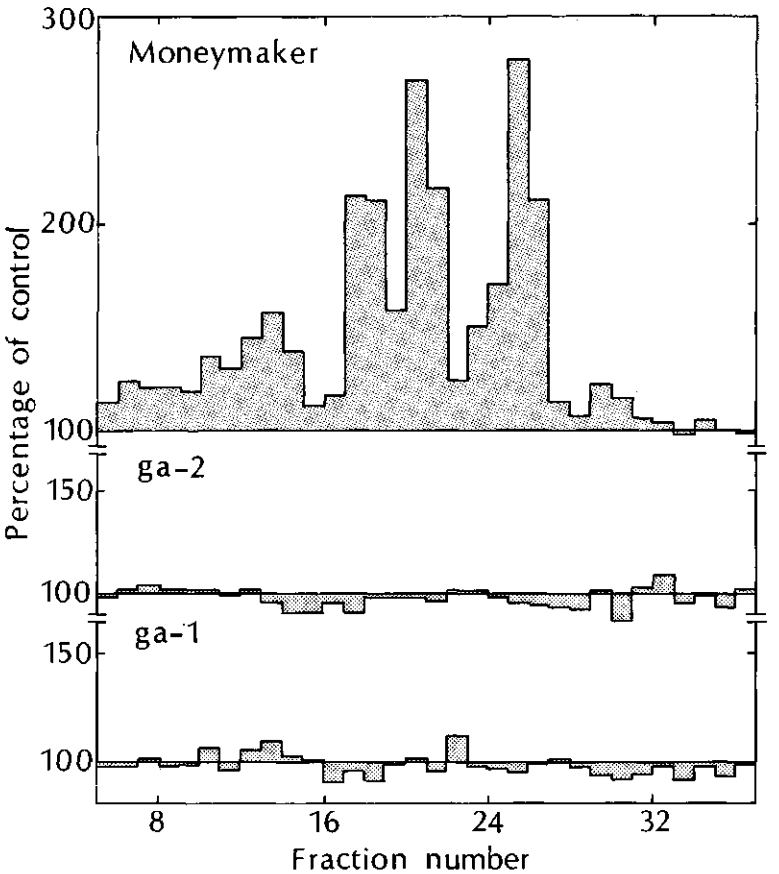

Fig. 2. Response in maize-d-5-bioassay to extracts of immature wild-type Moneymaker, ga-1 and ga-2 fruits. For each genotype 20 g dry weight (lyophilized) of immature fruits up to 3 cm in diameter were extracted and the acidic phase was subjected to reverse phase HPLC. The fraction number represents the gradient from 20% to 100% methanol. Each fraction was applied to four d-5 maize plants. The first + second leaf sheaths were measured after one week. The data are expressed as percentage of control (untreated 1^{st} + 2^{nd} leaf sheath = 41 mm). (Original graph courtesy of Professor J.A.D. Zeevaart, reproduced with permission).

The two GA-deficient lines and the ABA-deficient line, used in the present study, were isolated in a background of the cv. Moneymaker by Professor Van der Veen and Mrs Bosma of the Department of Genetics, Agricultural University, Wageningen (Koornneef et al. 1981, 1985). The mutations were induced by treatment of seeds with ethylmethanesulfonate.

GA-deficient mutants. Mutants of the genotypes ga-1/ga-1 and ga-2/ga-2 were selected in the M_2 population as non-germinating, GA_{4+7}-responsive seeds (Koornneef et al. 1981). The plants that develop from ga-1 or ga-2 seeds after GA-stimulated germination are extreme dwarfs with dark-green leaves and short internodes. Spraying the plants with 10 µM GA_{4+7} reverts the dwarfs phenotypically to wild-type plants (Fig. 1). The lack of endogenous GA in the ga-1 and ga-2 mutant has been demonstrated by Professor J.A.D. Zeevaart, Michigan State University, USA (Fig. 2).

ABA-deficient mutant. The ABA-deficient mutant, genotype sit^w/sit^w, was selected for wilting in the M_2 generation. The ABA level in the turgid leaves of sit^w plants is about 10% of that in turgid leaves of wild-type plants, water-stressed mutant leaflets do not accumulate ABA, in contrast to wild-type (Cornish and Zeevaart 1985).

In this thesis the role of endogenous GA during tomato seed germination was studied by comparing the germination of GA-deficient and wild-type seeds (Chapter 2). Special attention was paid to the weakening of the endosperm layers opposing the radicle tip. An analysis of the enzymatic hydrolysis of the endosperms is presented in Chapter 3. The role of endogenous GA in seed and fruit development was studied by comparison of seeds and fruits developing on ga-1 plants after pollination with either ga-1 or wild-type pollen (Chapter 4). A similar study was conducted on the role of endogenous ABA in seed development (Chapter 5). The influence of endogenous ABA on seed germination was studied in relation to GA-production and GA-deficiency (Chapter 6). A model for the role of endogenous ABA and GA in tomato seed germination is presented in Chapter 7, followed by a summary of the thesis in Chapter 8.

Chapter 2

Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants.

In collaboration with C.M. Karssen.
The text of this chapter is accepted for publication in Planta.

Abstract. Germination of tomato seeds c.v. Moneymaker is compared to the germination of seeds of the gibberellin-deficient dwarf mutant line ga-1, induced in the same genetic background. Germination of tomato seeds is absolutely dependent on the presence of either endogenous or exogenous gibberellins (GAs). GA_{4+7} is 1000-fold more active than commercial GA_3 in inducing germination of the ga-1 seeds. Red light, a pre-incubation at 2 °C, and ethylene do not stimulate germination of ga-1 seeds in the absence of GA_{4+7}. Fusicoccin stimulates independently, however.

Removal of the endosperm and testa layers opposing the radicle tip causes germination of ga-1 seeds in water. The seedlings and plants that develop from the detipped ga-1 seeds exhibit the extreme dwarfy phenotype that is normal to this genotype. Measurements of the mechanical resistance of the surrounding layers show that the major action of GAs is directed to the weakening of the endosperm cells around the radicle tip. In wild-type seeds this weakening occurs in water before radicle protrusion. In ga-1 seeds a similar event is dependent on GA_{4+7}, while fusicoccin also has some activity. Simultaneous incubation of de-embryonated endosperms and isolated axes shows that wild-type embryos produce an endosperm-weakening factor that is absent in ga-1 axes and is probably a GA. Thus, an endogenous GA facilitates germination in tomato seeds by weakening the mechanical restraint of the endosperm cells to permit radicle protrusion.

Introduction

Since the first report on the stimulative effect of gibberellic acid (GA_3) on lettuce seed germination (Lona 1956), similar results have been described for a large number of species. Nevertheless, definite proof that the promotive effect of GA_3 and other GAs on germination reflects the action of endogenous GAs within the seed is still absent. The well documented GA-induced mobilization of reserves in graminaceous seeds is a post-germination event (e.g. Ashford and Gubler 1984). In a few species a causal relationship has been shown between the effect of chilling on the release of dormancy and the increase of GA-like activity (Pinfield and Davies 1978, Taylor and Wareing 1979).

An alternative approach involves manipulation of endogenous GA levels by chemical or genetic means and observation of the resulting changes in germination and dormancy. Inhibition of seed germination by inhibitors of GA biosynthesis like diethylene glycol disulfide (Gafni and Shechter 1981) or 2-chloroethyltrimethylammonium chloride (chlormequat) (Hepher and Roberts 1985) have been reported. However, caution is required in interpreting results of inhibitor studies since secondary effects on e.g. sterol biosynthesis and cell division have been demonstrated (Douglas and Paleg 1974; Nitsche et al. 1985).

GA-deficient dwarfy mutants have been isolated in rice (Murakami 1972), barley (Hopp et al. 1981) and maize (Phinney and Spray 1982). Effects of the GA deficiency on the germination behaviour of their seeds have not been reported, however. Recently, GA-deficient mutants were isolated in Arabidopsis thaliana (Koornneef and Van der Veen 1980) and tomato (Koornneef et al. 1981, 1985). Germination of the Arabidopsis ga-1 mutant absolutely depends on the application of exogenous GAs (Karssen and Łaçka 1986).

The monogenic dwarf tomato mutants of the genotypes ga-1/ga-1 (line W335 = V335 = Ve335) and ga-2/ga-2 (line W270 = Ve270), were obtained after treatment of seeds of the cultivar 'Moneymaker' with ethylmethanesulfonate, followed by selection for non-germinating GA_{4+7}-responsive seeds in the M_2 population (Koornneef et al. 1981). The plants that develop after GA-stimulated germination of the mutant seeds are extreme dwarfs with dark-green leaves and very short internodes (Zeevaart 1984). In the mutant flower, the corolla and stamens do not elongate and the style is misshapen, both microsporocytes and macrosporocytes are initiated but they degenerate (Koornneef et al. 1985, Nester and Zeevaart 1986). Spraying the plants with 10 µM GA_{4+7} reverts the dwarfs phenotypically to wild-type plants, that develop flowers and produce fruits and seeds. Feeding studies with various GAs and precursors suggest that the GA-biosynthetic pathway in the ga-1 mutant is blocked prior to ent-kaurene and in the ga-2 mutant between kaurenoic acid and GA_{12} (Zeevaart 1984).

The present study analyzes the role of endogenous GAs in tomato seed germination with the use of the ga-1 mutant. Seeds of the cultivar 'Moneymaker' served as GA-producing controls. In particular the influence of GA-deficiency on the weakening of the endosperm opposing the tip of the radicle was examined. This process has been reported to be stimulated by exogenous GA_{4+7} in structurally related pepper seeds (Watkins and Cantliffe 1983). The stimulative actions of ethephon, fusicoccin (Nelson and Sharples 1980) and red light on germination was also studied in the presence or absence of endogenous GAs.

Material and methods

Seed material. Wild-type tomato , Lycopersicon esculentum Mill., cv. Moneymaker and the GA-deficient genotypes ga-1/ga-1 and ga-2/ga-2 were obtained from Professor J.H. van der Veen of the Department of Genetics, Agricultural University Wageningen. Plants for seed production were raised in a greenhouse during the summers of 1983 and 1985. GA-mutant plants were sprayed once a week with a solution of 10 µM GA_{4+7} (Berelex, ICI, U.K.) on the top and flowerbud regions to stimulate shoot growth and development of petals and anthers.

Seeds were isolated from mature fruits and incubated in 1% HCl for 1 h to remove the remnants of the mucilagenous locular tissue. Thereafter the seeds were rinsed with tap water, dried at room temperature and stored in closed plastic containers in a refrigerator at 7 °C until use. Comparison between wild type and mutant were always made with seed lots from the same harvest date.

Germination conditions. Triplicates of 50 seeds were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll no. 595) moistened with 1.5 ml of distilled water or test solution. GA_3 (Sigma, USA) and GA_{4+7} (a gift of ICI, UK), were dissolved in 1 M KOH and diluted with distilled water, the pH of the stock solutions was adjusted to 7.0 with 1 M HCl. Thiomersal (BDH, UK), in a concentration of 0.25 $mg/1^{-1}$ was added to prevent fungal growth. The Petri dishes with seeds were placed in closed plastic boxes and incubated at 26 °C in the dark unless mentioned otherwise. Visible radicle protrusion was used as a criterion for germination, it was normally counted after 7 days.

Red light (620-700 nm, 2.6 $W.m^{-2}$) was obtained by filtering irradiation from 6 red fluorescent tubes (Philips TL 20 W/15) by 3 mm plexiglas (Red 501, Röhm & Haas). Illuminated seeds were irradiated intermittently for 10 min per hour during the first 24 h after the start of imbibition.

Incubation of seed parts. Aseptical isolation of de-embryonated seed parts was performed in a laminar flow cabinet under white fluorescent light. The surface of dry seeds was sterilized by a 1 min incubation in 1% sodium hypochlorite, followed by a rinse with sterile tap water. The sterilized seeds were transferred to sterile Petri dishes with sterile distilled water or test solution. Solutions were sterilized with a Millex-GV filter unit (Millipore S.A., France). After 2 to 3 hours of dark imbibition the seeds were cut in halves. From the placental seed half (Fig. 1) all embryo parts were carefully removed with tweezers.

In studies on the behaviour of isolated axes, we used the radicle with adjacent part of the hypocotyl that was removed from the placental seed half (Fig. 1). Duplicates of 10 or 11 de-embryonated placental seed halves or isolated axes were further incubated in sterile Petri dishes with one layer of sterile filter paper and 1.5 ml sterile test solution. The Petri dishes were closed with parafilm and placed in the dark at 26 °C.

Puncture force determination. To measure the mechanical restraint of the layers opposing the radicle tip, seeds were cut in halves after 2 h of imbibition and the axes were removed from the placental seed halves. In one experiment also the testa was carefully removed, using tweezers. The seed halves were placed on a steel needle of 0.4 mm diameter with the tip ground in the shape of the radicle tip (Fig. 1). The needle was attached to the crosshead of an Instron 1122 universal testing instrument (Instron Ltd., U.K.). The needle with the seed half moved downwards with a speed of 5 $mm.min^{-1}$ to a hole in a PVC block, placed on a 20 N load cell. The diameter of the hole was such that the needle could pass, whereas needle plus seedhalf were obstructed. In all experiments reported, the same needle and counterhole were used. The puncture force needed to break through the layers opposing the radicle was recorded and taken as a measure for the mechanical restraint of the seed layers.

Statistics of the puncture force data. In the first two experiments, the puncture force was determined after incubation of intact seeds for different periods. Incubation periods of 20 h or more led in some cases to germination of part of the seeds. For the puncture force measurements only non-germinated seeds were used. To correct for this partly selective sampling and taking into account the number of germinated seeds, a median was calculated using the following procedure:

The n seeds that had already germinated were removed from the Petri dish which had a total number of 50 seeds. From the $50-n$ non-germinated seeds, 10 seeds were tested for their puncture force. The observed values were ordered

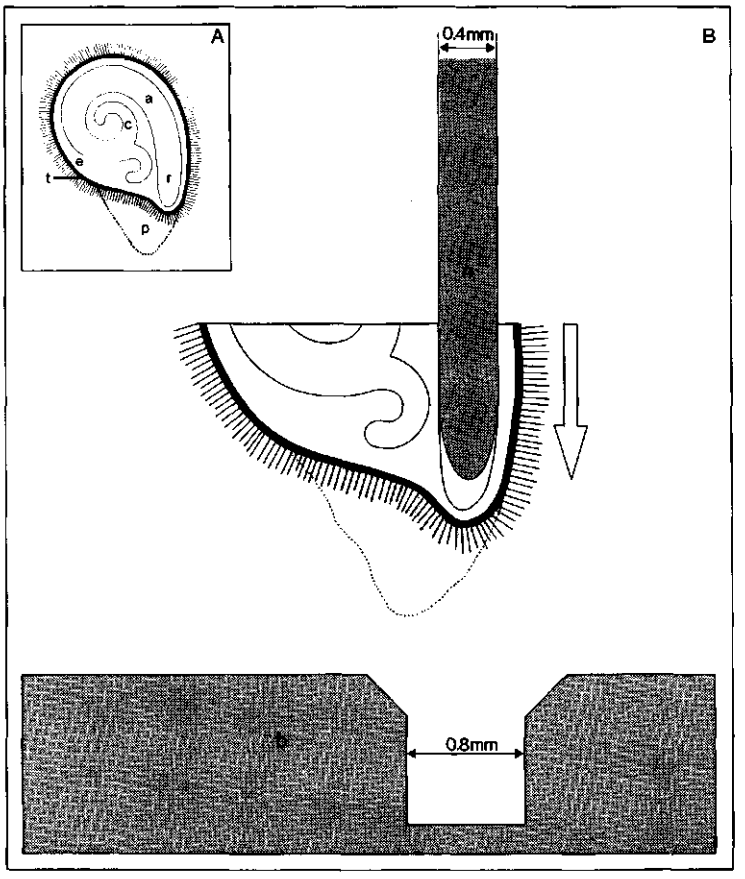

Fig. 1. Schematic presentation of (A) a section through a tomato seed and (B) the measurement of the puncture force. In **A** the curled embryo is shown with cotyledons (c), axis (a) and radicle (r) surrounded by endosperm (e), testa (t) and remnants of placental tissue (p). In **B** the placental seed half is shown, which is attached to the crosshead of an Instron 1122 Universal testing instrument, the radicle is replaced by a needle (n). Needle plus seedhalf move downwards to a block (b) with counterhole. See text for further description of the measurement.

by size, whereafter their rank number i (1 - 10) in the censored sample was replaced by a plotting position p for the complete sample. This p was calculated by the formula $p = 51^{-1}[n+i(51-n)11^{-1}]$ and plotted with the corresponding observed puncture force values on normal probability paper, from which the median was read.

The experiments with de-embryonated seed halves were performed with two duplicates of 10 or 11 seed halves per treatment. The similarity of the duplicate samplings was calculated using the Student-T-test, with a confidence level of 2.5 % on both sides. In the absence of significant differences, the duplicates were combined and the mean with standard deviation was calculated. A possible significant difference between two treatments was also calculated using the Student-T-test, but then with a confidence level of 5 % at one side.

Ethyl acetate extraction. In one experiment 100 isolated axes were incubated in water under intermittent (10 $min.h^{-1}$) red irradiation for 24 hours, followed by 2 days dark incubation. Thereafter, the incubation liquid was collected, and axes and filter paper were washed twice with distilled water. The incubation liquid was adjusted to pH 2.5 with 1 M HCl and extracted twice with equal volumes of water-saturated ethyl acetate. The water fraction was neutralized with 1 M KOH and sterilized through a Millex-GV filter. The two ethyl acetate fractions were pooled, evaporated to dryness at 25 °C and the residue was dissolved in 2 ml methanol. This methanol extract was transferred to two sterile Petri dishes with filter paper and allowed to evaporate till dryness. Thereafter 1.5 ml sterile water was added.

Results

Wild-type seeds germinated in water, but germination of ga-1 and ga-2 seeds was absolutely dependent on application of either GA_{4+7} or GA_3, the latter being 1000-fold less effective, however (Fig. 2). The ga-1 and ga-2 lines showed no difference in sensitivity to the exogenous GAs, therefore further experiments were restricted to ga-1 seeds only.

The GA requirement of ga-1 seeds could not be replaced by a pre-incubation at 2 °C, an irradiation with red light, or the application of ethylene in the atmosphere (Table 1). However, fusicoccin stimulated the ga-1 seeds in the absence of endogenous GAs. Application of GA_{4+7} could be replaced by removal of endosperm and testa layers opposing the radicle (detipping). From detipped ga-1 seeds, seedlings grew out to the dwarfy ga-1 phenotype.

The germination of detipped ga-1 seeds in water suggested that the action of GAs was located in the layers surrounding the tip of the radicle. The

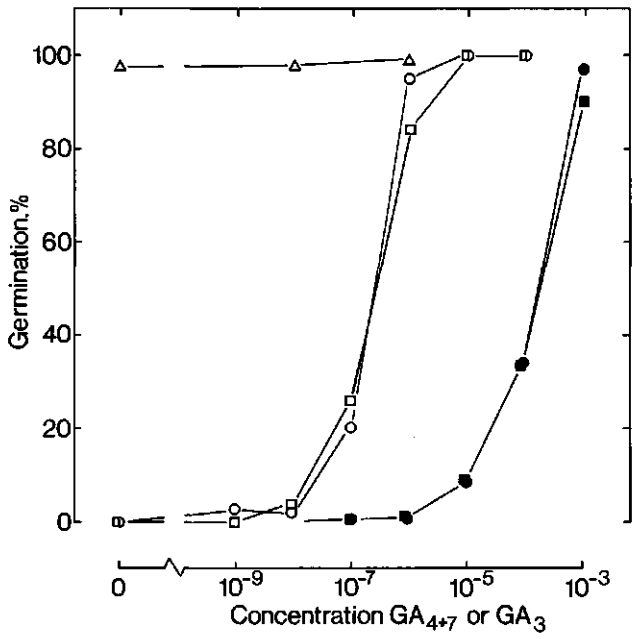

Fig. 2. The effect of GA_{4+7} and GA_3 on the germination of wild-type (△), ga-1 (○, ●) and ga-2 (□, ■) seeds; GA_{4+7}: open symbols, GA_3: closed symbols. Germination was recorded after 7 days incubation at 26 °C in darkness.

puncture force needed to break through these layers after different incubation periods is shown in Fig. 3. In wild-type seeds the puncture force decreased from the 12^{th} h of incubation onwards, which is well in advance of radicle protrusion that started around 20 h. During the early hours of incubation the puncture force required by the ga-1 and wild-type seeds was equal. In water the mechanical restraint of the ga-1 seed layers did not decrease: but in 10 µM GA_{4+7} ga-1 seeds behaved like wild-type seeds. The weakening and germination of wild-type seeds, incubated in GA_{4+7}, started earlier compared to that of the ga-1 seeds (Fig. 3), which might be due to endogenous GA production in the wild-type seeds.

In ga-1 seeds the decrease of the mechanical resistance of endosperm plus testa was compared to that of endosperm alone. In the presence of the testa and remnants of the placenta, the required force had to be about 0.1 N higher. Therefore, the weakening was restricted to the endosperm (Fig. 4). Similar observations were made with wild-type seeds in water or GA_{4+7} (data not

Table 1. Effects of pretreatments with red light or chilling and of treatments with GA_{4+7}, ethylene or fusicoccin, or detipping on the germination of ga-1 seeds at 26 °C. In detipped seeds, the testa plus endosperm layers opposing the radicle were removed after 2 h of imbibition. Germination was scored after 7 days of incubation.

Incubation medium	Condition	Germination, %
water	dark	0
water	red 24 h 10 $min.h^{-1}$	0
water	dark, 2 °C 7 d	0
GA_{4+7} 10 µM	dark	99
ethylene 8 $µl.l^{-1}$	red 24 h 10 $min.h^{-1}$	0
fusicoccin 10 µM	dark	65
water	dark, detipped	100

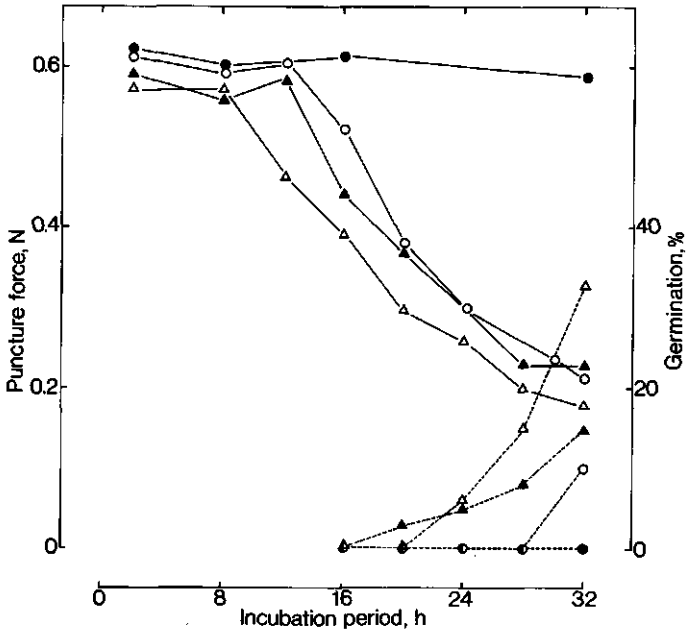

Fig. 3. Changes with time of the median force required to puncture the layers opposing the radicle tip (solid lines) and of germination (broken lines) of wild-type (▲, △) and ga-1 (●, ○) seeds incubated in water (closed symbols) or 10 µM GA_{4+7} (open symbols). See Material and Methods for the calculation of the puncture force.

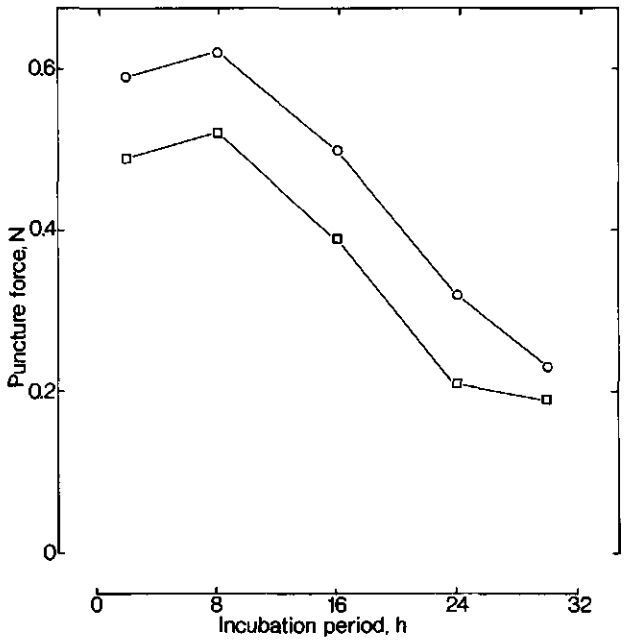

Fig. 4. Changes with time of the puncture force required to disrupt the ga-1 endosperm (□) or the ga-1 endosperm plus testa (○) in the region opposing the radicle tip during incubation in 10 µM GA_{4+7} at 26 °C. With the testa also remnants of the placental tissue were removed. Endosperms were isolated immediately before the measurements.

shown). In the previous time-course experiments puncture forces were measured directly after isolation of the layers from intact seeds. In order to investigate whether the weakening of the endosperm depended on the presence of the embryo, all further isolations were performed at around the 3rd h of imbibition and the isolated de-embryonated seed halves (placental ends) and embryonic axes were further incubated separately.

The experiments with de-embryonated seed halves clearly showed that the two genotypes only differed in the embryo. During incubation of embryoless seed halves the weakening of the endosperm resistance depended both in wild type and ga-1 absolutely on exogenous GA_{4+7} (Fig. 5), whereas in intact seeds such dependency only occurred in ga-1 (Fig. 3). The sensitivity of the embryoless seed halves of both genotypes was similar (Fig. 5). In the absence of the embryo the endosperm weakening occurred much slower than in intact seeds. The puncture force decreased to the minimal value of 0.2 N in 72 instead of 32

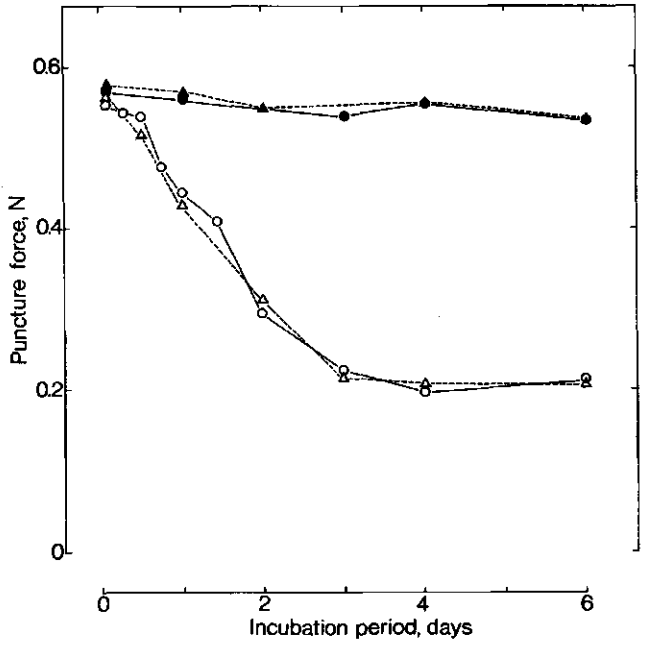

Fig. 5. Changes with time of the puncture force required to disrupt in de-embryonated seed halves the layers that opposed in the intact state the tip of the radicle . The seed halves of wild type (▲, △) and ga-1 (●, ○) were isolated after 2 h of incubation in water and thereafter incubated in water (closed symbols) or 10 µM GA_{4+7} (open symbols) at 26 °C in darkness.

hours (Figs. 3, 5). Fusicoccin was inactive when the seeds were first sterilized with hypochlorite, but it could mimic GA_{4+7} to some extent in non-sterilized de-embryonated seed halves. In a concentration of 100 µM fusicoccin the puncture force declined to 0.42 N in 48 hours (data not shown).

The previous experiment demonstrated that only wild-type embryos produce a factor that induces weakening of the endosperm, whereas that factor is missing in ga-1 embryos. Additional evidence was found when de-embryonated seed halves and isolated axes were incubated together, in different combinations, in a small volume of water (Table 2). Weakening of both wild-type and ga-1 endosperms only occurred when wild-type axes were present, it failed in the presence of ga-1 axes.

Table 2. Mean puncture force (± SD) of 11 de-embryonated seed halves of the ga-1 mutant or wild type, incubated for 7 d with 50 axes of ga-1 mutant or wild type in 1.5 ml water at 26 °C in the dark. The experiment was carried out in duplicate.

axes	Mean puncture force ± SD (N) de-embryonated seed halves	
	Ga-1/Ga-1	ga-1/ga-1
Ga-1/Ga-1	0.28 ± 0.08	0.22 ± 0.08
ga-1/ga-1	0.56 ± 0.06	0.58 ± 0.07

SD = standard deviation

Preliminary experiments were directed to the nature of the weakening factor. The incubation medium of 100 wild-type axes was acidified and mixed with ethyl acetate. Tests of the acidic water phase and the ethyl acetate phase on the endosperm weakening in de-embryonated ga-1 seed halves showed that the factor was ethyl acetate soluble (data not shown).

Discussion

The present experiments with wild-type and GA-deficient seeds clearly show that the germination of tomato seeds absolutely depends on the presence of either endogenous or exogenous GAs. A similar conclusion was reported from studies with the GA-deficient lines of Arabidopsis thaliana (Koornneef and van der Veen 1980, Karssen and Laçka 1986). Thus, in these species the stimulative action of exogenous GAs reflects the action of naturally occurring hormones.

Our experiments do not provide information about the nature of the endogenous GAs. Zeevaart (1984) has shown that GA biosynthesis in tomato shoots follows the early-13-hydroxylation pathway leading from GA_{53} to GA_1 and GA_8. GA_1 is probably the only endogenous GA that is biologically active *per se* in the control of elongation growth in many higher plants (Phinney 1985). In immature tomato fruits the GA biosynthesis may also follow a presumptive pathway of non-13-hydroxylated GAs (Zeevaart 1985) leading to GA_9 and GA_4. It is shown in Fig. 2 that exogenous GA_{4+7} is much more active than GA_3. This

might indicate that GA_4 and or GA_7 are either biologically active or are easily converted to an active GA in the endosperm. Conversion of GA_4 to GA_1 and GA_{34} has been reported for seedlings of dwarf rice (Durley and Pharis 1973) and germinating pine pollen (Kamienska et al. 1976). However, Durley et al. (1976) showed that lettuce seeds cv. Grand Rapids did not convert [^{3}H]GA_4 to other GAs prior to, or immediately following, visible germination. They concluded that GA_4 alone can promote radicle expansion. However, substantial conversion of [^{3}H]GA_4 to [^{3}H]GA_1 occurred during the extension of the lettuce hypocotyl. The activity of the commercial GA_3 may be due to the presence of minor quantities of another active GA.

The stimulation of tomato seed germination by red light or ethylene, as reported by Nelson and Sharples (1980), is probably dependent on the presence of endogenous GAs, since both treatments failed to stimulate the germination of GA-deficient seeds (Table 1). Fusicoccin, however, stimulated the germination of tomato seeds independently of endogenous GAs. This observation is in contrast to the results obtained with celery seeds where fusicoccin only stimulated germination in the presence of exogenous GA_{4+7} (Thomas and Sambrooks 1985). It has been postulated that fusicoccin stimulates cell growth by promotion of cell wall loosening, mediated by an induced decline of the extracellular pH (Marré 1979). Therefore, the failure of fusicoccin to stimulate tomato seed germination after sterilization with hypochlorite might be explained by an antagonistic effect of some residual hypochlorite on the fusicoccin-induced proton extrusion.

The present data make clear that the main action of GAs during germination is directed to the weakening of the endosperm cells surrounding the radicle tip. Endosperm weakening never occurred in ga-1, neither in intact nor in deembryonated seed halves, without addition of GA_{4+7} (Figs. 3, 5). Since in wild type weakening was only independent of exogenous GA in intact seeds, it is strongly suggested that an endogenous GA is produced in the embryo of wild-type seeds and diffuses from embryo to endosperm. The results of the reciprocal combination of isolated seed parts support this suggestion (Table 2). Unequivocal evidence that the diffusable factor is a GA is not presented. But the factor is ethyl acetate-soluble and is thus certainly not an endosperm-hydrolysing enzyme. Since fusicoccin is not a natural occurring factor in tomato seeds, the weakening stimulative action of this compound certainly does not mimic an endogenous event.

Although the embryo most likely produces a GA, growth of the embryo itself seems not absolutely dependent on GA. When endosperm and testa were removed in the area opposing the radicle tip, embryo elongation occurred in ga-1 without addition of GA_{4+7} (Table 1), albeit at a reduced rate. Probably, the rate of cell elongation is reduced in the growing radicle as is generally observed in

the area opposing the radicle tip, embryo elongation occurred in ga-1 without addition of GA_{4+7} (Table 1), albeit at a reduced rate. Probably, the rate of cell elongation is reduced in the growing radicle as is generally observed in mature dwarf mutant plants. It cannot be excluded that the growth of ga-1 embryos and plants in the absence of exogenous GAs is the result of endogenous GAs at concentrations below the detection level of the bioassay. However, such a small leakyness of the ga-1 allele is not likely since mature ga-1 plants maintained their extreme dwarf habitus during at least one year, while all plant parts were still sensitive to application of GA_{4+7} (non published results).

Evidently, endogenous GAs are not required for the development of the mechanical restraint of the endosperm during seed formation since the resistance during the early hours of incubation was similar in seeds of both genotypes (Fig. 3).

An effect of embryonic GAs on endosperm cells has been shown in different seed systems. Well documented is the transport of GAs from embryo tissues to aleurone cells in barley and other cereal seeds (e.g. Ashford and Gubler 1984). In seeds of dicotyledons, e.g., celery, indirect evidence suggests that embryo GA also acts on endosperm tissue (Jacobsen et al. 1976, Jacobsen and Pressman 1979). In these systems, the action of GA is either a post-germination event (barley) or it is required for the pre-emergence growth of embryos at the expense of endosperm breakdown (celery). It is proven here for the first time that endogenous GAs regulate seed germination by weakening of the mechanical restraint of the layers that surround the radicle. Previously Watkins and Cantliffe (1983) suggested such a role of GA in pepper seeds, because incubation of intact seeds in GA_{4+7} enhanced the weakening of the endosperm layers.

In lettuce seeds hydrolysis of the endosperm cell walls was also stimulated by exogenous GA, but required the presence of the cotyledons (Halmer and Bewley 1979). The hydrolysis occurred exclusively as a post-germinative phenomenon (Bewley et al. 1983) and thus pointed to a function in mobilization of storage carbohydrates. Obviously, endosperm weakening in tomato will involve a certain degradation of the cell walls, but this occurs prior to germination. Total degradation of the cell walls is probably not required for germination, the process might be limited to a specific loosening of cell wall rigidity, causing sufficient weakening to permit protrusion of the radicle. Therefore, the promotive effect of GAs on the weakening of endosperm cell walls opposing the radicle might be different from the total post-germination hydrolysis of the cell walls in the rest of the endosperm. In lettuce seeds, Georghiou et al. (1983) observed structural modifications in a restricted area of the endosperm opposite the radicle tip, prior to emergence of the radicle.

Chapter 3

Gibberellin-induced hydrolysis of endosperm cell walls in gibberellin--deficient tomato seeds prior to radicle protrusion.

In collaboration with B. Kieliszewska-Rokicka and C.M. Karssen.
The text of this chapter is submitted for publication in Planta.

Abstract

The weakening of the mechanical restraint of the endosperm layer in tomato seeds, a prerequisite for germination, is studied with the use of seeds of the GA-deficient ga-1 mutant. Incubation of ga-1 endosperms, including part of the testa, in 10 µM GA_{4+7}, results within 12 h in the release of fructose, glucose, galactose and mannose into the incubation medium. Only small amounts of sugars diffuse out of the endosperms during incubation in water. Chemical hydrolysis of endosperm cell walls of ga-1 seeds reveals that they are mainly composed of mannose, and smaller quantities of glucose and galactose. GA_{4+7} induces in the endosperms the production of endo- β-mannanase activity, that is not detectable during incubation in water. It also induces an increase of mannohydrolase and α-galactosidase activities compared to the water controls. No cellulase activity is found. It is concluded that in tomato seeds the weakening of endosperms prior to radicle protrusion is mediated by a GA-induced enzymatic degradation of the mannan-rich cell walls.

Introduction

The embryo in tomato seeds is completely surrounded by a rigid endosperm layer. In a previous study it was shown that weakening of the mechanical restraint of the endosperm cells opposing the top of the radicle is a prerequisite for germination (Chapter 2). The study of the seeds of the GA-deficient ga-1 mutant proved that the weakening absolutely depended on gibberellins (GA). In wild-type seeds endosperm weakening is induced by a diffusible embryo factor being most likely a GA. It was hypothesized that GA induces enzymatic hydrolysis of the relatively thick cell walls of the endosperm. The mutants are used in present chapter to study the cell-wall composition of tomato endosperms and the effect of exogenous GA on the weakening process in the absence of endogenous GA. The release of sugars from isolated endosperms and the activity of cellulose- or (galacto-)mannan-degrading enzymes in GA-deficient endosperms: cellulase (EC 3.2.1.4), endo-β-mannanase (EC 3.2.1.78), α-galactosidase (EC 3.2.1.22) and a mannohydrolase is studied. The term mannohydrolase will be used since is has not been determined wether this enzyme is a β-mannosidase or an exo-β-mannanase (Reese 1977). Mannans or galacto-mannans are the major components of endosperm cell walls and function as reserve carbohydrates in seeds of lettuce, fenugreek, date and many other species (see Matheson 1984 and Halmer 1985 for recent reviews).

Material and methods

Seed material

Seeds of the GA-deficient ga-1 mutant of tomato, Lycopersicon esculentum Mill., cv. Moneymaker, were obtained from Professor J.H. van der Veen of the Department of Genetics, Agricultural University Wageningen.

Isolation and incubation of endosperms

De-embryonated ga-1 seed halves, for short indicated as half endosperms although they also contained part of the testa, were aseptically isolated in a laminar-flow cabinet under white fluorescent light. The surface of dry seeds was sterilized by a 1 min incubation in 1% sodium hypochlorite, followed by a rinse with sterile tap water. After 2 to 3 h of incubation in sterile distilled water, the seeds were cut in halves. The placental seed halves, from which all embryo parts were carefully removed with tweezers, were used in the experiments. Samples of 50 half endosperms were rinsed three times with

sterile water and aseptically incubated in closed erlenmeyer flasks with 2 ml of either distilled water or 10 µM GA_{4+7}, sterilized with a Millex-GV filter unit (Millipore S.A., France). After incubation for different periods of time in a slowly shaking waterbath at 26 °C, the incubation liquid was analyzed for sugars released and the endosperms were used for enzyme determinations.

Derivatization and analysis of the sugars

After addition of 50 µg 2-deoxy-D-glucose as an internal standard, the samples were evaporated to dryness at 60 °C. To the dry samples 100 µl water-free pyridine and 50 µl 'Deriva Sil' (Chrompack, NL) were added, thereafter the tubes were sealed and left for 30 min at room temperature to permit derivatisation of the sugars. The resulting trimethylsilyl-derivates (TMS-derivates) were analyzed either directly or after a period of storage at -80 °C. The method used resulted for most sugars in at least two derivates, due to α- and β-anomeric forms. The derivatized samples were analyzed by gas chromatography, performed on a Perkin Elmer 8320 gas chromatograph equipped with a flame ionization detector and an internal integrator. A CP Sil 5CB fused silica column, length 50 m, inner diameter 0.32 mm and film thickness 0.13 µm was used. The split ratio of the sample was 1:50. After an initial temperature of 175 °C, the oven temperature was heated to 185 °C at a rate of 0.4 $°C.min^{-1}$. The injector temperature was 275 °C and the inlet pressure of the carrier gas helium 85 kPa, resulting in a linear gas velocity of 270 $mm.s^{-1}$. The detector temperature was 300 °C.

Isolation and hydrolysis of endosperm cell walls

After removal of testa and embryo, batches of 10 endosperms were ground with water in a mortar and passed through a french pressure cell at 100 kN to disrupt the endosperm cells. After centrifugation the pellet was washed twice with 5 ml 70% ethanol and twice with chloroform-methanol (2:1 v/v). The remaining cell-wall material was dried and dissolved in 75 µl sulphuric acid (72% w/v) during 3h at room temperature. Thereafter it was diluted with 1.0 ml distilled water and hydrolyzed in vials closed with teflon-coated caps for 1 h at 120 °C. The hydrolysate was neutralized with bariumcarbonate, centrifuged, filtered and analyzed for sugars.

Enzyme extraction

Endosperms that had been incubated in water or in GA_{4+7} for different periods of time, were homogenized in 1.5 ml 0.2 M phosphate/0.1 M citrate buffer pH

5.0, for extraction of endo- β-mannanase, mannohydrolase and α-galactosidase activities. For extraction of cellulase activity, endosperms that had been incubated 24 h in 10 μM GA_{4+7} were homogenized in water . The homogenates were centrifuged in a microfuge (Eppendorf, FRG) at 15,600 x g for 15 min. The supernatant was used as enzyme preparation and assayed either immediately or after storage at -20 °C.

Enzyme assays

Endo-β-mannanase activity was assayed viscosimetrically. Locust-bean galactomannan (Sigma Chemical Co, USA) was purified according to Halmer et al.(1975) and dissolved in distilled water. The assay mixture consisted of 10.0 ml galacto-mannan substrate, 5.0 ml phosphate/citrate buffer (pH 5.0; 0.2/0.1 M) and 200 μl enzyme preparation. Decrease of viscosity of the assay mixture in a glass Oswald viscosimeter at 35 °C, was monitored by measuring the decrease in time of flow of the viscosimeter-graduated volume (10 ml) during 30 min. Initial flow time of the assay mixture was about 185 seconds, flow time of distilled water was 30 seconds. Control incubation was performed with boiled enzyme preparation.

To assay mannohydrolase activity, 250 μl phosphate/citrate buffer (pH 5.0; 0.2/0.1 M), 50 μl 10 mM p-nitrophenyl-β-D-mannopyranoside (Boehringer Mannheim GmbH, FRG) and 200 μl enzyme preparation were mixed and incubated for 2 h at 35 °C. The reaction was stopped by addition of 2.5 ml 0.1 M Na_2CO_3. Extinctions were read at 400 nm. Activities were calculated on the basis of p-nitrophenol released. In control incubations extraction buffer was used instead of enzym preparation.

To assay α-galactosidase activity, 250 μl phosphate/citrate buffer (pH 5.0; 0.2/0.1 M), 50 μl 10 mM p-nitrophenyl-α-D-galactopyranoside (Sigma Chemical Co, USA) and 200 μl enzyme preparation were mixed and incubated for 10 min at 35°C. The reaction was stopped by addition of 2.5 ml 0.1 M Na_2CO_3. Extinctions were read at 400 nm. Activities were calculated on the basis of p-nitrophenol released. Enzyme preparation was replaced by extraction buffer in controls.

Cellulase activity was also assayed viscosimetrically. The assay mixture consisted of 10 ml 0.5 % (w/v) carboxy methyl cellulose (BDH, UK) dissolved in 25 mM citrate buffer (pH 4.8) and 200 μl enzyme preparation (water-extracted). Decrease in viscosity of the assay mixture in a glass Oswald viscometer at 45 °C, was monitored by measuring the decrease in time of flow of the viscometer-graduated volume during 150 min.

Results

Release of sugars from incubated endosperms

De-embryonated ga-1 seed halves, for short indicated as endosperms, were incubated in water or 10 µM GA_{4+7}. During 12 h of incubation in water only low amounts of sugars diffused from the endosperms (Tab. 1). On the contrary, incubation in 10 µM GA_{4+7} strongly stimulated the release of mannose, fructose, glucose, and galactose into the incubation liquid. The release started between 6 and 12 h of incubation and after 12 h mannose was the most abundant sugar in the diffusate.

Release of oligosaccharides into the incubation medium could not be detected by the applied method of analysis.

Table 1. Amounts of monosaccharides detected in diffusates from 50 de-embryonated seed halves of ga-1 tomato seeds, dissected after 3 h of imbibition in distilled water, rinsed, and incubated in distilled water or 10 µM GA_{4+7} for different periods of time.

incubation medium	time	released sugars, µg.(50 half endosperms)$^{-1}$ ± SD			
		mannose	fructose	glucose	galactose
H_2O	2 h (n=2)	trace	trace	2.4 ± 1.4	0.6 ± 0.2
	12 h (n=3)	0.5 ± 0.2	1.5 ± 0.3	1.6 ± 1.4	2.1 ± 1.4
	24 h (n=4)	0.4 ± 0.1	2.8 ± 2.1	4.5 ± 3.8	2.8 ± 1.9
GA_{4+7}	6 h (n=5)	0.4 ± 0.2	0.6 ± 0.1	1.7 ± 0.2	3.7 ± 0.6
	12 h (n=5)	32 ± 5	11 ± 3	10 ± 3	15 ± 3
	24 h (n=6)	141 ± 51	59 ± 25	39 ± 14	16 ± 4

SD = standard deviation

Cell wall hydrolyzates

Endosperm cell walls of ga-1 seeds were isolated and chemically hydrolyzed to study the origin of the released sugars. A typical profile of the gas-chromatografic analysis of the derivatized hydrolysate is presented in Fig. 1. and quantitative calculations of the identified sugars are presented in Tab. 2. The polysaccharides of the endosperm cell walls consist mainly of mannose, glucose and galactose. Minor quantities of arabinose, rhamnose, ribose, fructose and two unidentified components were also present in the hydrolysate.

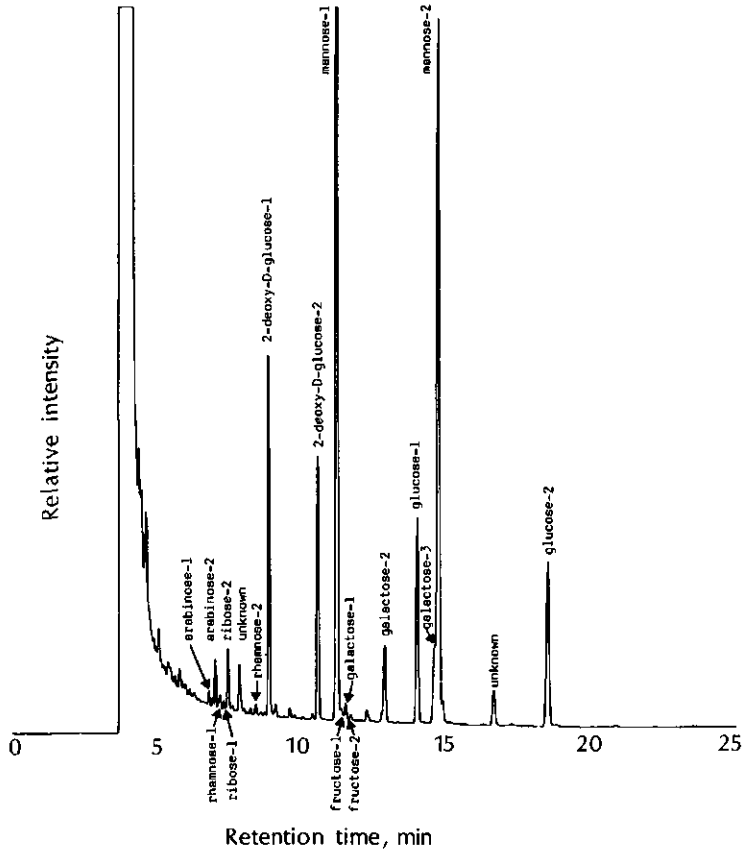

Fig.1. Gas-chromatographic profile of TMS-derivates of sugars produced on chemical hydrolysis of ga-1 endosperm cell walls. 2-Deoxy-D--glucose was added as an internal standard.

According to the retention times, these unidentified peaks were not derived from xylose. Mannose represented about two third of the amount of sugar units in the endosperm cell walls.

Table 2. Sugar composition of hydrolyzates of endosperm cell walls of ga-1 tomato seeds. The values represent the mean ± SD of 4 samples, in $\mu g.endosperm^{-1}$.

mannose	40.6 ± 2.6
glucose	12.4 ± 1.1
galactose	2.9 ± 0.4
arabinose	1.2 ± 0.3
ribose	1.1 ± 0.2
fructose	0.8 ± 0.2
rhamnose	0.6 ± 0.1

SD = standard deviation

Enzyme activity in incubated endosperms

The activities of three galacto-mannan-hydrolyzing enzymes were analyzed in extracts of half endosperms incubated for different periods of time in water or 10 µM GA_{4+7}.

Endo- β-mannanase activity could not be detected in half endosperms at the start of incubation, or during further incubation in water (Fig. 2). Incubation in GA_{4+7} induced activity of the enzyme from 6 h onwards.

Activity of mannohydrolase was present in low levels at the start of the incubation and doubled during 24 h incubation in water (Fig. 3). Incubation of the endosperms in GA_{4+7} induced an about 4-fold stronger increase of the activity than in the water control.

The activity of α-galactosidase was already high at the start of the incubation and hardly changed during further incubation in water (Fig. 4). Incubation in GA_{4+7} increased the activity with about 20 %.

Water extracts of endosperms incubated 24 h in 10 µM GA_{4+7}, contained no cellulase activity. A commercial cellulase (Yakult Hansha Co., Ltd.) preparation, incubated under the same conditions, was highly active (data not shown).

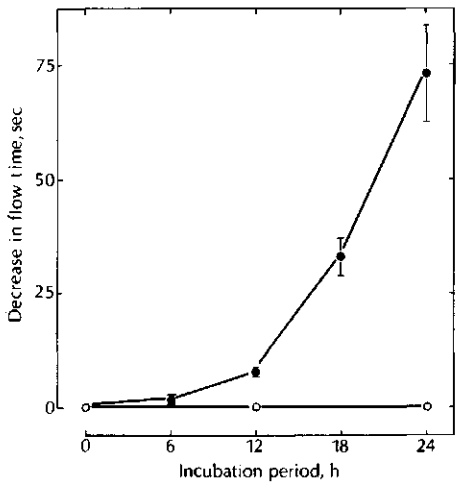

Fig. 2. Endo- β-mannanase activity extracted from endosperms after incubation in water (○) or 10 μM GA_{4+7} (●). Each point represents the mean of at least three independent samples. Error bars = SD. The incubation time was preceded by 3 h of incubation in water. Activity is expressed as a decrease in flow time of the assay mixture in the viscosimeter after 30 min.

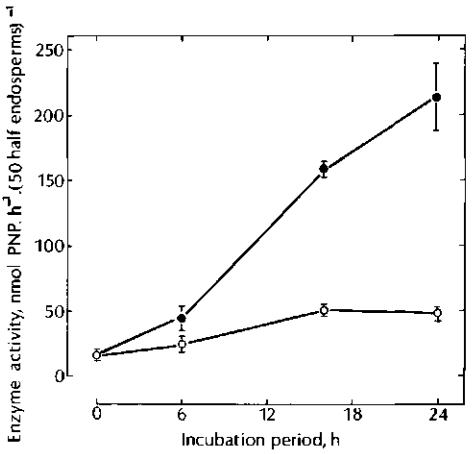

Fig. 3. Mannohydrolase activity extracted from endosperms after incubation in water (○) or 10 μM GA_{4+7} (●). Each point represents the mean of at least three independent samples. Error bars = SD. The incubation time was preceded by 3 h of incubation in water.

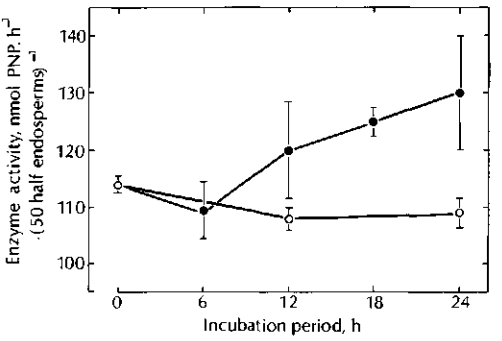

Fig. 4. α-Galactosidase activity extracted from endosperms after incubation in water (○) or 10 μM GA_{4+7} (●). Each point represents the mean of at least three independent samples. Error bars = SD. The incubation time was preceded by 3 h of incubation in water.

Discussion

The occurrence of relatively large quantities of mannose and of smaller quantities of galactose in the hydrolysate of cell walls of tomato endosperm (Fig. 1, Tab. 2), indicates that mannans, possibly galacto-mannans, are the main components of these cell walls. Glucose, that is also present in considerable quantities in the hydrolysate, will originate from cellulose and possibly also from glucose units in the mannan chains. Galacto-glucan-mannans have been found in the endosperms of species of Liliaceae and Iridaceae (Dea and Morrison 1975). Fructose is only a minor component of the cell walls (Tab. 2). The substantial quantities of fructose that diffused out of the endosperms upon incubation in GA_{4+7} (Tab. 1), originated probably from sucrose, or its galactolyzed derivates such as raffinose, stored in the cytoplasm of the endosperm cells. Hydrolysis of the cytoplasmatic carbohydrates seems also GA-induced since they do not diffuse from water-incubated endosperms. Part of the glucose and galactose in the diffusate may also originate from this cytoplasmic source. Lack of cellulase activity after 24 h incubation means that the glucose in the diffusate does not originate from cellulose hydrolysis. The glucose units did not originate from starch hydrolysis, since tomato seeds do not contain starch, as was confirmed by the absence of a blue staining reaction with I_2KI reagent (data not shown).

Thus, all mannose, part of the galactose, and possibly also a part of the glucose, that is present in the diffusate 12 h after the start of the incubation, is the result of enzymatic hydrolysis of the cell walls.

Hydrolysis of galacto-mannans to mannose and galactose requires activity of the enzymes endo-β-mannanase, mannohydrolase and α-galactosidase (Reese and

Shibata 1965). In tomato an active α-galactosidase could be isolated from the endosperms directly from the start of incubation in water (Fig. 4). In water also mannohydrolase showed some activity which slightly increased during incubation (Fig.3). Nevertheless, a significant release of mannose into the incubation liquid did not occur (Tab. 1). Apparently for *in situ* action the enzymes require either GA and/or the availability of proper substrates resulting from endo-β-mannanase activity. The last explanation was shown valid for lettuce seeds, where mannohydrolase and α-galactosidase are incapable of releasing mannose or galactose from native cell walls without prior cleavage by endo-β-mannanase (Bewley et al. 1983). In tomato endosperms endo-β-mannanase activity was only detected after incubation in GA_{4+7} (Fig. 2). Application of GA_{4+7} also resulted in release of galactose and glucose (Tab. 1). Therefore, the increase in manohydrolase and α-galactosidase activities in endosperms incubated in GA_{4+7} may be either due to a direct GA-induced de-novo synthesis or activation, or - indirectly - to the presence of substrates released by endo-β-mannanase.

The GA_{4+7}-induced endo-β-mannanase activity occurred in isolated endosperms between 6 and 12 h after the start of incubation (Fig. 2). The first signs of endosperm weakening were observed in the same period of time (Chapter 2). Both endo-β-mannanase activity and endosperm weakening were absent during incubation of the endosperms in water (Fig. 2, Chapter 2). It is concluded that the GA-induced endosperm weakening in tomato is mediated by the induction of endo-β-mannanase activity, resulting in a partial digestion of the endosperm cell walls.

In tomato seeds the cell walls of the endosperm not only serve as storage food, they also play an essential role in the regulation of dormancy and germination. Hydrolysis of the endosperm cell walls starts prior to germination, resulting in sufficient weakening to permit radicle protrusion. A relation between cell wall-hydrolytic activity (cellulase) and endosperm weakening was also shown in seeds of *Datura ferox* (Sanchez et al. 1986). In *Datura ferox* far-red irradiation inhibits both cellulase activity and germination, comparable to the inhibition by far-red of endosperm weakening in tomato (Groot, S.P.C., unpublished results).

The regulation of starch mobilization in gramineceous seeds is well documented (see for a recent review Halmer 1985). However, the hydrolysis of starch is a post-germination event (Upadhyaya et al. 1982). Also the hydrolysis of galacto-mannans and mannanas in the endosperm of fenugreek and lettuce starts after radicle protrusion (Meier and Reid 1982, Bewley et al. 1983). In fenugreek galacto-mannan breakdown can occur in the absence of the embryo, but hydrolysis is strongly stimulated by the presence of the embryonic axis or exogenous GA_{4+7} (Reid and Meier 1972, Spyropoulos and Reid 1985). The

post-germinative stimulation of Endo- β-mannanase production in lettuce endosperms requires the presence of both the embryonal axis, or exogenous GA, and the cotyledons (Halmer and Bewley 1979).

The present experiments with the GA-deficient tomato mutant demonstrate the induction and increase, by GA of cell wall-hydrolizing enzyme activities in endosperm cells as a requirement for radicle protrusion.

Chapter 4

The role of endogenous gibberellin in seed and fruit development of tomato: studies with a gibberellin-deficient mutant.

In collaboration with J. Bruinsma and C.M. Karssen.
The text of this chapter is submitted as a paper to Physiologia Plantarum.

Abstract

The role of endogenous gibberellin (GA) in seed and fruit development was studied with the use of the GA-deficient ga-1 mutant of tomato. Flowers of the ga-1 mutant are abnormal and sterile, but parthenocarpic fruit development is observed occasionally on the dwarf plants. A single application of GA_{4+7} restores the fertility of the mutant flowers and results in seed set. Development of GA-producing and GA-deficient seeds in GA-deficient fruits was compared by pollination of ga-1/ga-1 flowers with wild-type or ga-1 pollen, respectively. In ga-1/ga-1 seeds dehydration starts about one week earlier than in Ga-1/ga-1 seeds. Ultimate fresh and dry weights of mature Ga-1/ga-1 seeds are higher than those of ga-1/ga-1 seeds and show negative correlations with the total number of seeds per fruit. Total content and composition of seed proteins are not influenced by the GA-deficiency. Germination of the mature seeds depends on embryonal GA synthesis and is not influenced by maternal GA production. Final fresh weight of the ga-1/ga-1 fruits is positively correlated with the number of seeds per fruit. In these fruits, the minimum number of seeds for growth above the parthenocarpic level is about 10 or 35 in the presence of Ga-1/ga-1 or ga-1/ga-1 seeds, respectively. Fruits containing GA-producing seeds reach a higher fresh weight than those containing GA--deficient seeds, and their ripening is delayed by one week. It is concluded that gibberellin is indispensable for the development of fertile flowers and for seed germination, but only promoting in later stages of fruit and seed development.

Introduction

Immature seeds are rich sources of gibberellins (GAs) and have been used in many studies concerning quantification and characterization of endogenous GAs (see Khan 1982, MacMillan 1984, Pharis and King 1985 for recent reviews). In fact, a great deal of our knowledge about GA-biosynthesis and metabolism is derived from studies with immature seeds (e.g., Sponsel 1985) or cell-free systems derived from them (e.g., Graebe et al. 1980).

Various suggestions have been made about the role of endogenous GAs during seed development: (1) GAs are required for growth and development of the seed, (2) fruit growth and development is controlled by GAs derived from seeds, (3) GAs interact with other plant hormones to regulate seed growth and development, and (4) GAs or GA-conjugates produced during development serve as storage forms to be utilized during germination and growth (Karssen 1982a, Khan 1982). These suggestions are mainly based on correlations between changes in GA-like activity, mostly determined by bio-assay, and certain phases of seed development (e.g., Sponsel 1980, Barendse et al. 1968). Care must be taken, however, since not all GAs that are active in certain bioassays are also physiologically active in seeds. Moreover, the molecular forms of the GAs that are present in the seeds vary considerably, even between related species (Sponsel 1980). It has also to be taken into account that almost all quantitative and qualitative determinations were performed with whole seeds, neglecting possible differences in concentration and sensitivity between seed parts.

A regulatory role of endogenous GAs in seed development was doubted by Zeevaart (1966). He sprayed plants of Pharbitis nil at anthesis with chlormequat, which resulted in a decrease of GA-like activity to almost undetectable levels. Nevertheless, no effect on seed growth was observed. An alternative approach is the modification of endogenous GA levels by genetic means. Mostly, GA deficiency is looked for among mutants with dwarfed shoot growth. Complete phenotypic reversion by GA applications and the demonstration of lesions for all or for certain specific GAs are essential additional criteria to identify dwarfs as GA-deficient mutants (Hedden and Phinney 1979, Potts and Reid 1983). In tomato and Arabidopsis thaliana, GA-deficient mutants have been isolated by selecting for seeds that do not germinate without an application of GAs (Koornneef and Van der Veen 1980, Koornneef et al. 1981, Karssen et al. 1987). The seedlings that develop from GA-treated mutant seeds show dwarf growth which can be reverted to normal growth by GA sprays.

Recently Barendse et al. (1986) used GA-deficient Arabidopsis mutants to study the role of endogenous GAs during fruit and seed development. They found no influence of the GA-deficiency on seed growth. The rate of fruit growth,

however, was clearly influenced by GAs originating from both maternal and embryonic tissues.

In this paper the role of GAs during seed development was studied using the GA-deficient ga-1 mutant of tomato (Koornneef et al. 1981, Groot and Karssen in press). The GA-biosynthetic pathway in this mutant is blocked prior to ent-kaurene and GAs could not be detected by the maize-d5 bioassay in either shoots or immature fruits (Zeevaart 1984, see Fig. 2 in Chapter 1).

Material and methods

Plant material

Seeds of wild-type tomato, Lycopersicon esculentum Mill., cv. Moneymaker, and of the GA-deficient mutant ga-1/ga-1 in this genetic background, were supplied by Professor J.H. Van der Veen, Department of Genetics, Agricultural University Wageningen. Mutant seeds were germinated in a Petri dish with 10 µM GA_{4+7} (mixture of GA_4 and GA_7, a gift of ICI, UK). After germination, the seedlings were planted in pot soil and cultivated in the greenhouse at a minimum temperature of 21 °C during the photoperiod and 19 °C at night. The photoperiod was maintained at 16 h by additional illumination from high-pressure mercury halide lamps (Philips HPI/T) at 18 $W.m^{-2}$.

Seedlings and plants were sprayed once a week with about 2 ml 10 µM GA_{4+7} on the top of each plant, to stimulate development of normal flowers. Self-pollination of the flowers was stimulated by vibration. Crosses were made by emasculation of the flowers before opening of the corolla, immediately followed by pollination with Ga-1 or ga-1 pollen. Self-pollinations and crosses were performed at the same plants, pollinated flowers being tagged. Fruits and seeds were sampled at different moments after pollination.

Fresh and dry weight determinations

Seed fresh weight was determined on samples of 20 seeds that were removed from the fruits and briefly blotted with filter paper. Dry weight was determined after drying the seeds during 1 h at 130 °C.

Protein determinations

For determination of total protein content, samples of 10 developing seeds were ground in a cold mortar with 4 ml 50 mM KH_2PO_4, pH 7.5, containing 1.5 mM NaCl. After 1 h at 4 °C, the extract was centrifuged in a microfuge (Eppen-

dorf, FRG) at 15,600 x g. The protein content in the supernatant was determined according to Bradford (1976), using bovine gamma globulin (Biorad laboratories, FRG) as a standard.

Also for qualitative protein determinations samples of 10 seeds were ground in a cold morter with 4 ml extraction buffer. This buffer consisted of 65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycine, and 2.5% 2-mercaptoethanol (v/v). After 4 h at 4 °C, the extract was centrifuged in the microfuge. The resulting supernatant was mixed with an equal volume of sample buffer, 62.5 mM Tris, pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 5% glycerol (v/v) and some bromophenol blue. The samples were heated and analyzed in 13% polyacrylamide gels containing 0.1% SDS (SDS-PAGE) (Laemmli 1970). After electrophoresis, gels were stained with 0.25% (w/v) Coomassie Brilliant Blue in methanol:acetic acid: water (5:1:4, v/v) and destained in methanol:acetic acid:water (5:7:88, v/v).

Germination experiment

Seeds were imbibed in water, irradiated for 1 day with intermittent red light, followed by an incubation at 2 °C for a period of 5 days. After this dormancy-breaking treatment, seeds were incubated in water at 26 °C in the dark for 7 days. Seeds that had not germinated in water were further treated with 10 µM GA_{4+7}.

Results

On dwarf ga-1 plants no normal flowers were formed. Flower buds developed, but flower development stagnated, particularly in the corolla and stamen, and buds finally aborted. A single spray with 10 µM GA_{4+7}, applied on the newly formed buds, was sufficient to complete flower development; fruits and seeds developed upon fertilization without requiring an additional GA spray. Occasionally, fruit development was observed on dwarf ga-1 plants without application of GAs (Fig. 1). These fruits were parthenocarpic, due to the sterility of the ga-1 flowers.

In wild-type tomato GAs are produced in different plant parts. In this study, we concentrated on the role of seed-produced GA with regard to fruit and seed development. Therefore the development of GA-producing Ga-1/ga-1 seeds and GA-deficient ga-1/ga-1 seeds was compared in ga-1/ga-1 fruits. The seeds were raised by pollination of ga-1/ga-1 flowers with Ga-1 and ga-1 pollen, respectively. Since both types of seeds developed simultaneously on the same mother plant, differences of environmental factors were excluded.

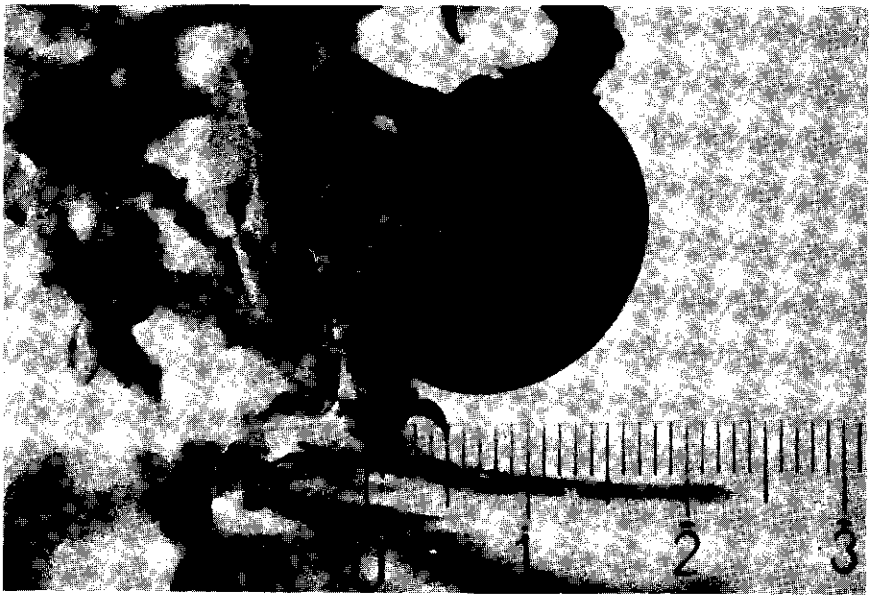

Fig. 1. Mature ga-1/ga-1 fruit which had developed on a dwarf GA-deficient plant of about 1 year old, without application of exogenous GAs. The parthenocarpic fruit had ripened normally. Ruler is in cm.

Fruit development

Ripening of the ga-1/ga-1 fruit was delayed by the presence of Ga-1/ga-1 seeds. The orange-red colour appeared after eight weeks, one week later than with the fruits without GA-producing seeds (data not shown). The seed genotype also influenced the ultimate fresh weight of the ga-1/ga-1 fruits (Fig. 2). Fruit weight depended on the number of seeds per fruit. When Ga-1/ga-1 seeds were present fruit weight strongly increased from a number of about 10 seeds per fruit. With ga-1/ga-1 seeds this critical number was about 35 seeds; moreover, the increase in fruit weight was smaller.

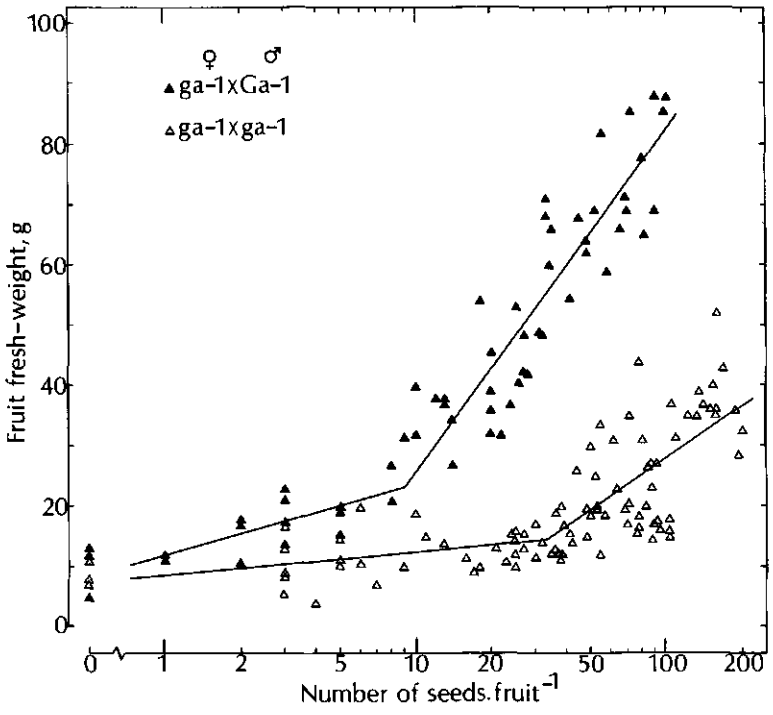

Fig. 2. Fresh weight of ripe fruits in relation to the logarithm of the number of seeds per fruit. The fruits developed on ga-1/ga-1 plants and contained either GA-deficient ga-1/ga-1 (△) or GA-synthetizing Ga-1/ga-1 (▲) seeds.

Seed development

The number of seeds that developed in ga-1/ga-1 fruits was low following pollination of emasculated flowers with either ga-1 or Ga-1 pollen, 50 to 60 % of the fruits contained less than 40 seeds. When ga-1/ga-1 flowers were self-pollinated, about 50 % of the fruits contained 75 seeds or more. Evidently, emasculation and/or artificial pollination reduced seed set. The development of ga-1/ga-1 fruits and seeds resulting from either self-pollination or artificial pollination with ga-1 pollen was equal in every respect.

Fresh and dry weights were compared during seed development of both genotypes (Fig. 3). The seeds were collected at random from fruits containing different numbers of seeds. Fresh weight was clearly influenced by the genetic constitution of the seeds, ga-1/ga-1 seeds reached a lower maximum fresh weight than Ga-1/ga-1 seeds and started to dehydrate earlier. Dry weight of

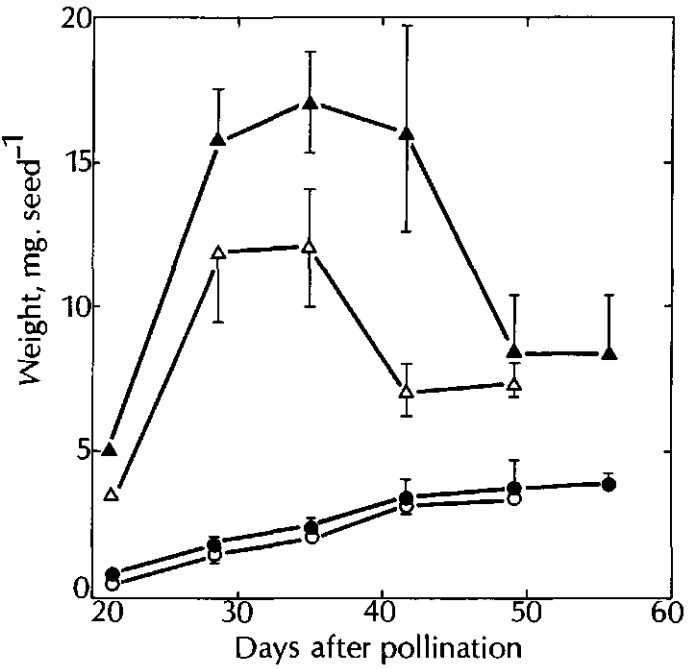

Fig. 3. Courses of fresh (▲, △) and dry (●, ○) weights during development of Ga-1/ga-1 (▲, ●) and ga-1/ga-1 (△, ○) seeds on GA-deficient ga-1/ga-1 motherplants.

these randomly collected seeds of both genotypes, developed at a similar rate and dry matter accumulation continued during dehydration of the seeds (Fig. 3).

The earlier dehydration of ga-1/ga-1 seeds was reflected in an earlier firmness of endosperm cells and an earlier collapse of testa cells (Table 1).

Table 1. Morphological characteristics of ga-1/ga-1 and Ga-1/ga-1 seeds, developing in ga-1/ga-1 fruits.

age in dap	seed genotype					
	ga-1/ga-1			Ga-1/ga-1		
	testa	endosperm	embryo	testa	endosperm	embryo
21	thick	milky	torpedo	thick	milky	torpedo
28	thick	mucous	curved	thick	mucous	curved
35	partly thin	soft-dough	curled	partly thin	soft-dough	curled
42	thin	firm	curled	partly thin	soft-dough	curled
49	thin	firm	curled	thin	firm	curled
56	thin	firm	curled	thin	firm	curled

The endosperm cells developed from a milky appearance via a mucous and a soft-dough constituency to a firm and relative dehydrated stage. This final stage was reached in ga-1/ga-1 seeds at 42 dap and in Ga-1/ga-1 seeds at 49 dap. The testa develops from maternal integument cell layers and, therefore, lacked the capacity to produce GAs in both genotypes. At 21 dap the cells of the outer epidermis of the outer integument were elongated and the testa formed a thick layer around endosperm and embryo. Gradually the testa cells collapsed because their cell walls disappeared and only secondary thickenings on the walls remained as hairs. The collapse of the testa cells started at the placental

base of the seeds at 35 dap and was completed at 42 and 49 dap in ga-1/ga-1 and Ga-1/ga-1 seeds, respectively. Embryo development was not visibly affected by the lack of GA production. In both genotypes embryos developed from a torpedo shape at 21 dap via a U-shaped (curved) form to the final, curled appearance.

Final fresh and dry weights of mature seeds of both genotypes were compared in relation to the number of seeds per fruit (Fig. 4). Dry and fresh weights of Ga-1/ga-1 seeds were higher than those of ga-1/ga-1 seeds. The weight of mature seeds showed a negative correlation with the number of seeds per fruit.

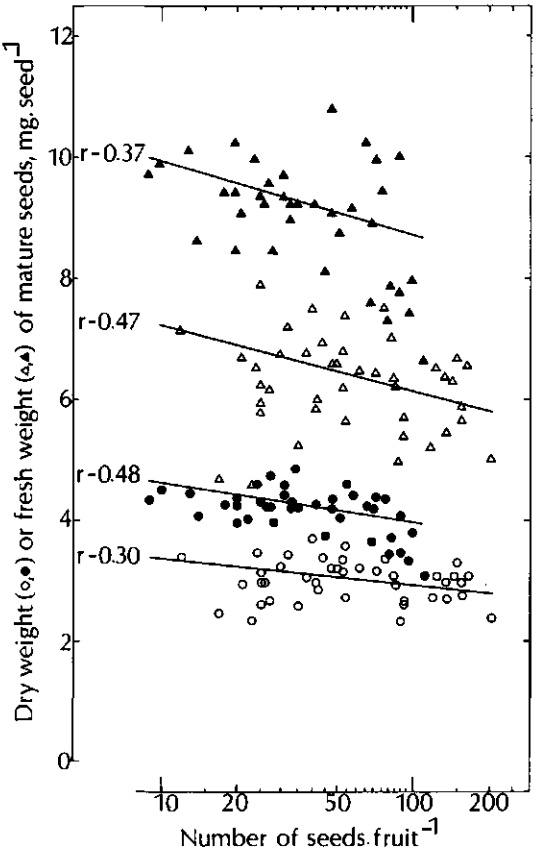

Fig. 4. Fresh weights (▲, △) and dry weights (●, ○) of mature seeds in relation to the logarithm of the number of seeds per fruit. The Ga-1/ga-1 (▲, ●) and ga-1/ga-1 (△, ○) seeds developed on GA-deficient ga-1/ga-1 motherplants.

Table 2. Changes in protein content during development of ga-1/ga-1 and Ga-1/ga-1 seeds in ga-1/ga-1 fruits (n=3). Protein content is expressed as equivalents of bovine gamma globuline standard.

developmental stage, dap	protein content, mg.g dry weight^{-1} (± SD)	
	ga-1/ga-1	Ga-1/ga-1
21	319 ± 6	320 ± 31
28	349 ± 11	377 ± 8
35	437 ± 5	465 ± 4
42	494 ± 20	502 ± 17
49	501 ± 20	536 ± 37
56		499 ± 14

SD = standard deviation

Ga-1/ga-1 and ga-1/ga-1 seeds showed no difference in total protein content during seed development (Tab. 2). Extracts of mature seeds of both genotypes showed also no qualitative difference in the major protein bands on SDS-PAGE (Fig. 5).

Germination

Germination of GA-deficient ga-1/ga-1 seeds depends on the application of GAs, whereas wild-type seeds germinate in water (Groot and Karssen in press). It was studied whether the germination characteristics of the seeds depended on the genotype of the embryo and endosperm, or were influenced by the maternal tissues. GA-deficient and GA-producing seeds were raised in fruits of both genotypes, by self-pollination of Ga-1/Ga-1, ga-1/ga-1 and Ga-1/ga-1 plants and by the cross ga-1/ga-1 x Ga-1/Ga-1 (Table 3).

Ga-1/ga-1 seeds always germinated in water, also when the mother plant was GA-deficient. Germination of seeds with a GA-deficient embryo absolutely depended on the application of GA_{4+7}, irrespective of the genotype of the mother plant. Thus, germination characteristics are exclusively determined by the genotype of the embryo.

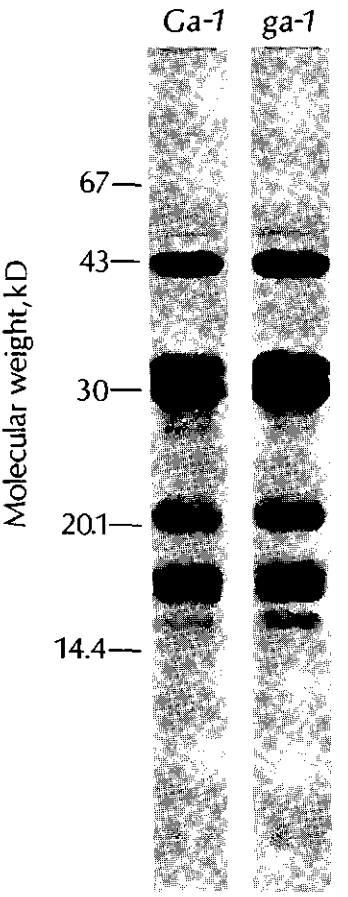

Fig. 5. SDS-PAGE pattern of polypeptides extracted from mature ga-1/ga-1 (ga-1) or wild-type (Ga-1) seeds, developing in ga-1/ga-1 fruits.

The segregation between normal and dwarf seedlings in the offspring of self-pollinated Ga-1/ga-1 plants approached a 3 : 1 ratio. It shows that ga-1 pollen and ovules are completely fertile upon development in GA-producing flowers, without the need for application of GA_{4+7}.

Table 3. Germination of seeds derived from self-pollination of either homozygous wildtype (Ga-1/Ga-1), mutant (ga-1/ga-1) or heterozygous (Ga-1/ga-1) plants, or from the cross ga-1/ga-1 (♀) x Ga-1/ga-1 (♂). Seeds were imbibed in water, irradiated for 1 day under intermittent red light, followed by an incubation at 2 °C for a period of 5 days. After this dormancy-breaking treatment, seeds were incubated in water at 26 °C in the dark for 7 days. Seeds that had not germinated in water were further treated with 10 μM GA_{4+7}.

parent(s)	progeny	phenotype seedling	germination in water	germination in GA_{4+7}	not germinated	total
Ga-1/Ga-1 Selfing	Ga-1/Ga-1	normal	145	0	5	150
ga-1/ga-1 Selfing	ga-1/ga-1	dwarf	0	150	0	150
ga-1/ga-1 x Ga-1/Ga-1	Ga-1/ga-1	normal	144	0	6	150
Ga-1/ga-1 Selfing	Ga-1/Ga-1 Ga-1/ga-1	normal	185	0		185 [a]
	ga-1/ga-1	dwarf	0	53		53 [a]
	unknown (died)		8	2	2	12

[a] χ^2(normal:dwarf = 3:1) = 0.947, dF = 1, p = 0.33.

Discussion

The present study clearly shows that the effect of GA-deficiency strongly differs during the subsequent phases of the generative development in tomato. Whereas flower fertility and seed germination were completely blocked in the absence of endogenous GA, the growth of seeds and fruits still proceeded. Occasionally, spontaneous parthenocarpic fruit development occurred on ga-1 plants (Fig. 1).

The poor development of corolla and stamen in GA-less flowers was also observed in GA-deficient Arabidopsis plants (Koornneef and Van der Veen 1980). Nester and Zeevaart (1986) recently reported a similar inhibition of flower development in the GA-deficient ga-2 mutant of tomato. They observed aberrations during pollen and ovule development, resulting in both male and female sterility. GAs have been associated with the development of both female and male reproductive structures. Applied GA_3 stimulates pistil development in, e.g., castor bean, maize and Hyoscyamus, and promotes stamen development in

flowers of Bryophyllum, Cannabis, Cucumis and tomato (Pharis and King 1985). Association of abnormal stamen development with reduced levels of endogenous GAs was also found with the stamenless-2 tomato mutant (Shawney 1974). Normal stamen development in the stamenless-2 mutant can be achieved by application of GA_3 to the mutant flowers (Sawney and Greyson 1973). Our observations show that either maternal GA production or a single spray with GA_{4+7} resulted in the development of fertile ga-1 pollen and ovules in Ga-1/ga-1 and ga-1/ga-1 flowers, respectivily (Tab. 3). GA-dependent development of the corolla, observed in the ga-1 mutant, has also been shown for flowers of Lamium amplexicaule. The corollas of these flowers failed to grow when immature buds were cultivated in the absence of GA (Lord and Mayers 1982).

Development of GA-deficient seeds and fruits occurred after a single spray on the flower bud with GA_{4+7}. The observed development of small parthenocarpic fruits on ga-1 plants without a GA spray (Fig. 1) proves that GAs are not absolutely required for tomato fruit development. Because of the sterility of the flowers, seed set could not occur without a spray of GA_{4+7}. The present experiments do not exclude that the very early phase of seed development occurred with the aid of residual amounts of GAs, which remained after the flower-inducing spray with GA_{4+7}. A requirement for GAs during the very early phase of embryo development was reported for Phaseolous coccineus (Cionini et al. 1976). In vitro culture of young embryo's of P. coccineus, excised at the heart-shape or early cotyledonary stages, needed the presence of either the GA-rich suspensor or of exogenous GA_3, whereas in later stages application of GA_3 could be omitted.

An effect of residual GA_{4+7} on the later stages of ga-1 seed development is most unlikely, the stimulation of elongation growth by a GA_{4+7} spray in the ga-1 mutant lasts about one week. Complete seed development occurred after a single spray. If the same plant was sprayed with GA_{4+7} at subsequent occasions to initiate normal development of the younger flower buds, the developing fruits were shielded. Moreover, we observed no differences between seeds and fruits developing due to the first or the last spray in a sequence of weekly applications of GA_{4+7}. If exogenous GAs accidentally should have entered the developing fruits, it is expected that they primarily should replace maternal GAs. An effect of maternal GA is seen when the growth of seedless fruits in wild-type Moneymaker, as studied by Varga and Bruinsma (1976), is compared with the GA-deficient fruits in the present study (Fig. 2). Average fresh weights of parthenocarpic fruits were 40 and 10 g, respectively. It can therefore be excluded that exogenous GAs accidentally influenced the growth of the GA-deficient fruits in our experiments.

Although tomato fruits and seeds grew in the absence of endogenous GAs, they developed better when GAs were produced. This was not only true for the

stimulation of parthenocarpic fruit growth by maternal GA, but also for fruit and seed development under influence of seed-produced GA. Halfway development, the GA-containing seeds reached a considerably higher fresh weight than the deficient seeds (Fig. 3). Only when the final fresh and dry weights of the seeds were expressed in relation to number of seeds per fruit it was seen that Ga-1/ga-1 seeds became heavier than ga-1/ga-1 seeds (Fig. 4). These ultimately higher dry and fresh weights may be connected with the extended developmental period (Tab. 1), allowing prolonged dry-matter accumulation and retarded dehydration. At higher numbers of seed per fruit, the weight per seed decreases (Fig. 4), apparently by increasing competition for the limited supply of nutrients.

Barendse et al. (1986) studied seed development in Arabidopsis thaliana using GA-deficient lines. They observed no effect of maternal or embryonal GA on the weight of mature seeds. However, in their experiments seeds were collected at random from fruits with different numbers of seeds per fruit, as in our Fig. 2.

Endogenous GAs had no influence on total protein content per g dry weight during seed development (Tab. 2). Also no qualitative differences in the most abundant proteins were observed (Fig. 5). These major protein bands most likely represent storage proteins since they disappear after germination of the seeds (unpublished results). The method used was not sufficiently discriminating to elucidate possible influences of endogenous GA on the occurrence of less abundant proteins.

Fruit ripening occurred in the absence of both maternal and embryonal GA (Fig. 1). But in the presence of Ga-1/ga-1 seeds development of ga-1/ga-1 fruits lasted about one week longer than with GA-deficient seeds. Inhibition of ripening by applied GAs has been described for fruits of several species (review by McGlasson et al. 1978). Treatment of tomato fruits with GA_3 delayed both the loss of chlorophyll and the increase of carotenoids (Abdal-kader et al. 1966, Dostal and Leopold 1967, Khudari 1972). We report here for the first time a role of endogenous GA in fruit ripening. Our results indicate that the effect of applied GAs on tomato fruit ripening reflects the role of endogenous, seed-produced GA during the last period of fruit development.

Varga and Bruinsma (1976) reported that in tomato cv. Moneymaker the ultimate fresh weight of the fruits show a logarithmic relationship with the number of seeds per fruit, provided the fruits contained more than 8 seeds. With fewer seeds per fruit, fruit weight hardly surpassed the 40 g of the average partenocarpic fruit. A similar relationship was found in the ga-1/ga-1 fruits containing Ga-1/ga-1 seeds (Fig. 2), albeit that fruit weight was on the average 30 g lower than in wild-type fruits. Varga and Bruinsma concluded that fruit growth profited indirectly from the sink activity exerted by its

developing seeds. The present data suggest that the sink activity is related to the ability of the seeds to produce GA. In GA-deficient seeds the sink activity is much lower, therefore fruit growth is only stimulated above the parthenocarpic level with more than 35 seeds per fruit (Fig. 2).

Germination of ga-1/ga-1 seeds completely depended on application of GAs, irrespective whether the seeds had developed in GA-deficient or GA-producing fruits (Table 3). Thus maternal GA had neither directly, by accumulating in free or bound form in the seeds, nor indirectly an effect on the germination capacity of the seeds. Germination apparently requires embryo-synthesized GA.

In conclusion, endogenous GA stimulates the development of fruits and seeds of tomato, but their presence is not indispensable except, perhaps, immediately after fertilization. In contrast, the development of fertile flowers and the germination of seeds are absolutely dependent on the presence of endogenous GA.

Chapter 5

The role of endogenous abscisic acid in seed development of tomato: Studies with an abscisic acid-deficient mutant.

In collaboration with R. Dijkman, E. Vermeer, I.I. van Yperen and C.M. Karssen.

Abstract

The role of endogenous abscisic acid (ABA) in seed development was studied with the use of the ABA-deficient sit^{W} mutant of tomato. The sit^{W} mutation causes a strong reduction of the endogenous ABA level in the developing seed.

Reciprocal crosses of wild type and the sit^{W} mutant show a dual origin of ABA. The genotype of the mother plant regulates the ABA content present in the testa which shows a peak half-way seed development. The genotype of the embryo and endosperm is responsible for a second ABA fraction, present in these tissues. This second fraction reaches its peak during the second half of seed development.

The strong reduction of endogenous ABA level in the developing sit^{W}/sit^{W} seed does not change the final fresh and dry weights of the seed nor the accumulation and composition of storage proteins. The onset of dormancy depends on endogenous ABA, particularly on the fraction in embryo plus endosperm, the influence of maternal ABA is minor. Non-dormant sit^{W}/sit^{W} seeds do not germinate precociously within the fruit, but viviparous germination of sit^{W}/sit^{W} seeds is observed when ripe fruits remain attached to the mother plant. The absence of vivipary in wild-type seeds is primarily a function of osmotic control by the fruit.

Introduction

Developing seeds accumulate ABA in large quantities, but temporary. Upon maturation of the seeds the ABA content generally decreases (Bewley and Black 1978, King 1982). It has been suggested that endogenous ABA plays a role in the increase of the sink activity of the developing seeds (Dewdney and Mcwha 1979, Tietz et al. 1981, Ackerson 1984a), in the stimulation of synthesis of specific storage proteins (Triplett and Quatrano 1982, Schroeder 1984, Bray and Beachy 1985), in the dehydration at the end of development (King 1976) and in the prevention of precocious germination (Ackerson 1984b). All these suggestions were based on correlations between changes in the endogenous ABA level and certain developmental or physiological events, or on the effect of applications of ABA to developing seeds. However, such correlations may not be causal and the responses to externally applied ABA may not imitate those elicited by changes in endogenous levels. Moreover, most of these studies were carried out in vitro with isolated seeds or seed parts.

In a limited number of species endogenous ABA levels have been manipulated by genetic means. It has been shown in Arabidopsis thaliana with the use of mutants with strongly reduced levels of endogenous ABA or with a reduced sensitivity to exogenous ABA, that seed dormancy is induced during seed development by embryonal ABA (Karssen et al. 1983, 1987, Karssen and Łaçka 1986).

ABA deficiency is always accompanied by severe water loss of the mutant plant in reaction to water stress (Koornneef 1986). This is probably the reason why mutants totally devoid of ABA have not been described so far, they would be so seriously impaired that the mutation probably would be lethal. Mutants with reduced ABA levels were induced and isolated in Arabidopsis thaliana (Koornneef et al. 1982), maize (Smith et al. 1983), potato (Quarrie 1982) and pea (Wang et al. 1984). Three different ABA-deficient tomato mutants, notabilis (not), flacca (flc) and sitiens (sit), were isolated by Stubbe (1957, 1958, 1959). Another tomato mutant at the sitiens locus (sit^W) was isolated by Van der Veen and Bosma (Koornneef et al. 1985). The sit^W mutation was obtained by selecting for wiltiness among the offspring of ethylmethanesulphonate-treated seeds of the tomato cv. Moneymaker (Koornneef et al. 1985). The ABA level in turgid leaves of sit^W plants is about 10% of that in turgid leaves of the wild type cv. Rheinlands Ruhm; water-stressed mutant leaflets do not accumulate ABA, in contrast to wild type (Cornish and Zeevaart 1985). As a result stomata do not close and the plants wilt rapidly under water stress. Since sit^W is a recessive mutation, heterozygous plants are phenotypically similar to wild-type plants.

So far the tomato mutants have been mainly used in studies on ABA bio-

synthesis and on the relationship between ABA and stomatal behaviour and water stress (Bowman et al. 1984; Tal and Nevo 1973; Neil and Horgan 1985; Cornish and Zeevaart 1985). In the present experiments the ABA-deficient sit^{W} mutant was used to study the presence and the role of ABA in the development of tomato seeds. The development of homozygous ABA-deficient seeds was compared with heterozygous ABA-producing Sit/sit^{W} seeds, both developing in sit^{W} fruits. The seeds were obtained by pollination of sit^{W} flowers with sit^{W} or wild-type pollen, respectively.

Material and Methods

Plant material and culture

Seeds of wild-type tomato Lycopersicon esculentum Mill., cv. Moneymaker (Sit) and the ABA-mutant sit^{W} were supplied by prof. Van der Veen, Department of Genetics, Agricultural University, Wageningen. Plants for seed production were cultivated under two different conditions. For developmental studies, plants were cultivated in a heated greenhouse on hydroponic culture. The nutrient solution was based on the universal nutrient solution of Steiner (1969), and consisted of tap water with 882 mg $Ca(NO_3)_2.4H_2O$, 444 mg KNO_3, 134 mg KH_2PO_4, 473 mg $MgSO_4.7H_2O$, 154 mg K_2SO_4 and 30 µl concentrated H_2SO_4 per liter. The solution was supplemented with iron chelate and micro elements: 19.2 mg FeNa-EDTA, 2.69 mg H_3BO_3, 506 µg $ZnSO_4.7H_2O$, 78 µg $CuSO_4.5H_2O$, 126 µg $Na_2MoO_4.2H_2O$ per liter. The nutrient solution was changed about every 3 weeks.

Minimum temperature in the greenhouse was 21 °C during the day and 19 °C during the night. The photoperiod was maintained at 16 h by additional illumination from high-pressure mercury halide lamps (Philips HPI/T) at 18 $W.m^{-2}$. Self-pollination of the flowers was stimulated by vibration. Crosses were made by emasculation of the flowers before opening of the corolla, immediately followed by pollination with Sit or sit^{W} pollen. Self-pollinations and crosses were performed on the same plants, pollinated flowers being tagged. Seeds were sampled at different moments after pollination.

Seeds used in germination experiments were produced either under conditions described above (condition 1), or from plants cultivated on soil in a non-heated greenhouse under natural summer conditions, whereby the older leaves below the clusters with ripe fruits were removed regularly (condition 2). Under this latter condition sit^{W} plants were sprayed once or twice a week with about 2 ml 10 µM ABA to reduce wilting.

Seeds used for germination experiments were isolated from ripe fruits and incubated in 1% HCl for 1 h to remove the remnants of the mucilagenous locular

tissue. Thereafter the seeds were rinsed with tap water, dried at room temperature and stored in closed plastic containers in a refrigerator at 7 °C until use. Comparison between wild type and mutant always occurred with seed lots from the same harvest date.

Gas chromatographic ABA determination

Triplicate samples of seeds or seed parts were weighed and frozen in liquid nitrogen. The seeds were ground in a mortar and extracted overnight in 1 ml 80% methanol containing 10 $mg.l^{-1}$ 2,6-di-t-4-butylmethylphenol (BHT). The extract was centrifuged and the pellet washed twice with 80% methanol. The combined supernatants were passed through a Millipore AP 25 prefilter and a Sep Pak C18 cartridge (Waters Ass.) to remove insoluble and apolar components. The filtrate was evaporated to dryness at 40 °C under a stream of charcoal-washed air. The residue was taken up in 200 µl ethylacetate and mixed with 200 µl 0.1 M phosphate buffer (pH 3). After centrifugation the ethylacetate phase was transferred to a 1 ml glass vial. The remaining buffer was again partitioned against 200 µl ethylacetate. The second ethylacetate phase was combined with the first one. The ethylacetate was removed by a stream of air at room temperature and the residue dissolved in 100 µl methanol containing 2,3,5-tri-iodobenzoic acid (100 $\mu g.l^{-1}$) as an internal standard for the gas-chromatographic determination. The sample was methylated by adding 200 µl diethylether with diazomethane. After methylation the sample was dried again under a stream of air and redissolved in 100 µl ethylacetate.

From this sample 2.5 µl was injected in a Perkin Elmer 8320 gas chromatograph equipped with an electron capture detector and an internal integrator. A CP Sil 5CB fused silica WCOT column was used, length 50 m, inner diameter 0.32 mm, film thickness 0.13 µm. The split valve was opened after 0.75 min and after 3 min at 80 °C the oven was heated to 140 °C with a rate of 30 $°C.min^{-1}$, after 4 min at 140 °C the oven temperature was increased to 200 °C with a rate of 5 $°C.min^{-1}$ and kept at that temperature for 19 min. The injector temperature was 275 °C and the inlet pressure of the carrier gas, helium, was 85 kPa, resulting in a linear gas velocity of 0.27 $m.s^{-1}$. The detector temperature was 350 °C. Recovery was determined with the use of ^{14}C-ABA that was added to the first extraction medium. It was shown to be at least 95%.

Fresh and dry weight determinations

From individual, mature fruits 20 seeds were removed and briefly blotted on filter paper. Fresh and dry weights of the samples were determined by weighing, to 0.1 mg, the seeds in little vials before and after oven-drying at

130 °C during 1 h.

Protein determinations

For determination of total protein content samples of 10 developing seeds were ground in a cold mortar with 4 ml buffer of 50 mM KH_2PO_4, pH 7.5, containing 1.5 mM NaCl. After 1 h at 4 °C, the extract was centrifuged in a microfuge (Eppendorf, FRG) at 15,600 x g. The protein content in the supernatant was determined according to Bradford (1976), using bovine gamma globulin (Biorad laboratories, FRG) as a standard.

Also for qualitative protein determinations samples of 10 seeds or seed parts were ground in a mortar with 4 or 2 ml extraction buffer, respectively. This buffer consisted of 65 mM Tris-HCl pH 6.8, 2% SDS, 10% glycine and 2.5% 2-mercaptoethanol (v/v). After 4 h at 4 °C, the extract was centrifuged in the microfuge. The resulting supernatant was mixed with an equal volume of sample buffer, 62.5 mM Tris pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 5% glycerol (v/v) and some bromophenol blue. The samples were heated and analyzed in 13% polyacrylamide gels containing 0.1% SDS (Laemmli 1970). Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue in methanol:acetic acid:water (5:1:4, v/v) and destained in methanol:acetic acid:water (5:7:88, v/v).

Osmotic potential of the fruit juice

Fruit juice was collected from ripe fruits, 10 days after they had turned from orange to red. The juice was filtered and the osmotic potential of the juice was determined with an osmometer (Knauer, FRG), calibrated with mannitol solutions, calculated according to Michel et al. (1983).

Germination experiments

Triplicates of 50 seeds were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher & Schüll no. 595) moistened with 1.5 ml of distilled water or test solution. GA_{4+7} (a gift of ICI, UK), was dissolved in 1 M KOH and diluted with distilled water, the pH of the stock solutions was adjusted to 7.0 with 1 M HCl. Thiomersal (BDH, UK), in a concentration of 0.25 $mg.l^{-1}$, was added to prevent fungal growth. The Petri dishes with seeds were placed in closed plastic boxes and incubated at 26 °C in the dark. Visible radicle protrusion was used as a criterion for germination, it was normally counted after 7 days.

Red light (620-700 nm, 2.6 $W.m^{-2}$) was obtained by filtering irradiation from 6 red-fluorescent tubes (Philips TL 20 W/15) by 3 mm plexiglas (Red 501,

Röhm & Haas). Illuminated seeds were irradiated intermittently for 10 $min.h^{-1}$ during the first 24 h after the start of imbibition.

Results

ABA levels in developing seeds

Cultivation on hydroponic culture and protection against direct sunlight prevented wilting, but did not reverse the other characteristics of the ABA deficiency. Flower buds of the sit^W mutant developed normally like wild-type flowers. The production of pollen was reduced, however.

Developing wild-type seeds showed a peak in ABA content of 36 $ng.seed^{-1}$ about 30 days after pollination (dap) (Fig. 1). In mature seeds only 1.8 $ng.seed^{-1}$ remained. The amount of ABA in developing sit^W/sit^W seeds was strongly reduced during all phases of development, it reached only 1 ng at 30 dap and 0.2 ng at maturity. The Sit/sit^W seeds contained an intermediate amount of ABA, reaching a maximum value of 10 ng around 40 dap. A comparison of ABA content (Fig. 2A) and fresh weight (Fig. 2B) in intact seeds and in seed parts of wild-type during seed development showed that the early peak in both parameters roughly coincided. It was also demonstrated that both peaks depended for the greater part on the integuments. From half-way development the fresh weight of the integuments, now becoming testa, decreased sharply and so did their ABA content. In the intact seed the loss of both fresh weight and ABA was partly compensated at first by the increase of both parameters in embryo and endosperm, which reached a peak around 40 dap. Thereafter, the ABA content of all seed parts was strongly reduced, whereas some water was left in the seeds (Fig. 2, 3).

The ABA content of embryo plus endosperm from sit^W/sit^W seeds at 35 dap was compared to that of the same parts of seeds developing on Sit/sit^W plants after pollination with sit^W pollen. In the latter case all fruits and other maternal tissues will be heterozygous and thus contain ABA, whereas half of the seeds will be sit^W/sit^W and half Sit/sit^W. However, the genotype of developing seeds could not be recognized. Individual seeds of the cross contained at 35 dap between 3.1 and 11.1 ng ABA per embryo plus endosperm, without a sharp distinction into two groups. Moreover, the ABA content always surpassed that of seeds from self-pollinated sit^W/sit^W plants, which ranged from 0.8 to 2.9 ng per embryo plus endosperm (cf. Fig. 1).

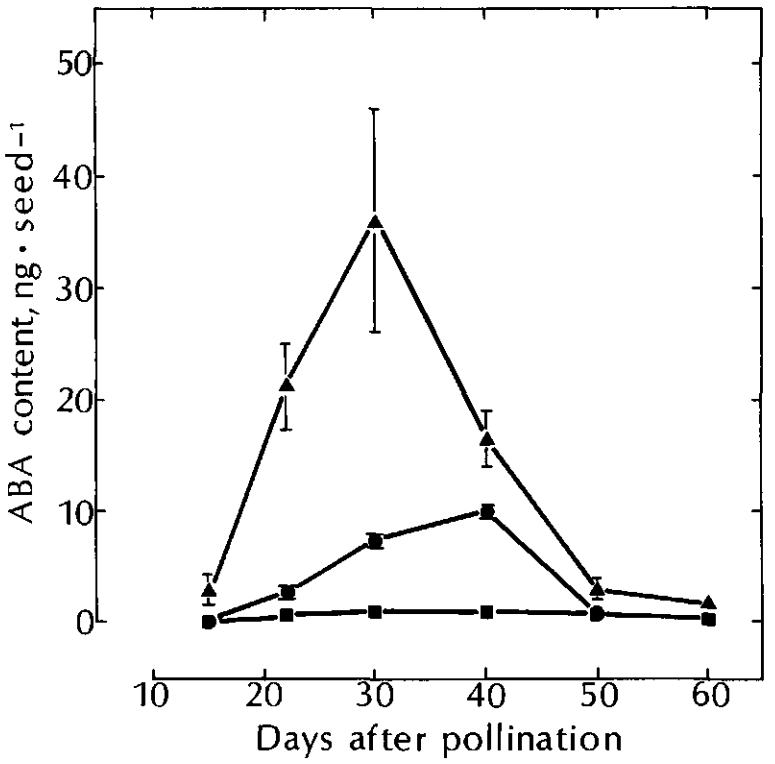

Fig. 1. ABA content in seeds developing on self-pollinated Sit/Sit (▲) or sit^W/sit^W (■) plants or on sit^W/sit^W plants pollinated with Sit pollen leading to Sit/sit^W seeds (●). Vertical bars indicate SD (n=3).

Fresh and dry weights

It has been shown before (Chapter 3) that the fresh and dry weights of mature tomato seeds were influenced by the number of seeds per fruit because of competition. Therefore, fresh and dry weights of mature seeds of different genotypes were plotted against the number of seeds per fruit (Fig. 3). The results show that the ABA deficiency did not affect fresh and dry weights of mature seeds. Fresh weight of wild-type, sit^W/sit^W and Sit/sit^W seeds was about 7 mg per seed. Fruits with low seed numbers contained somewhat heavier seeds on the average. Dry weight of seeds of all genotypes was 3.5 mg per seed on the average. The number of seeds per fruit only slightly influenced seed dry weight.

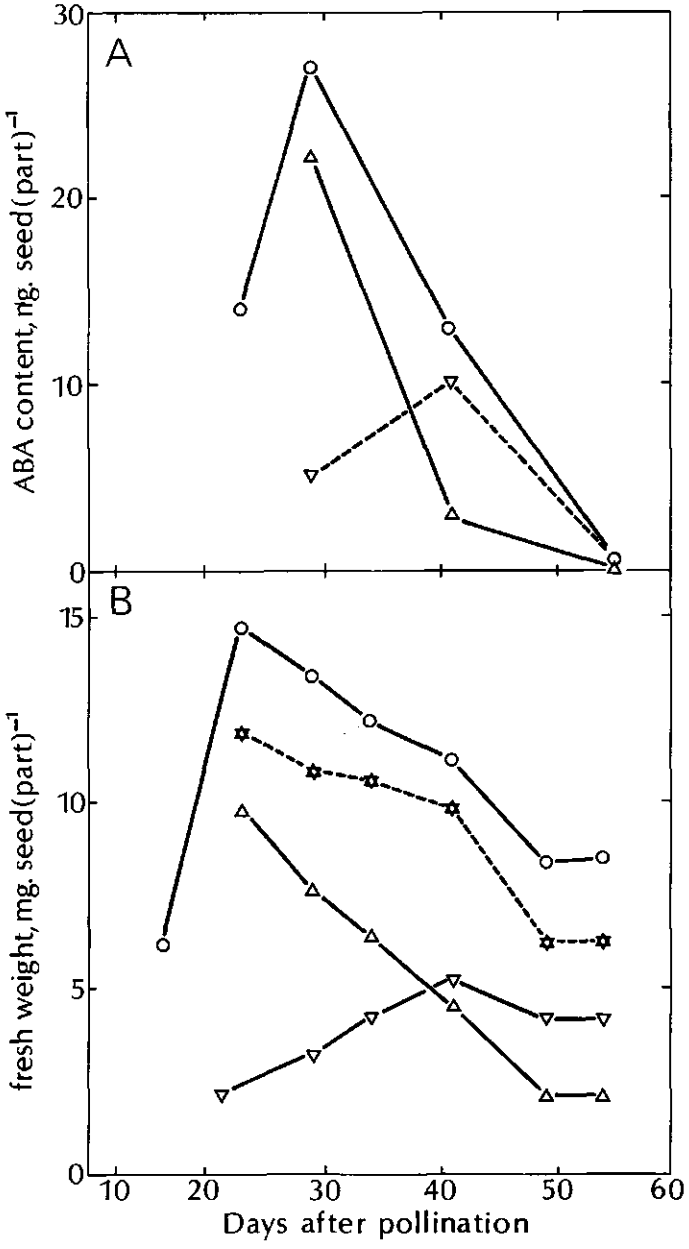

Fig. 2. ABA content (A) and fresh weight (B) of developing Sit/Sit seeds or seed parts. **A.** ABA content determined in intact seeds (○) and isolated testa (△) was used to calculate the amount in embryo plus endosperm (▽). **B.** Fresh weights were determined of intact seeds (○), isolated testa (△) and embryo plus endosperm (▽). The asterix (✡) represents the calculated sum of isolated testa and embryo plus endosperm, indicating loss during isolation.

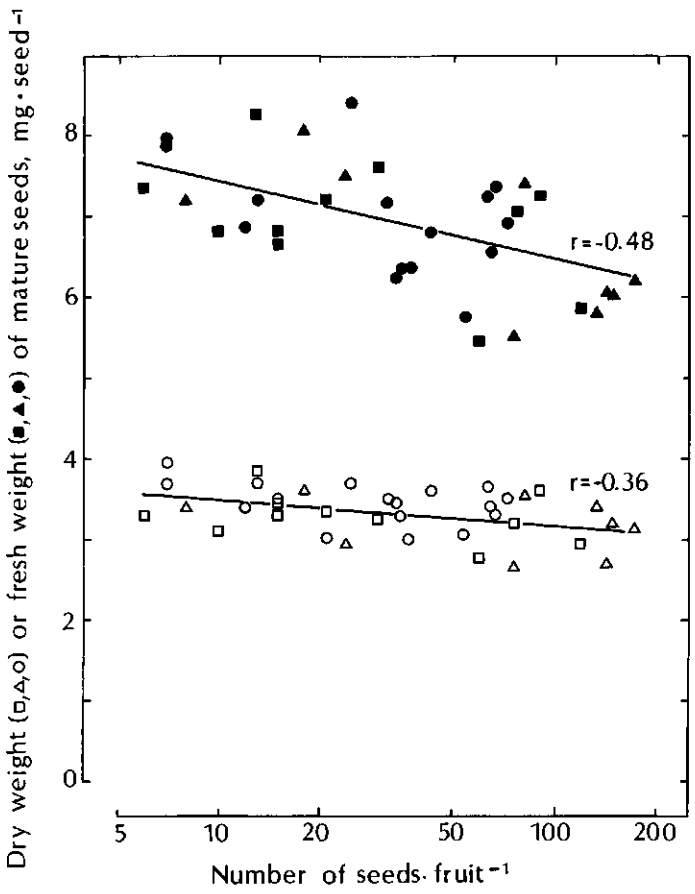

Fig. 3. Dry weight (open symbols) and fresh weight (closed symbols) of mature seeds in relation to the number of seeds per fruit. The $\underline{sit^W/sit^W}$ (□,■) and $\underline{Sit/sit^W}$ (○,●) seeds developed on $\underline{sit^W/sit^W}$ mother plants after self-pollination or pollination with Sit pollen, respectively. The Sit/Sit (△,▲) seeds derived from self-pollination of Sit/Sit plants.

Proteins

Mature, wild-type seeds were separated in embryo, endosperm and testa. The seed parts were extracted with SDS and analyzed by gel electrophoresis. The gel, presented in Fig. 4A, shows that embryo and endosperm exhibited similar patterns with regard to the major protein bands. The 6 pronounced protein bands most likely represent storage proteins, since they were less present in extracts of germinated seeds (data not shown). In extracts of the testa only

one unique protein band with a low molecular weight was visible (Fig. 4A). Light-microscopical examination of the testa revealed that most of the Coomassie Brilliant Blue-stainable material of the testa was located in the inner integumental layer.

Gelelectrophoretic patterns of proteins in mature Sit/Sit and sit^W/sit^W seeds are presented in Fig. 4B. No qualitative differences in protein pattern could be observed by this method. SDS-extracts of Sit/Sit, Sit/sit^W and sit^W/sit^W seeds of different developmental stages showed also no differences

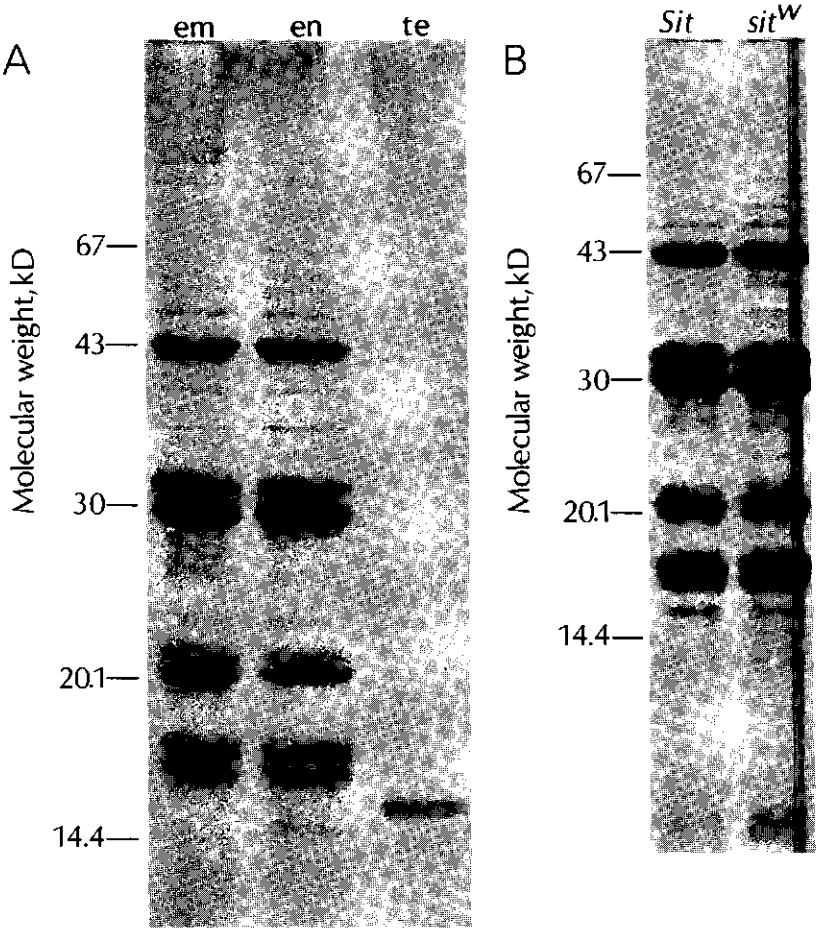

Fig. 4. SDS-polyacryamide gelelectrophoretic pattern of polypeptides extracted from A: mature wild type seedparts (em = embryo, en = endosperm, te = testa); or from B: mature Sit/Sit (Sit) or sit^W/sit^W (sit^W) seeds derived after self-pollination.

in the pattern of the major protein bands (data not shown).

Wild type, Sit/sit^W and sit^W/sit^W seeds from different developmental stages were also extracted with buffer plus salt, for quantitative determinations of protein content. The results indicate that lower levels of maternal ABA initially depressed protein accumulation, but the effect disappeared in the course of development (Fig. 5). Developing Sit/sit^W and sit^W/sit^W seeds did not differ in protein content.

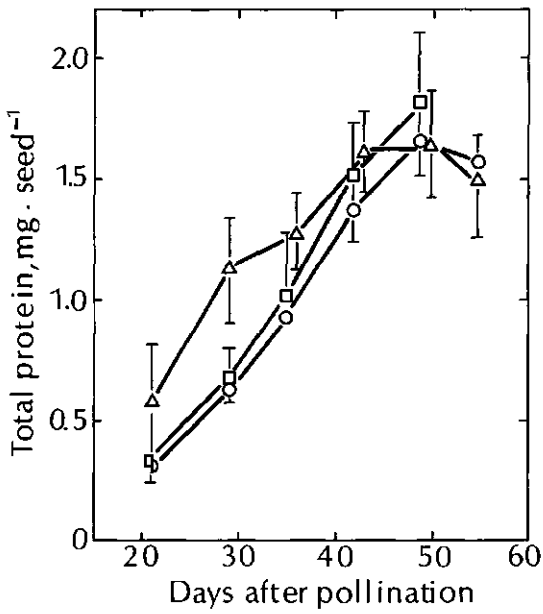

Fig. 5. Courses of protein content in seeds developing on self-pollinated Sit/Sit (△) or sit^W/sit^W (□) plants or on sit^W/sit^W plants pollinated with Sit pollen leading to Sit/sit^W seeds (○). Protein weight is expressed as equivalents of bovine gamma globuline. Vertical bars represent SD (n=3).

In a second experiment, performed with seeds harvested 2 months later, extracts were made from mature seeds of all three genotypes (Tab. 1). The amount of proteins was somewhat less compared to the levels found in mature seeds of the previous experiment. Again, there was no difference between the three genotypes.

Table 1. Total protein content extractable with phosphate buffer plus salt from mature seeds of different genotypes. Data represent the mean of 3 samples per genotype, collected from individual fruits.

genotype		proteincontent ± SD	
motherplant	seed	mg.seed^{-1}	mg.(g fresh weight)$^{-1}$
Sit/Sit	Sit/Sit	1.21 ± 0.11	189 ± 11
sit^{w}/sit^{w}	Sit/sit^{w}	1.19 ± 0.23	176 ± 20
sit^{w}/sit^{w}	sit^{w}/sit^{w}	1.28 ± 0.11	166 ± 1

Viviparous germination

Despite strongly reduced ABA levels, sit^{w}/sit^{w} seeds did not germinate prematurely in the fruits. However, when ripe sit^{w}/sit^{w} fruits remained attached to the plant for at least 2 weeks after ripening, viviparous germination of the mutant seeds was observed (Fig. 6,7), it did not occur in wild-type seeds (Fig. 7).

An attempt to study vivipary of heterozygous seeds in ABA-deficient fruits by crossing sit^{w}/sit^{w} x Sit/Sit was not successful because an insufficient number of fruits was produced. In fruits of self-pollinated Sit/sit^{w} plants about one quarter of the seeds germinated viviparously when the fruits remained attached to the mother plant (Fig. 7). A test of the seedlings that developed from the viviparous seeds, revealed that they all had the wilty phenotype and therefore the sit^{w}/sit^{w} genotype. The osmotic potential and the ABA content of the locular tissue of the ripe fruit of Sit and sit^{w} are shown in Tab. 2. The ABA content of the sit^{w}/sit^{w} fruits was about 10 % of the level found in wild-type fruits. The osmotic potential of the fruit juice was similar for both genotypes.

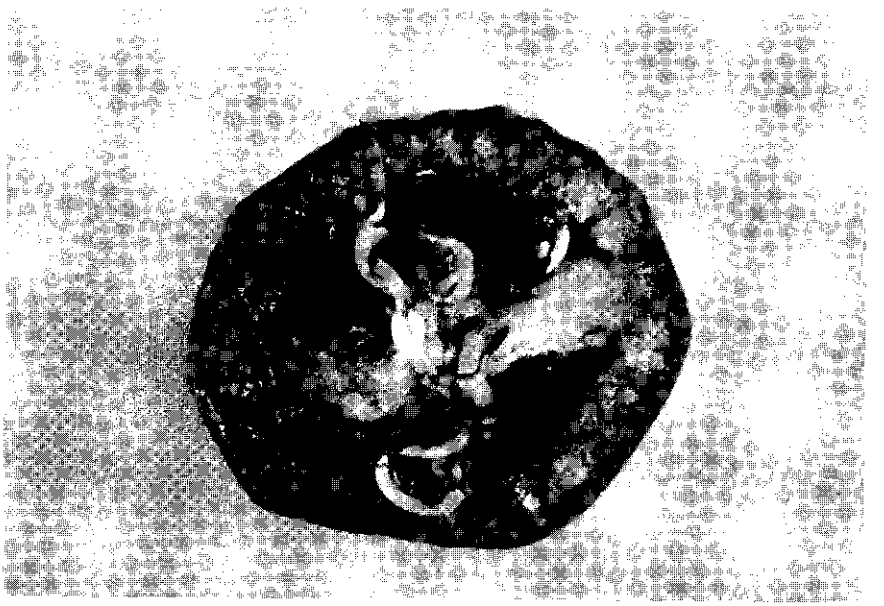

Fig. 6. Viviparous germinating mature $\underline{sit^W/sit^W}$ seeds in a ripe $\underline{sit^W/sit^W}$ fruit.

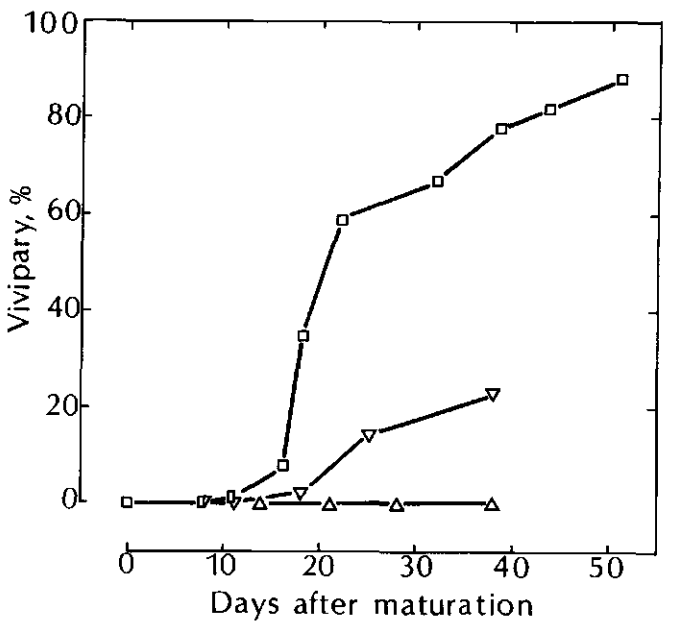

Fig. 7. Percentage of seeds germinated within ripe fruits derived from self-pollination of $\underline{Sit/Sit}$ (△), $\underline{sit^W/sit^W}$ (□) and $\underline{Sit/sit^W}$ (▽) plants.

Table 2. ABA content and osmotic potential of the juice of ripe Sit/Sit and Sit/sit^{W} fruits.

	genotype of the fruit	
	Sit/Sit (n=2)	sit^{W}/sit^{W} (n=2)
ABA content, µM	0.84 ± 0.18	0.08 ± 0.01
Osmotic potential, MPa	-0.85 ± 0.11	-0.93 ± 0.07

Development of dormancy

The development of dormancy in wild-type seeds was strongly influenced by cultivation conditions and plant handling. Seeds used in the previous experiments were produced on plants raised in pots or on hydroponic culture in a heated greenhouse with 16 h daylength without defoliation (condition 1 in Tab. 3). Under those conditions wild-type plants produced seeds that germinated directly after harvest for 15% at 26 °C in darkness. Cultivated on soil in a

Table 3. Germination of freshly harvested seeds produced under different conditions. See text for the description of the conditions of cultivation.

production	genotype	germination, %
condition 1	Sit/Sit seeds on Sit/Sit plants	15
	Sit/sit^{W} seeds on sit^{W}/sit^{W} plants	57
	sit^{W}/sit^{W} seeds on sit^{W}/sit^{W} plants	97
condition 2	Sit/Sit seeds on Sit/Sit plants	82
	sit^{W}/sit^{W} seeds on sit^{W}/sit^{W} plants	96

non-heated greenhouse at natural summer conditions, whereby the older leaves were routinely removed (condition 2 in Tab. 3), wild-type seeds germinated for 82% directly after harvest. These latter conditions come close to commercial seed productions in tomato. A first result of a further analysis of these data points to defoliation as an important factor. When wild-type plants at condition 1 were defoliated halfway the development of certain fruits, seeds from those fruits germinated directly after harvest for 75% (data not shown).

Freshly harvested $\underline{sit^W/sit^W}$ seeds from both cultivation conditions germinated to a high percentage, while $\underline{Sit/sit^W}$ seeds, that had been formed in a $\underline{sit^W/sit^W}$ fruit, took an intermediate position (Tab. 3). We also studied the germination characteristics of the seeds developing after self-pollination of $\underline{Sit/sit^W}$ (Fig. 8). All seeds that gave rise to wilting seedlings had ger-

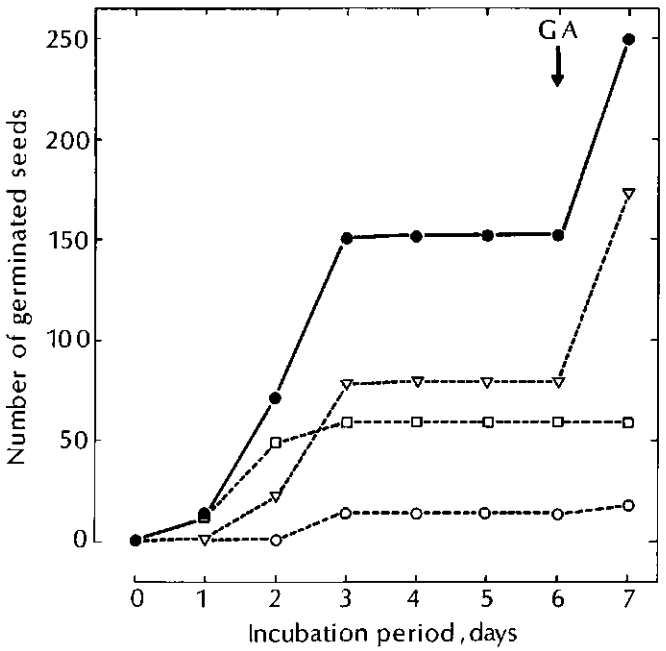

Fig. 8. The influence of the genotype of the embryo on the germination of freshly harvested mature seeds from self-pollinated $\underline{Sit/sit^W}$ plants. Seeds were collected from ripe fruits and incubated in water the next day. All seeds that had not germinated on the 6^{th} day were subsequently treated with 10 µM GA_{4+7}. Germinated seeds (●) were removed from from the Petri dishes, transplanted in soil and checked for rapid wilting of seedlings under water stress: wilting (□) or non-wilting (▽) seedlings. The phenotype of some seedlings (7%) could not be classified (○), since they did not survive the transplantation.

minated within 3 days. The seeds that formed normal seedlings germinated for about 45% in water, the remainder of the seeds required GA_{4+7} to germinate. Thus, sit^{w}/sit^{w} seeds also showed a lack of dormancy after development in ABA-rich maternal tissues. The segregation of 59 wilting versus 173 non-wilting seedlings in the offspring of self-pollinated Sit/sit^{w} plants (Fig. 8) is very close to a 1 : 3 ratio and indicates an equal contribution of Sit and sit^{w} pollen and ovules to the fertilization.

Germination of dormant wild-type seeds was stimulated by a low temperature pretreatment, particularly in combination with red light, by GA_{4+7}, and by several weeks of dry storage (Tab. 4).

Table 4. Influence of a cold pretreatment, after-ripening and different incubation conditions on the germination of freshly harvested, wild-type seeds, produced at condition 1 (see text for description). Seeds were incubated at 26 °C in water or 10 µM GA_{4+7}, and 7 days in darkness (dark) or 1 day under intermittant red followed by 6 days darkness (red). nt = not tested.

pretreatment	Germination, %		
	incubation conditions		
	H_2O, dark	H_2O, red	GA_{4+7}, dark
none	11	35	100
6 d 2 °C, imbibed	31	95	nt
7 weeks 7 °C, dry	77	nt	100

Discussion

Two sources of ABA

The present data clearly demonstrate that in developing tomato seeds endogenous ABA originates both from zygotic and maternal tissues. They are in accordance with previous studies on seeds of Arabidopsis thaliana (Karssen et

al. 1983). The ABA that occurs in Sit/sit^{W} seeds developing in sit^{W}/sit^{W} fruits (Fig. 1) can only originate from embryo and endosperm since the fruit and the integuments are ABA-deficient. This conclusion is strongly supported by the resemblance between the courses of ABA content in intact Sit/sit^{W} seeds, developing in sit^{W}/sit^{W} fruits (Fig. 1) and in embryo plus endosperm of wild-type seeds (Fig. 2A). The high rise in ABA content around 30 dap only occurred in Sit/Sit seeds, i.e. when both zygotic and maternal tissues had the capacity to produce ABA. Therefore, this rise has to be of maternal origin. The analysis of seed parts in Sit/Sit showed indeed that the rise around 30 dap was mainly present in the (maternal) integuments (Fig. 2A). Smith (1935) described that in tomato seeds the cell walls of the outer integument collapse half-way development, leaving only the secondary thickening on the radial cell walls which develop into hairs. This collapse most probably causes the dramatic reduction in fresh weight which occurs in tomato much earlier than in seeds of most other species. The loss of water is accompanied by a reduction of maternal ABA (Fig. 2). Therefore, the ABA rather dissipates into the fruit tissue than that it is metabolically removed. It can not be excluded that maternal ABA entered the zygotic endosperm and embryo in the course of development. Athough a comparison between total ABA in Sit/sit^{W} seeds developing in ABA-deficient maternal tissues (Fig. 1) and the ABA content of developing Sit/Sit endosperms and embryos (Fig. 2A) does not support this, the measurements of ABA in embryo plus endosperm of individual seeds demonstrated that at 35 dap maternal ABA from Sit/sit^{W} fruits and integuments increased the ABA content of sit^{W}/sit^{W} embryo and endosperm.

Role of ABA in seed development

Application of exogenous ABA to developing intact seeds of wheat and barley stimulated the import of assimilates and the growth of the seeds (Dewdney and McWha 1978, Tietz et al. 1981, Ackerson 1984a). King and Patrick (1982) expressed some doubt about the reliability of the applied techniques, however. Exogenous ABA has also often been applied during in vitro cultures of isolated embryos. In cultivation medium without ABA embryos germinate precociously. Addition of ABA not only prevented growth but, according to some studies, also stimulated the synthesis of certain specific proteins such as legumin in field-bean cotyledons (Barrat 1986), the β-subunit of β-conglycinin in soybean embryos (Bray and Beachy 1985), or agglutinin in wheat embryos (Triplett and Quatrano 1982). ABA also caused continuation of the transcription of mRNAs for storage proteins (Eisenberg and Mascarenhas 1985). The effect of ABA is not specific, however. During in vitro cultures of developing rapeseed embryos high osmotic concentrations of mannitol could replace ABA in the inhibition of

germination and the stimulation of storage-protein synthesis (Crouch et al. 1985). Barrat (1986) obtained similar results with field-bean cotyledons. In rapeseed embryos the osmotic effect is more rapid than ABA and is not mediated by an increase of ABA levels in the cultivated embryos (Finkelstein and Crouch 1986).

In the present experiments ABA-deficient tomato seeds do not exhibit any of these changes. Neither fresh and dry weights (Fig. 3), nor the content and composition of reserve proteins (Fig. 4, 5) were affected. The relatively higher protein content early in development of Sit/Sit seeds is more likely due to the better condition of the wild-type mother plant than to endogenous ABA, since there is no difference in protein content between sit^W/sit^W and Sit/sit^W seeds that developed on the same mother plants. Moreover, sit^W/sit^W seeds did not germinate precociously in any experiment. Therefore, our experiments with tomato present serious doubts whether a role of endogenous ABA in seed development was simulated in studies with exogenous ABA.

Role of ABA in induction of dormancy

The present experiments clearly prove that endogenous ABA plays an important role in the induction of dormancy during seed development. The results are in good agreement with the studies on the role of ABA in dormancy induction in Arabidopsis thaliana (Karssen et al. 1983). Particularly the ABA fraction that is produced in embryo and endosperm is decisive: Sit/sit^W seeds from sit^W/sit^W fruits showed inhibited germination whereas sit^W/sit^W seeds from the same fruits as well as from ABA-rich Sit/sit^W fruits (Fig. 8) germinated directly after harvest. However, maternal ABA may add to the action of zygotic ABA because Sit/Sit seeds were more dormant than Sit/sit^W seeds from sit^W/sit^W fruits. Translocation of maternal ABA from the folliage to the seeds might explain the dormancy-antagonizing effect of defoliation indicated in the present study. It is well-known that leaves produce large amounts of ABA, particularly during water stress. Hoad (1978) found in lupin that the level of ABA in the phloem sap increased with water stress and, concomitantly, the ABA content of the developing seeds. A small increase of the ABA levels in both testa and cotyledons, in reaction to water stress, was also reported for developing soybean seeds (Brenner et al. 1986).

Vivipary

The capacity of mature seeds to germinate viviparously in ripe fruits, that were still attached to the mother plant, is primarily a function of the degree of dormancy of the seeds (Fig. 7). The ABA-induced dormancy of tomato seeds

will be analysed in the next chapter. It will be shown that sit^{w}/sit^{w} seeds have the capacity to germinate in a solution of 0.5 MPa more negative osmotic potential than Sit/Sit seeds, whereas seeds of both genotypes have the same sensitivity to exogenous ABA. The different sensitivity to osmotic stress is the most likely explanation why mature sit^{w}/sit^{w} seeds do germinate readily, in both sit^{w}/sit^{w} and heterozygous fruits, whereas wild-type seeds in wild-type fruits do not germinate (Fig. 6, 7). The osmotic potential of the fruit tissues is about -0.9 MPa in both types of fruits (Tab. 2), a potential that only inhibits the germination of wild-type seeds (Chapter 6). Ripe Sit/Sit fruits contain a 10-fold higher ABA concentration than sit^{w}/sit^{w} fruits (Tab. 2), a level that is in itself, however, not inhibitory to seeds of both genotypes (Chapter 5). Thus the primary control of viviparous germination is of an osmotic nature. The conclusions are in accordance with those of Dörffling (1970), who also postulated a secondary role of ABA in the prevention of vivipary in tomato. Reduced dormancy and viviparous germination have also been reported for seeds of the ABA-deficient flacca tomato mutant (Weyers 1985).

In sit^{w}sit^{w} fruits germination of immature seeds was never observed (Fig. 7). In Arabidopsis similar observations were explained by lack of water due to dehydration of fruits and seeds (Karssen et al. 1983). In tomato, such an explanation is unlikely, since even mature seeds still contain considerable quantities of water (Fig. 2, 3). Therefore, some other ABA-independent factor must prevent precocious germination. In tomato seeds germination depends on a GA-stimulated weakening of the endosperm layer opposing the radicle tip (Chapter 2, 5). We presume that precocious germination is prevented by either a limitation of synthesis or transport of the essential GA, or by reduced sensitivity of the responding endosperm cells to this hormone.

In conclusion, the present study has shown that the action of endogenous ABA during seed development of tomato is limited to the induction of dormancy. All other aspects of seed growth, such as fresh and dry weight and accumulation of reserve proteins are not influenced by the reduction of endogenous ABA. Although the effects of exogenous ABA on cultivated embryos of many species do not imitate the action of endogenous ABA, they clearly show that immature seeds have the potency to react to ABA.

Chapter 6

Differences in dormancy and germination between seeds of wild-type and abscisic acid-deficient mutants in tomato.

In collaboration with W.P.J.M. van Ham, A.J. Stolte and C.M. Karssen.

Abstract

The ABA-induced seed dormancy in tomato is studied by comparing seeds of the ABA-deficient sit^{w} seeds with wild-type seeds cv. Moneymaker. Wild-type seeds that were raised on plants in a heated greenhouse without regular defoliation of the older leaves develop dormancy in contrast to sit^{w} seeds. In dormant wild-type seeds the neccesary weakening of the endosperm resistance in front of the radicle does not occur and germination is inhibited. A few weeks of afterripening at dry storage relieves this difference between wild-type and sit^{w} seeds. Stored seeds of both lines do still differ in the sensitivity of germination and embryo elongation to osmotic inhibition. The critical osmotic potential for 50% inhibition in sit^{w} seeds is -0.5 MPa more negative than in wild-type seeds.

Seeds of the GA-deficient ga-1 mutant, that absolutely require GA for endosperm weakening and germination, germinate in water after removal of the endosperm and testa layers in front of the radicle (detipping). In osmotic solutions the radicle elongation of detipped seeds of different lines is increasingly inhibited in the order sit^{w}, wild-type, ga-1 sit^{w}, and ga-1.

Freshly harvested wild-type seeds contain about 10-fold higher levels of endogenous ABA than sit^{w} seeds. Dry storage reduced the levels by half and imbibition in water caused a further reduction. Comparison with the minimal levels of penetrated ABA that are required for inhibition of germination made it unlikely that the different behaviour of wild type and sit^{w} depend on the actual levels in mature seeds.

It is concluded that the about 30-fold higher ABA levels of wild-type seeds during seed development has caused profound differences in the seeds that are still noticeable during germination. At harvest the action of developmental ABA interferes with the stimulation of endosperm hydrolysis, in stored seeds it inhibits the cell elongation capacity.

Introduction

Previous studies with ABA-deficient mutants of Arabidopsis thaliana and tomato provided clear evidence that the induction of dormancy during the development of seeds on the mother plant depends on a rise of endogenous ABA (Karssen et al. 1983, Chapter 5). Upon maturation of the seeds the ABA levels strongly decreased. Therefore, it was doubted whether endogenous ABA was also responsible for the maintenance of dormancy in the mature seed (Karssen et al. 1983). Bonamy and Dennis (1977) expressed similar doubts. They showed that in peach seeds the ABA level diminished much more rapidly during incubation at 20 °C than at 5 °C, whereas dormancy was only relieved at 5 °C.

Therefore, it was hypothesized that the inhibitory effect of ABA on germination is somehow fixed during seed development and does not depend on the actual presence of ABA for its expression in the mature seed (Karssen and Łaçka 1986). It is the aim of the present study to localize and analyse this ABA-induced dormancy in tomato seeds by comparing seeds of the ABA-deficient sit^{w} mutant with wild-type cv. Moneymaker seeds. The isolation and phenotypic characterization of the sit^{w} mutant have been described in Chapter 5.

In the present analysis special attention is given to the cell-expansion force of the embryo because recent studies with rapeseed have shown that application of ABA inhibits cell expansion by interfering with the process of cell-wall loosening (Schopfer and Plachy 1985). The present paper also studies whether the ABA-deficiency interferes with GA-induced processes in the seeds. Studies with GA-deficient ga-1 mutants have shown that the germination of tomato seeds absolutely depends on GA (Chapter 2). This hormone induces a weakening of the mechanical restraint of the endosperm cells, thereby permitting radicle protrusion. The study of the interaction between the two hormones in the control of germination is enabled by the availability of the recombinant of the sit^{w} and the ga-1 mutants.

Material and methods

Seed material

Wild-type tomato (Lycopersicon esculentum Mill. cv. Moneymaker), and the ABA-deficient line sit^{w}, the GA-deficient line ga-1, and the recombinant ga-1 sit^{w} were obtained from Professor J.H. van der Veen of the Department of Genetics, Agricultural University, Wageningen. Plants for seed production were raised at two locations. At small scale, seeds were produced year-round in a heated greenhouse with minimum temperatures of 21 °C (day) and 19 °C (night) and a photoperiod of 16 h was maintained by additional illumination from high-

-pressure mercury halide lamps (Philips HPI/T) at 18 $W.m^{-2}$. The plants were cultivated in pot soil. The *sit*^{W} plants were protected against direct sunlight and sprayed regularly with tap water to prevent wilting. At a larger scale seeds were produced in a non-heated greenhouse during summer and autumn, without additional illumination. The plants were cultivated in soil and shielded against direct sunlight. At this location the older leaves below the clusters with ripe fruits were removed regularly. The *sit*^{W} and *ga-1 sit*^{W} plants were sprayed once or twice a week with about 2 ml 10 µM ABA (Fluka, FRG) to reduce wilting. Of the *ga-1* plants, the top and flower-bud regions were sprayed once a week with a solution of 10 µM GA_{4+7} (Berelex, ICI, UK) to stimulate shoot growth and development of fertile flowers.

Seeds were isolated from ripe fruits and incubated in 1% HCl for 1 h to remove the remnants of the mucilagenous locular tissue. Thereafter the seeds were rinsed with tap water, dried at room temperature and stored in closed plastic containers in a refrigerator at 7 °C until use. Comparison between wild type and mutant always occurred with seed lots from the same harvest date.

Germination conditions

Triplicates of 50 seeds were sown in 5 cm glass Petri dishes on one layer of filter paper (Schleicher and Schüll No. 595) moistened with 1.5 ml of 0.25 $mg.l^{-1}$ thiomersal (BDH, UK) to prevent fungal growth, with or without growth regulators or osmotic material. ABA (Fluka, FRG) and GA_{4+7} (a gift of ICI, UK), were dissolved in 1 M KOH and diluted with distilled water, the pH of the stock solutions was adjusted to 7.0 with 1 N HCl. The osmotic potential of the polyethylene glycol (PEG) (Serva, FRG) (mol mass 6,000) solutions was calculated according to the formula of Michel (1983). The PEG solutions were refreshed every two days. The Petri dishes with seeds were placed in closed plastic boxes and incubated at 26 $\pm$ 1 °C in the dark unless mentioned otherwise. In experiments with intact seeds visible radicle protrusion was used as the criterion for germination, unless mentioned otherwise. When the layers opposing the radicle tip were removed (detipped seeds) a certain minimum protrusion was taken as criterion.

ABA determinations

The isolation of ABA and its determination by gaschromatography are described in Chapter 5.

Puncture force determinations

The force needed to puncture the endosperm plus testa layers opposing the radicle tip was determined and taken as a measure for the mechanical restraint of these seed layers. The puncture force determinations and statistical calculations of the data were performed according to the previous description (Chapter 2).

Results

Alleviation of dormancy

During incubation in water the force required to puncture the layers opposing the tip of the radicle and the percentage germination were followed in wild-type and *sit*^{W} seeds produced in the heated greenhouse (Fig.1).

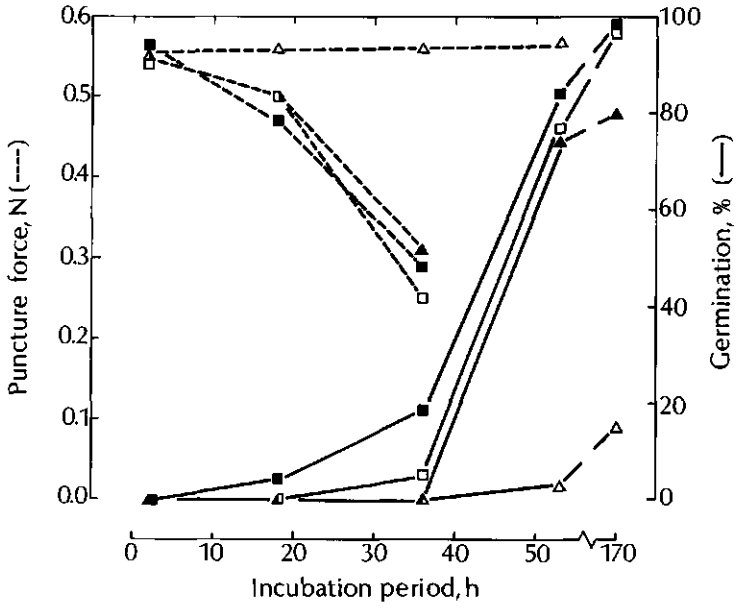

Fig. 1. Changes with incubation time of the median force required to puncture the layers opposing the radicle tip (broken lines) and of germination (solid lines) of wild-type (△, ▲) and *sit*^{W} (□, ■) seeds. Seeds were incubated either 1 day after harvest (open symbols) or after 8 weeks of dry storage at 7 °C (closed symbols).

It was shown before that at that location wild-type seeds develop dormancy (Chapter 5). The seeds were tested directly after harvest or after 8 weeks of dry storage at 7 °C. At the start of incubation the endosperm resistance of both genotypes was similar, it was not affected by dry storage. When wild-type seeds were incubated directly after harvest only a few seeds germinated and the puncture force did not decrease during incubation. Apparently, the dormant state of these seeds also prevented endosperm weakening. In wild-type seeds dry storage induced the capacity to weaken the endosperm which was followed by full germination. Both processes occurred at the same rate as in the non-dormant *sit*^{W} seeds before and after dry storage.

ABA levels

The levels of endogenous ABA were determined in seeds of both genotypes that had been raised in the heated greenhouse (Tab. 1). Freshly harvested seeds of wild type contained about 10-fold higher ABA levels than fresh *sit*^{W} seeds. Dry storage reduced the levels by half and imbibition in water caused a further strong reduction in the wild-type seeds.

Table 1. ABA content of wild-type and *sit*^{W} seeds. The seeds were extracted at harvest (non-dried), after 1 year dry storage at 7 °C, or after 1 year storage followed by 18 h imbibition of 50 seeds in 1.5 ml water. Data represent the mean ± standard deviation of duplicate samples of 50 seeds.

line	extraction	ABA content, ng.seed^{-1}
wild type	- at harvest	1.8 ± 0.2
	- after 1 year storage	0.8 ± 0.1
	- after 1 year storage plus 18 h imbibition	0.1 ± 0.0
sit^{W}*sit*^{W}	- at harvest	0.2 ± 0.0
	- after 1 year storage	0.1 ± 0.0

Inhibition of germination of stored seeds

Since the dormancy of wild-type seeds was rapidly relieved during dry storage (Fig. 1), the main part of our experiments could only be performed with stored non-dormant seeds that fully germinated in water and darkness at 26 °C for both genotypes. Therefore, it was tested whether seeds of wild type and *sit*^{W} differed in their response to less favourable conditions, such as incubation in ABA or osmotic inhibition. In these experiments seeds were used that were produced on large scale in the non-heated greenhouse, wild-type seeds raised at that location developed less dormancy compared to seeds from the heated greenhouse (Chapter 5).

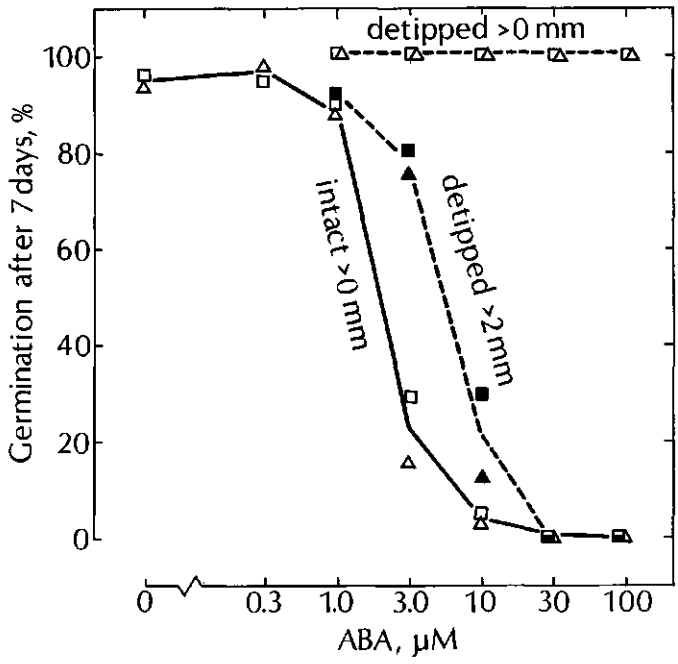

Fig. 2. Effect of exogenous ABA on the germination, or growth, of intact (solid line) or detipped (broken lines) seeds of wild type (△, ▲) and *sit*^{W} (□, ■). In detipped seeds endosperm plus testa layers opposing the radicle tip were removed in the dry state. The criterion for germination was either visible radicle protrusion (open symbols) or radicle growth surpassing 2 mm (closed symbols).

Effect of exogenous ABA.

Exogenous ABA inhibited the germination of after-ripened seeds of both genotypes to the same extent (Fig. 2). The same was true for the ABA inhibition of embryo growth studied in detipped seeds, where the interfering mechanical restraint of endosperm and testa was removed in the area opposing the radicle. Following detipping the radicle protruded in all seeds to a limited extent of about 1 mm. This early protrusion was not affected by ABA that only inhibited the further extension of the radicle (Fig. 2). The inhibition of radicle growth required higher ABA concentrations than the prevention of germination, 50 % inhibition occurred in both genotypes at about 2 µM and 5 µM, respectively.

Exogenous ABA also inhibited endosperm weakening in de-embryonated ga-1 seed-halves which was induced by incubation in 0.1-0.2 µM GA_{4+7} (Tab. 2). Complete inhibition was achieved by simultaneous incubation in 1 µM ABA.

Table 2. Influence of incubation of de-embryonated, placental half of seeds in distilled water or GA_{4+7} with or without ABA on the required force to puncture these layers. The seed parts were isolated in daylight from intact seeds after 3 h incubation in distilled water. The puncture force was determined either directly or after additional incubation for 21 h in 1.5 ml test solution at 26 °C in darkness. The puncture force is expressed as the mean of two duplicate samples of 10 de-embryonated seed parts.

Incubation period (h)	Incubation medium GA_{4+7} (µM)	ABA (µM)	Puncture force ± SD (N) (n=20)
3	0	0	0.57 ± 0.07
24	0	0	0.58 ± 0.06
24	0.2	0	0.40 ± 0.08
24	0.2	0.3	0.53 ± 0.08
24	0.2	1.0	0.57 ± 0.05
24	0.2	3.0	0.57 ± 0.06
24	0	3.0	0.56 ± 0.06

SD = standard deviation

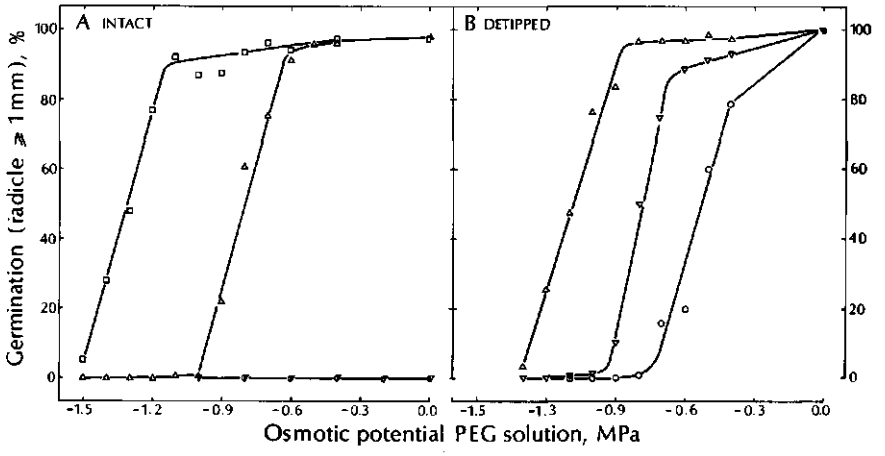

Fig. 3. Effect of osmotic potential of the incubation medium on the germination of intact (**A**) or detipped (**B**) seeds of wild type (▲), sit^{W} (□), ga-1 sit^{W} (▽) and ga-1 (○). In detipped seeds endosperm plus testa layers opposing the radicle tip were removed in the dry state. The criterion for germination was radicle growth surpassing 1 mm, which was scored after 12 days.

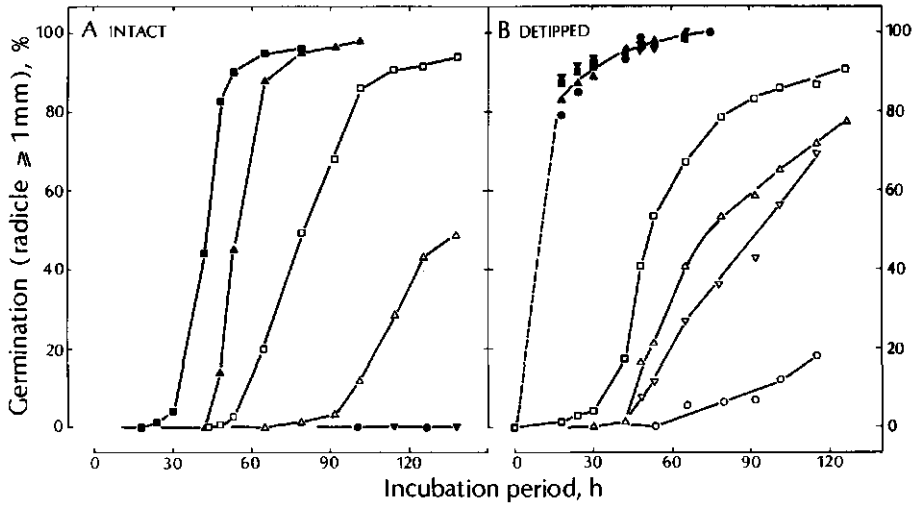

Fig. 4. Rate of germination of intact (A) or detipped (B) seeds of wild type (▲,△), sit^{W} (■,□), ga-1 sit^{W} (▼,▽) and ga-1 (●,○) in water (closed symbols) or -0.5 MPa PEG (open symbols). In detipped seeds endosperm plus testa layers opposing the radicle tip were removed in the dry state. The criterion for germination was radicle growth surpassing 1 mm.

Effect of osmotic inhibition.

Osmotic inhibition of the germination of after-ripened intact seeds and of the radicle extension from detipped seeds was studied in relation to both ABA- and GA-deficiencies. To prevent interference with the early protrusion that immediately followed detipping, a minimal extension of 1 mm was taken as criterion for both germination and radicle extension. In contrast to ABA, PEG also inhibited the early protrusion.

Seeds of *sit*^{W} germinated in PEG solutions of 0.5 MPa lower osmotic potential than wild-type seeds (Fig. 3A). In water *sit*^{W} germinated a few hours earlier than wild-type seeds, in -0.5 MPa PEG the germination of *sit*^{W} seeds was less delayed than wild-type seeds (Fig. 4A). Intact seeds of the GA-deficient *ga-1* line and of the recombinant *ga-1 sit*^{W} did not germinate in water or PEG (Fig. 3A, 4A).

In wild type, protrusion of the radicles occurred from detipped seeds at about 0.3 MPa lower PEG concentrations than from intact seeds (Fig. 3A,B). Unfortunately, seed-born infections prevented prolonged incubation of detipped *sit*^{W} seeds. After detipping also the radicles of GA-deficient seeds extended, their resistance to osmotic stress was about 0.6 MPa less than in wild type, the recombinant took an intermediate position (Fig. 3B). In water radicles of all genotypes extended at the same high rate (Fig. 4B). In -0.5 MPa PEG radicle protrusion from detipped seeds of GA-producing genotypes (*sit*^{W}, wild type) started earlier and occurred at a higher rate than that of GA-deficient genotypes (*ga-1 sit*^{W}, *ga-1*), whereas radicles of ABA-producing genotypes (wild type, *ga-1*) protruded later and at a slower rate than those of ABA-deficient genotypes (*sit*^{W}, *ga-1 sit*^{W}).

Discussion

The present data show that dormancy and germination of ABA-deficient tomato seeds differ from those of wild-type seeds, both directly after harvest and after a certain period of dry storage. It has to be discussed whether these differences are merely the result of strong dissimilarities in ABA levels during seed development or depend on the actual differences in ABA levels in the mature seeds (Tab. 1). During seed development wild-type seeds reached a peak of 36 ng.seed^{-1}. in contrast to 1 ng.seed^{-1} in *sit*^{W} (Chapter 5). Germination of non-dormant seeds of both lines was equally sensitive to exogenous ABA, 50% inhibition occurred at about 2 µM ABA (Fig. 2). At the end of imbibition tomato seeds have taken up about 4 µl water.seed^{-1} (unpublished data). In case of unobstructed diffusion each seed should have absorbed about

2 ng ABA from the 2 µM solution. However, Velasco and Stoner (1983) showed that in tomato seeds diffusion barriers exist that reduce the penetration of ABA to about 50 %. Thus the absorption from a 2 µM ABA will be about 1 $ng.seed^{-1}$. That amount approaches the 0.8 $ng.seed^{-1}$ of endogenous ABA in dry non-dormant seeds of the wild-type (Tab. 1). However, those seeds fully germinated in water at 26 °C. The lack of an inhibiting effect of endogenous ABA is most likely due to the considerable reduction of the ABA level during imbibition. It was shown in lettuce seeds that absorbed ABA, at a level comparable to the endogenous amount in dry seeds, was only inhibitory to germination if it was maintained throughout incubation (Karssen 1982c). We suppose that also in freshly harvested tomato seeds the endogenous ABA level will be reduced during imbibition, the inhibition of endosperm weakening continued, however, during at least 50 h (Fig. 1). Therefore, it is unlikely that actual ABA levels in mature seeds are responsible for the observed differences in dormancy and germination between wild-type and *sit*^{W} seeds.

Dormancy at harvest

It was shown before that the weakening of endosperm resistance absolutely depended on embryo-produced endogenous GA or on application of GA (Chapter 2). Therefore, the absence of endosperm weakening in freshly harvested wild-type seeds indicates that endogenous ABA influenced in some way the action of embryonal GA. This can be thought to be either an inhibition of GA-biosynthesis, a reduction of the sensitivity to GA or a direct inhibition of GA-induced endosperm hydrolysis. The first supposition is not likely, direct effects of ABA on GA production have not been reported. The second presumption is supported by experiments on *Arabidopsis thaliana*; seed of ABA-deficient lines were 100-fold more sensitive to GA_{4+7} than ABA-producing lines (Karssen and Laçka 1986). As we argued above, a direct inhibition by endogenous ABA of GA action during germination is not likely.

Different inhibition of nondormant seeds

During incubation in water after a period of dry storage at 7 °C, wild-type seeds showed similar endosperm weakening and germination as *sit*^{W} seeds (Fig. 1). They only differed in the moment that radicle protrusion from intact seeds started (Figs. 1, 4A). The differences were strongly enlarged when the seeds were submitted to osmotic stress. Seeds of the ABA-deficient line showed much higher resistance, germination occurred at more negative osmotic potentials (Fig. 3A), and at -0.5 MPa radicle protrusion occurred much earlier than in wild-type seeds (Fig. 4A). It is hypothesized that the high ABA levels that

occur during seed development in wild-type seeds cause an inhibition of cell elongation that can still be observed after long periods of dry storage. In seeds of Brassica napus applied ABA inhibited cell elongation at the site of cell-wall loosening without affecting the osmotic potential of the seeds (Schopfer and Plachy 1985). It can not be decided yet whether endogenous ABA similarly affected tomato seeds.

Separation of the actions of ABA and GA

The present data support the revision of the hormone balance theory of seed dormancy that was based on studies in Arabidopsis thaliana (Karssen and Laçka 1986). Also in tomato seed germination endogenous ABA and GA do not act simultaneously, the action of ABA being restricted to seed development, whereas GA is only required during germination. It was reported before that the absence of endogenous GA does not essentially interfere with seed growth and development (Chapter 4). The actions of ABA and GA are only related through the intermittant state of dormancy. ABA-induced dormancy resulted in a lower resistance to osmotic stress, which in intact seeds only became visible when seeds could produce GA (Figs 3A, 4A).In water radicle growth of GA-deficient genotypes could only occur in detipped seeds. GA-deficiency further decreased the resistance to osmotic inhibition (compare ga-1 to wild type in Figs 3B, 4B). The difference caused by ABA during development was maintained, however be it at less negative osmotic potentials (compare sit^{W} to wild type and ga-1 sit^{W} to ga-1 in Fig. 4B).

Apart from the separation of ABA and GA action in time, during development and during germination, respectively, the site of action of the two hormones is also partly separated. Endogenous ABA induces a difference in the growth potential of the embryo, whereas the main effect of endogenous GA is on the weakening of the endosperm resistance.

Chapter 7

General discussion

The present study forms a new demonstration of the usefulness of hormone mutants in the analysis of hormone action in growth and development of plants. In previous studies, for instance, a role of gibberellins in elongation growth and of abscisic acid in stomatal closure was proven by comparing plants of wild types and GA- or ABA-deficient mutants, respectively (Phinney 1985, Tal and Imber 1971). Our experiments were concentrated on the generative part of the life cycle of higher plants. In addition to parallel studies on Arabidopsis thaliana the role of GA and ABA was studied in the development of flowers, fruits and seeds and in seed germination of tomato. In tomato, like in Arabidopsis, GA- and ABA-deficient mutant lines were available for the experiments. Tomato seeds have the advantage of a much bigger size, therefore, the location of hormone synthesis and action in the different seed parts could be studied. This Chapter summarizes the main results of the thesis and discusses its contribution to the understanding of the role of hormones in seed development, dormancy and germination.

Flower development

It was shown in previous studies that application of GAs stimulates the development of pistils and stamen in flowers of many species (Pharis and King 1985). A regulatory role for GA in flower development was also suggested in experiments with the stamenless-2 tomato mutant that contains reduced levels of endogenous GA (Shawney 1974). The experiments with the GA-deficient mutants of tomato provided definite proof that GA is absolutely required for normal flower development. The ga-1 plants produce flower buds that do not develop into normal flowers, particularly the development of corolla and stamen is stagnated. A single spray with GA_{4+7} applied to newly formed buds is sufficient to complete flower development. Nester and Zeevaart (1986) recently observed in ga-2 mutants of tomato abberations in pollen and ovule development resulting in both male and female sterility.

ABA-deficient plants develop normal flowers (Chapter 5). Poor production of pollen, that is observed in these plants was associated with the weak condition of the sit^{w} plants.

Fruit development

Parthenocarpic fruit development was occasionally observed on dwarf ga-1 plants and fruit maturation occurs normally (Chapter 4). It was concluded that endogenous GA is not essensial for fruit development and maturation in tomato.

Nevertheless, GA production by seeds has a clear stimulative effect on fruit-growth. The development of GA-producing and GA-deficient seeds in GA-deficient fruits was compared by pollination of ga-1/ga-1 flowers with wild-type or ga-1 pollen, respectively. Fruits containing GA-producing seeds reach a higher fresh weight than those containing GA-deficient seeds and their ripening is delayed by one week.

A role of endogenous GA in fruit growth was also demonstrated by parallel studies in Arabidopsis thaliana. Barendse et al. (1986) showed that the rate of fruit growth was clearly increased by GAs originating from both maternal and embryonic tissues. Apart from these studies with hormone mutants no firm information had been gathered about a role of endogenous GA in fruit growth and development. Previous studies with applied GAs had only suggested a retarding effect of endogenous GA on tomato fruit maturation (McGlasson 1978).

Seed development

GA. Development of flowers, seeds and fruit on ga-1 dwarfs occurs after a single spray on the flower-bud with GA_{4+7} (Chapter 4). It can not be excluded that the very early phase of seed development occurs with the aid of residual amounts of GA_{4+7}. An effect on the later stages of seed development in ga-1 is most unlikely, because, the stimulation of elongation growth by a GA_{4+7} spray lasts only for one week. Therefore, it was concluded that GA is not essential for seed development in tomato, with a possible exception for initial stages. Endogenous GA has no influence on total protein content during seed development. Also no quantitative differences are observed in the most abundant proteins. Nevertheless, seeds grow better in the presence of endogenous GA, seed maturation is delayed by one week and fresh and dry weight are increased.

The present results raise serious doubts about the several roles that were suggested for endogenous GA during seed development (Karssen 1982a, Khan 1982). These suggestions were all based on correlations between the peak in GA-like activity, determined by bio-assay, and certain events during seed development. A role of GA was doubted before in studies on inhibitors of GA-biosynthesis (Zeevaart 1966) and in studies with GA-deficient mutants of Arabidopsis thaliana (Barendse et al. 1986).

ABA. Increases in dry-matter accumulation and stimulations of storage protein synthesis during seed development have often been associated with ABA. These conclusions were mainly based on the effect of applications of ABA to developing seeds (Dewdney and McWha 1979, Tietz et al. 1981, Triplett and Quatrano 1982, Ackerson 1984a, Schroeder 1984, Bray and Beachy 1985). Our experiments

with the ABA-deficient tomato mutant show that despite strong reductions of ABA content in the developing seeds, neither fresh and dry weight, nor the composition and content of reserve proteins are affected (Chapter 5). Also suggestions about a role of ABA in the prevention of precocious germination were not confirmed. ABA-deficient seeds did not germinate precociously in any experiment (Chapter 5). Therefore, also a role of endogenous ABA on seed growth and development is seriously doubted. Simulations of the action of endogenous ABA by application of ABA to isolated seeds or seed parts provide no reliable information on its regulatory role *in vivo*. The large variation of suggested functions of ABA based on that type of studies were not confirmed by the present data.

Dormancy induction

The present experiments clearly prove that seed-produced-ABA plays a decisive role in the induction of dormancy during seed development (Chapter 5). The onset of dormancy depends particularly on the ABA fraction produced in embryo and endosperm, the influence of maternal ABA is minor. The dual origin of ABA was shown by reciprocal crosses of wild-type and *sit*^{W} mutant. The genotype of the mother plant regulates the ABA content present in the testa which shows a peak half-way seed development. The genotype of the embryo and endosperm is responsible for a second ABA fraction, present in these tissues. This second fraction reaches its peak during the second half of seed development. The results on dormancy induction are in good agreement with similar studies on dormancy induction in seeds of *Arabidopsis thaliana* (Karssen et al. 1983).

Vivipary

Non-dormant *sit*^{W} seeds did not germinate precociously within the fruit but viviparous germination was observed when ripe fruits remained attached to the mother plant. The absence of vivipary in wild-type seeds is primarily a function of osmotic control by the seeds. The ABA level in wild-type fruits is not inhibitory to seeds of both genotypes. The osmotic potential of fruit tissue of both wild type and *sit*^{W} is -0.9 MPa (Chapter 5). Germination experiments with mature seeds showed that *sit*^{W} seeds do germinate in that potential and wild-type seeds do not (Chapter 6). Thus, the different level of ABA during seed development induces a different sensitivity to osmotic stress that in turn causes vivipary in ABA-deficient seeds. A similar correlation between reduced ABA levels and viviparous germination was found in the *flacca* mutant

of tomato (Weyers 1985) and in 5 out of 6 viviparous maize mutants (Neill et al. 1986).

Seed germination

The present experiments with GA-deficient tomato seeds prove that seed germination in this species is absolutely dependent on the presence of GA (Chapter 2). Studies on *Arabidopsis* reached a similar conclusion. Studies on tomato seeds also enabled an analysis of the location of GA action in the different seed parts. It was shown that the major action of GA is directed to the weakening of the endosperm cells around the radicle tip which facilitates protrusion of the radicle (Chapter 2). In *ga-1* seeds a similar event is dependent on exogenous GA_{4+7}. Simultaneous incubation of de-embryonated endosperms and isolated axes shows that wild-type embryos produce an endosperm-weakening factor that is absent in *ga-1* axes and thus probably is a GA.

GA-induced weakening of isolated *ga-1* endosperms is accompanied by the simulatenous induction of endo-β-mannosidase activity, the increase of α-galactosidase and mannohydrolase and the release of mannose, glucose, fructose and galactose into the incubation medium (Chapter 3). Chemical hydrolysis of endosperm cell walls reveals a composion of mainly mannose, and smaller quantities of galactose and glucose. It is concluded that in tomato seeds weakening of the endosperm is mediated by GA-induced enzymatic degradation of the mannan-rich cell walls.

GAs are stimulatory, but not essential for radicle growth (Chapter 6). Removal of the layers opposite the radicle tip causes germination of *ga-1* seeds in water (Chapter 2). Seedlings and plants that develop from the detipped *ga-1* seeds exhibit the extreme dwarfy phenotype that is normal to this genotype.

Maintenance and relief of dormancy

The endosperm resistance of freshly harvested wild-type seeds was not reduced during incubation in water and germination was blocked (Chapter 6). Seeds from *sit*^{W} did not show this form of dormancy. It is concluded that endogenous ABA interferes in some way with the action of GA.

The first form of dormancy was quickly relieved during low temperature incubation or dry storage. After dry storage seeds of wild-type and *sit*^{W} still showed considerable difference in the resistance to osmotic inhibition (Chapter 6). Determination of endogenous ABA levels in mature seeds revealed

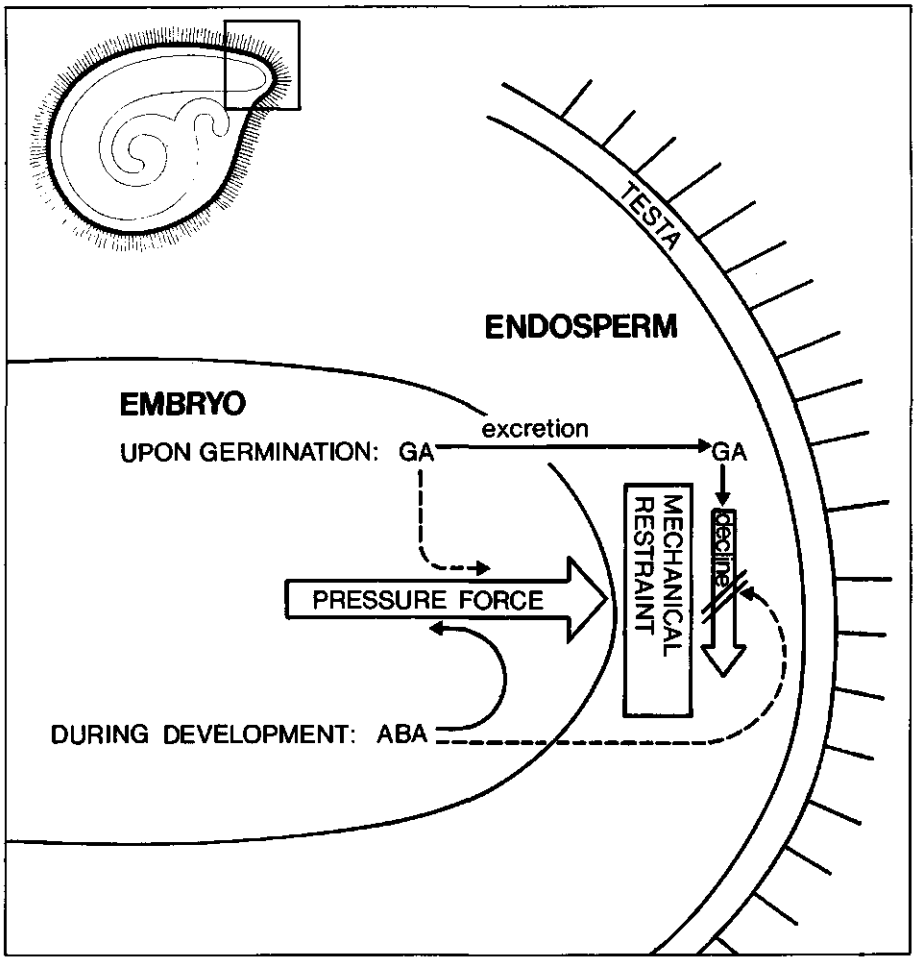

Fig. 1. A model of the action of ABA and GA in dormancy and germination of tomato seeds.

that dormancy and inhibition of germination of ripe seeds does not depend on actual ABA levels but reflects an effect of ABA action during seed development.

Model

The action of endogenous ABA and GA on dormancy and germination of tomato seeds is presented schematically in Fig. 1. The scheme visualises the separation in time and place of the action of the two hormones. ABA acts during seed development and induces an inhibition of the GA-induced endosperm weakening and a reduction of the growth potential of the embryo. The first effects diminishes rather soon during dry storage, but the latter effect of ABA is still visible after long periods of dry storage.

The absence of GA during seed development does not afford a change in germination or dormancy of mature seeds. GA is only active during germination, it acts primarily on the hydrolysis of the galactomannan-rich cell walls in the endosperm which results in a weakening of the mechanical restraint of those cell layers. GA is produced in the embryo and excreted to the endosperm cells. The resistance of the testa, in contrast to the endosperm, is not changed. A second effect of GA is the stimulation of the growth potential of the embryo.

The separation in time and place of ABA and GA action is a strong argument against the existance of a hormone balance for the regulation of seed dormancy.

Samenvatting

Zaden hebben een essentiële functie in de reproductie van hogere planten en dienen vaak voor de verspreiding van de soort. Voor de mens zijn ze van belang als bron van voedsel of grondstoffen. Vandaar dat veel aandacht besteed wordt aan de verbetering van de kwaliteit van zaden door middel van veredelingsonderzoek. Onderzoek aan de zaadontwikkeling en de zaadkieming kan een bijdrage leveren aan de verbetering van zaadkwaliteit. Met name plantehormonen worden een belangrijke rol toegedacht in de regulatie van de zaadkwaliteit. Men vermoedt dat ze van invloed zijn op de opslag en mobilisatie van reservestoffen en op de inductie en verbreking van kiemrust.

Deze studie is gericht op de rol van gibberellinen (GAs) en van abscisine zuur (ABA) bij de ontwikkeling en kieming van zaden van tomaat. Het onderzoek is uitgevoerd met behulp van tomatemutanten die niet of maar zeer gedeeltelijk in staat zijn deze hormonen te produceren en daarom met de termen GA-deficiënt of ABA-deficiënt aangeduid worden. Deze mutanten zijn geïnduceerd in de genetische achtergrond van de cultivar Moneymaker, welke in dit proefschrift met 'wild-type' wordt aangeduid. De ontwikkeling en kieming van zaden van deze mutanten is vergeleken met die van wild-type zaden. Zijdelings is ook naar de bloem- en vruchtontwikkeling gekeken.

Bloem ontwikkeling

Gibberelline (GA) blijkt essentieel te zijn voor de ontwikkeling van fertiele bloemen. GA-deficiënte planten vormen wel bloemknoppen, maar die ontwikkelen zich niet tot normale bloemen, met name de ontwikkeling van de bloemkroon en de meeldraden is duidelijk onderdrukt. Een enkele bespuiting met GA_{4+7} op nieuwe knoppen is genoeg om een normale ontwikkeling te verkrijgen (hoofdstuk 2). Aan tomateplanten die deficiënt zijn voor ABA is de bloemontwikkeling normaal. De geringere stuifmeelproductie die in deze bloemen plaatsvindt, is waarschijnlijk een indirect gevolg van de zwakke conditie van deze planten. Door gebrek aan ABA kunnen deze planten namelijk hun huidmondjes niet sluiten, waardoor de planten voortdurend staan te verdampen en snel last krijgen van watertekort (hoofdstuk 5).

Vrucht ontwikkeling

Een enkele keer ontstaan er parthenocarpe vruchten (dit zijn vruchten zonder zaden) aan GA-deficiënte planten, deze vruchten rijpen normaal. Hieruit kan geconcludeerd worden dat GA niet onmisbaar is voor de ontwikkeling en de rijping van de tomatevruchten. Productie van GA door de zaden heeft echter wel

rijping van de tomatevruchten. Productie van GA door de zaden heeft echter wel een stimulerend effect op de groei van de vruchten en het vertraagt het moment van vruchtrijping met een week (hoofdstuk 4).

Zaadontwikkeling

GA. De ontwikkeling van GA-producerende en GA-deficiënte zaden is vergeleken door bloemen van GA-deficiënte planten te bestuiven met respectievelijk wild-type of eigen stuifmeel. De experimenten hebben aangetoond dat ook voor de ontwikkeling van zaden GA-productie niet noodzakelijk is. Een uitzondering daarop is mogelijk de allereerste fase van de ontwikkeling, want een nagebleven effect van de GA-bespuiting die nodig is om de fertiliteit van de bloemen te herstellen, kan niet geheel uitgesloten worden. Het is echter onwaarschijnlijk dat die bespuitingen effect hebben op de latere onwikkelingsstadia, aangezien het effect van een GA-bespuiting op de strekkingsgroei niet langer dan een week duurt. Al is GA-productie voor de zaadontwikkeling niet essentieel, het heeft wel degelijk invloed: de zaadontwikkeling duurt een week langer en de uiteindelijk vers- en drooggewichten van de zaden worden hoger. De productie van reserve-eiwitten in het zaad wordt niet beïnvloed door GA (hoofdstuk 4).

ABA. In de literatuur worden de toename van het drooggewicht en de stimulatie van de synthese van reserve-eiwit gedurende de zaadontwikkeling vaak geassocieerd met een stijging van het ABA-gehalte. Die associaties zijn voor een groot deel gebaseerd op experimenten waarbij ABA werd toegediend aan geïsoleerde zaden of zaadonderdelen. Met behulp van de mutanten kon in onze experimenten gekeken worden naar de rol van het endogene ABA in een intacte situatie. Daarbij bleek dat noch de vers- en drooggewichten, noch de samenstelling en de hoeveelheid reserve-eiwitten beïnvloed worden door de ABA-deficiëntie (hoofdstuk 5). Ook de suggestie dat ABA een rol zou spelen in het verhinderen van de vroegtijdige kieming van onrijpe zaden werd niet bevestigd. ABA-deficiënte zaden kiemen namelijk niet in de vrucht voor ze rijp zijn (hoofdstuk 5). Het is daarom zeer de vraag of ABA wel een rol speelt bij de groei en ontwikkeling van zaden. De experimenten waarbij ABA wordt toegevoegd aan geïsoleerde zaden of zaadonderdelen, leveren zeer waarschijnlijk geen betrouwbare informatie over een rol van endogeen ABA in vivo.

Inductie van kiemrust

De in dit proefschrift gepresenteerde experimenten laten duidelijk zien dat ABA gedurende de zaadontwikkeling wèl een rol speelt bij de inductie van kiemrust. Met name de ABA-fractie in het embryo en endosperm is hier verantwoordelijk voor, de invloed van maternaal ABA is van een geringere betekenis. De aanwezigheid van deze twee bronnen in de zaden van tomaten is aangetoond met behulp van reciproke kruisingen tussen wild-type en de ABA--deficiënte mutant. Het genotype van de moederplant bepaalt het ABA-gehalte in de integumenten welke zich ontwikkelen tot zaadhuid. Een tweede fractie is aanwezig in embryo en endosperm en wordt door die weefsels zelf geproduceerd (hoofdstuk 5).

Vivipary

Onrijpe, ABA-deficiënte zaden kiemen niet in de vrucht voor ze rijp zijn. Indien de rijpe mutant-vruchten echter enkele weken aan de plant blijven hangen, treedt er vivipary op: de rijpe zaden kiemen in de vrucht (Fig. 6, hoofdstuk 5). Deze vivipary treedt niet op bij wild-type zaden. Het ABA-gehalte van het embryo en endosperm tijdens de ontwikkeling is hier hoogstwaarschijnlijk verantwoordelijk voor (hoofdstuk 5).

Kieming

Een tomatezaad is opgebouwd uit een gebogen liggend embryo omgeven door endospermweefsel en een zaadhuid. Om te kiemen zal het kiemworteltje door deze weefsels heen moeten breken. De experimenten hebben ondubbelzinnig aangetoond dat de kieming van tomatezaden afhankelijk is van GA-synthese. GA induceert vooral een verzwakking van het endosperm tegenover het kiemwortelpuntje. Met behulp van gezamelijke incubatie van losse endospermen en embryonale assen van het wild-type of van de GA-deficiënte mutant, is aangetoond dat de wild-type embryos een faktor produceren die de verzwakking van zowel wild-type endospermen als van GA-deficiënte endospermen induceert. Deze faktor wordt niet door GA-deficiënte embryonale assen geproduceerd. Het is daarom zeer waarschijnlijk dat deze faktor een GA is (hoofdstuk 2).

De door GA geïnduceerde verzwakking van geïsoleerde endospermen gaat gepaard met een gelijktijdige inductie van het celwand afbrekende enzym endo-β-mannosidase, terwijl de activiteit van twee andere celwand afbrekende enzymen, α-galactosidase en mannohydrolase onder invloed van GA toeneemt. Ook gelijk-

tijdig met de endospermverzwakking komen bij de incubatie van geïsoleerde endospermen in een GA-oplossing de suikers mannose, glucose, fructose en galactose vrij. Met behulp van chemische hydrolyse is aangetoond dat de celwanden van de endospermcellen bij tomaat voor een groot deel uit mannose bestaan en uit geringere hoeveelheden galactose en glucose. Op grond van deze informatie werd de conclusie getrokken dat de endospermverzwakking veroorzaakt wordt door een GA-geïnduceerde enzymatische afbraak van galactogluco-mannaan ketens in de celwanden (hoofdstuk 3).

Wanneer de endosperm- en testalagen tegenover het kiemwortelpuntje worden weggesneden, kunnen GA-deficiënte zaden ook kiemen, uiteindelijk kan dan zelfs een volledige dwergplant ontstaan (Fig 1, hoofdstuk 1). De groeisnelheid van het kiemworteltje is echter wel beperkt. Blijkbaar is GA niet essentieel voor de groei van het kiemworteltje, maar werkt het wel stimulerend (hoofdstuk 6).

Kiemrust

Zoals eerder genoemd, bezitten wild-type zaden kiemrust, ze kiemen slecht direct na de oogst. In hoofdstuk 6 is aangetoond dat in deze zaden de noodzakelijke verzwakking van het endosperm geblokkeerd is. ABA-deficiënte zaden bezitten deze vorm van kiemrust niet. Dus op de een of andere manier beïnvloedt het endogene ABA de GA-werking. Na een paar maanden droge bewaring verdwijnt deze vorm van kiemrust in de wild-type zaden. Ze kiemen dan volledig in water. Toch blijft er een verschil met ABA-deficiënte zaden: de wild-type zaden zijn veel gevoeliger voor remming van de kieming met behulp van een osmoticum. De bepaling van de ABA-gehaltes in rijpe en geïncubeerde zaden toonde aan dat de kiemrust niet veroorzaakt wordt door verschillen in actuele ABA gehaltes, maar veel eerder een effect is van de werking van ABA tijdens de zaadontwikkeling (hoofdstuk 6).

Model

Op basis van de gevonden resultaten kan een model gepostuleerd worden over de werking van endogeen ABA en GA met betrekking tot kiemrust en kieming van tomatezaden. Een schema van dit model is weergegeven in figuur 1 van hoofdstuk 7, het toont een scheiding in tijd en plaats van de werking van beide hormonen. ABA oefent haar werking uit tijdens de zaadontwikkeling, het veroorzaakt dan een remming van de later door GA-geïnduceerde endospermverzwakking en het veroorzaakt een reductie van de groeikracht van het embryo in een osmoticum. Het eerste effect verdwijnt vrij snel gedurende droge bewaring van

de zaden, het tweede effect is nog steeds zichtbaar na bewaring gedurende langere tijd.

De afwezigheid van GA tijdens de zaadontwikkeling heeft op zich weinig invloed op het kiemgedrag van rijpe zaden. Het is alleen actief gedurende de kieming. Door het embryo geproduceerd GA induceert dan de hydrolyse van de mannaanketens in de endospermcelwanden tegenover het kiemwortelpuntje. Die hydrolyse resulteert in een verzwakking van deze lagen, waardoor het kiemworteltje er door heen kan breken. Daarnaast stimuleert GA ook de groeikracht van het embryo.

De scheiding in tijd en plaats van de ABA- en GA-werking is een krachtig argument tegen de regulatie van zaadkieming door een hormoonbalans.

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Curriculum vitae

Op 31 juli 1956 ben ik in Oude Niedorp geboren. Na het behalen van het diploma VWO in 1974 werd aansluitend met de studie biologie aan de Universiteit van Amsterdam begonnen. In 1978/79 ben ik tijdelijk beleidsadviseur geweest van het College van Bestuur van die universiteit, op het gebied van studentenhuisvesting. Het doktoraaldiploma biologie werd met lof behaald in 1982, met als doktoraalvakken genetica, bijzondere dierkunde, plantenfysiologie en didaktiek der biologie. Hierna ben ik op pro deo basis een klein jaar werkzaam geweest bij de vakgroep Genetica aan de Universiteit van Amsterdam. Van oktober 1983 tot oktober 1986 was ik in dienst van de Nederlandse organisatie voor zuiver-wetenschappelijk onderzoek (ZWO) in het kader van een onderzoeksproject gesteund door de Stichting voor Biologisch Onderzoek in Nederland (BION). Het onderzoek werd uitgevoerd aan de vakgroep Plantenfysiologie van de Landbouwuniversiteit Wageningen. Sinds januari 1987 ben ik werkzaam bij het Instituut voor de Veredeling van Tuinbouwgewassen (IVT) te Wageningen.

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