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

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Abstract. The germination of seeds of tomato [*Lycopersicon esculentum* (L.) Mill.] cv. Moneymaker has been compared with that of seeds of the gibberellin-deficient dwarf-mutant line *ga-1*, induced in the same genetic background. Germination of tomato seeds was absolutely dependent on the presence of either endogenous or exogenous gibberellins (GAs). Gibberellin A_{4+7} was 1000-fold more active than commercial gibberellic acid in inducing germination of the *ga-1* seeds. Red light, a preincubation at 2° C, and ethylene did not stimulate germination of *ga-1* seeds in the absence of GA_{4+7}; however, fusicoccin did stimulate germination independently. Removal of the endosperm and testa layers opposite the radicle tip caused germination of *ga-1* seeds in water. The seedlings and plants that develop from the detipped *ga-1* seeds exhibited the extreme dwarfy phenotype that is normal to this genotype. Measurements of the mechanical resistance of the surrounding layers showed that the major action of GAs was directed to the weakening of the endosperm cells around the radicle tip. In wild-type seeds this weakening occurred in water before radicle protrusion. In *ga-1* seeds a similar event was dependent on GA_{4+7}, while fusicoccin also had some activity. Simultaneous incubation of de-embryonated endosperms and isolated axes showed that wild-type embryos contain 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.

Key words: Endosperm weakening – Germination (seed) – Gibberellin and seed germination – *Lycopersicon* (mutant, germination) – Mutant (gibberellin deficient).

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 gibberellins (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 such as diethylene glycol disulfide (Gafni and Shechter 1981) or 2-chloroethyltrimethylammonium chloride (chlormequat; Hepher and Roberts 1985) has been reported. However, caution is required in interpreting results of inhibitor studies since secondary effects on other processes, e.g. sterol biosynthesis and cell division, have been demonstrated (Douglas and Paleg 1974; Nitsche et al. 1985).

Gibberellin-deficient dwarfy mutants have been isolated in rice (Murakami 1972), barley (Hopp

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Abbreviations: GA(s) = gibberellin(s); GA_3 = gibberellic acid

et al. 1981) and maize (Phinney and Spray 1982). The 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 depends absolutely on the application of exogenous GAs (Karssen and Laçka 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 indicate 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.

Materials and methods

Seed material. Wild-type tomato [*Lycopersicon esculentum* (L.) 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. Gibberellin-mutant plants were sprayed once a week with a solution of 10 μM GA_{4+7} (Berelex; ICI, Bracknell, Berks., UK) on the top and flower-bud 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 5° C until use. Comparisons 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 (no. 595; Schleicher & Schüll, Dassel, FRG) moistened with 1.5 ml of distilled water or test solution. Gibberellic acid (Sigma, St. Louis, Mo., USA) and GA_{4+7} containing 40% A_4 and 42% A_7 (a gift of ICI, Bracknell, Berks., 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, Poole, Dorset, UK), in a concentration of 0.25 $mg \cdot l^{-1}$ 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 unless mentioned otherwise. Visible radicle protrusion was used as a criterion for germination, it was normally determined after 7 d.

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

Incubation of seeds parts. Aseptic isolation of de-embryonated seed parts was performed in a laminar-flow cabinet under white light from fluorescent tubes. 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 containing sterile distilled water or test solution. Solutions were sterilized with a Millex-GV filter unit (Millipore S.A., Molsheim, France). After 2–3 h of dark imbibition the seeds were cut into halves. From the placental seed-half (Fig. 1), all embryo parts were carefully removed with tweezers.

In studies on the behaviour of isolated embryo axes, we used the radicle with the 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 into halves after at least 2 h of imbition and the axes were removed from the placental seed-halves. In one experiment the testa was also 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., Highwycombe, UK). The needle with the seed-half moved downwards with a speed of 5 $mm \cdot min^{-1}$ 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 seed-half 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.

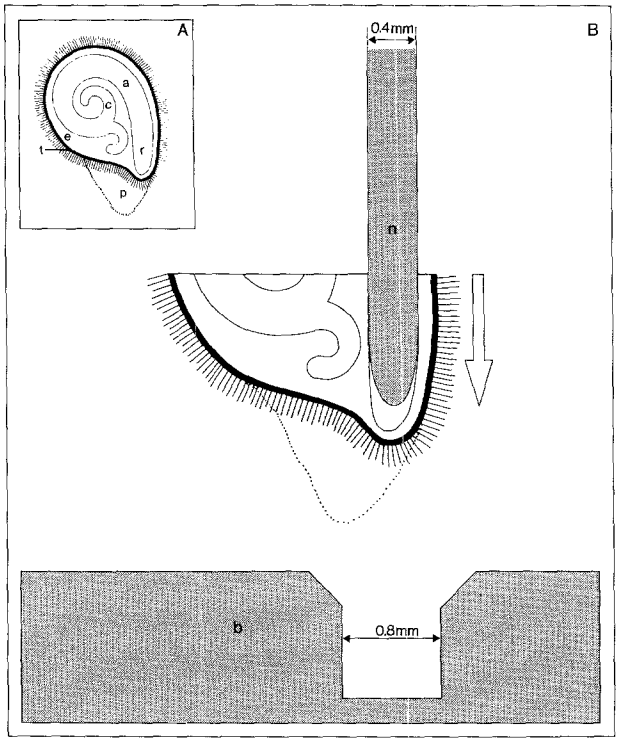

Fig. 1 A, B. Schematic presentation of **A** a section through a tomato seed and **B** the measurement of the puncture force. In **A** the curled embryo is shown with cotyledons (*c*), axis (*a*) and radicle (*r*) surrounded by endosperm (*e*), testa (*t*) and remnants of placental tissue (*p*). In **B**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

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 to take 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 by size; thereafter, 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 $p = 51^{-1}[n + i(51\text{-}n)11^{-1}]$ 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.

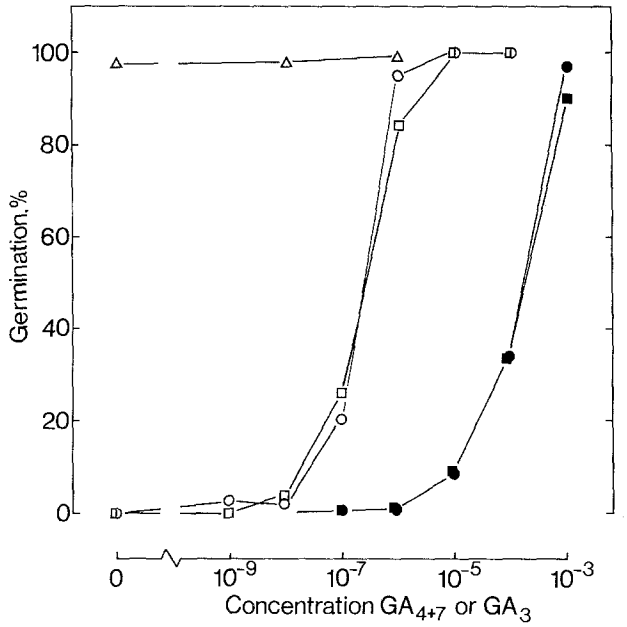

Fig. 2. The effect of GA_{4+7} and GA_3 on the germination of wild-type (△), *ga-1* (○, ●) and *ga-2* (□, ■) tomato seeds; GA_{4+7}, *open symbols* ; GA_3, *closed symbols*. Germination was recorded after 7 d incubation at 26° C in darkness

Ethyl-acetate extraction. In one experiment, 100 isolated axes were incubated in water under intermittent (10 min·h^{-1}) red irradiation for 24 h, followed by 2 d 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 aqueous 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 containing filter paper and allowed to evaporate till dryness. Thereafter, 1.5 ml sterile water was added.

Results

Wild-type tomato 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 than the former (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 germination of the *ga-1* seeds in the absence of endogenous GAs. Application of GA_{4+7} was not necessary when the endosperm and testa layers opposing the radicle were removed (detip-

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 the seeds of the dwarf mutant of tomato *ga-1*. In detipped seeds, the testa plus endosperm layers opposing the radicle were removed after 2 h of imbibition. Germination was scored after 7 d of incubation at 26° C

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

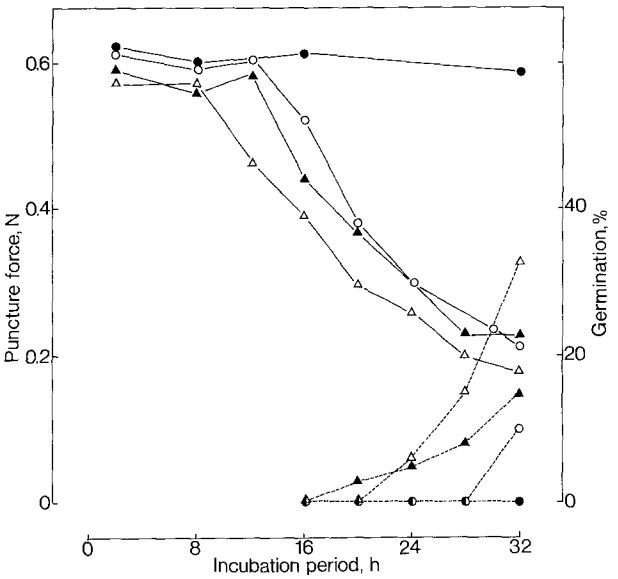

Fig. 3. Changes with time of the median force required to puncture the layers opposing the radicle tip (*solid lines*) and of germination (*broken lines*) of wild-type (△, ▲) and *ga-1* (○, ●) tomato seeds incubated in water (*closed symbols*) or 10 μM GA_{4+7} (*open symbols*). See *Materials and methods*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

ping). The seedlings growing from detipped *ga-1* tomato seeds were of the dwarfy *ga-1* phenotype.

The germination of detipped *ga-1* seeds in water indicated that the action of GAs was located in the layers surrounding the tip of the radicle. The 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 12th h of incubation onwards, well in advance of radicle protrusion which 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;

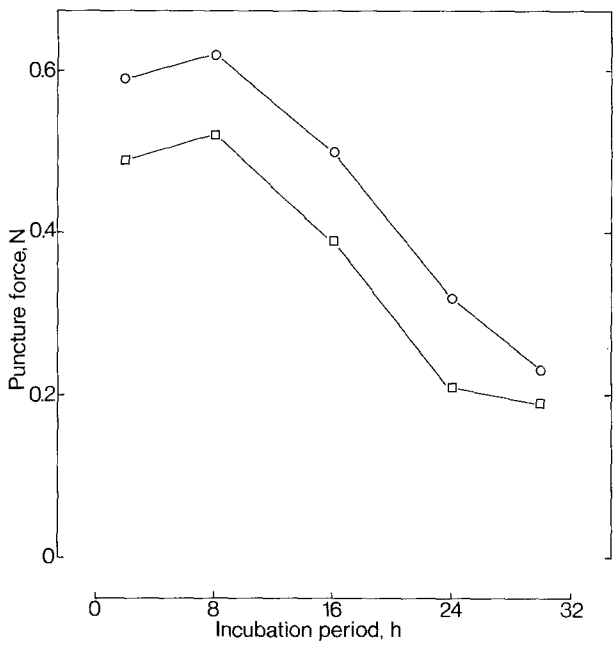

Fig. 4. Changes with time of the puncture force required to disrupt the endosperm (□) or endosperm plus testa (○) in the region opposing the radicle tip during incubation of *ga-1* tomato seeds in 10 μM GA_{4+7} at 26° C. Remnants of the placental tissue were also removed with the testa. Endosperms were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

however, 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 with these processes in *ga-1* seeds (Fig. 3); this difference might be the consequence of endogenous GA production in the wild-type seeds.

In *ga-1* tomato seeds the decrease of the mechanical resistance of endosperm plus testa during incubation was compared with that of the endosperm alone. In the presence of the testa and remnants of the placenta, the required force had to be about 0.1 N higher, indicating that the weakening process was restricted to the endosperm (Fig. 4). Similar observations were made with wild-type seeds in water or GA_{4+7} (data not shown). In the time-course experiments described so far, 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 subsequent isolations were performed at around the third hour of imbition and isolated de-embryonated seed-halves (placental ends) and embryonic axes were incubated separately for a further period of time.

The experiments with de-embryonated seed-halves clearly showed that the difference between

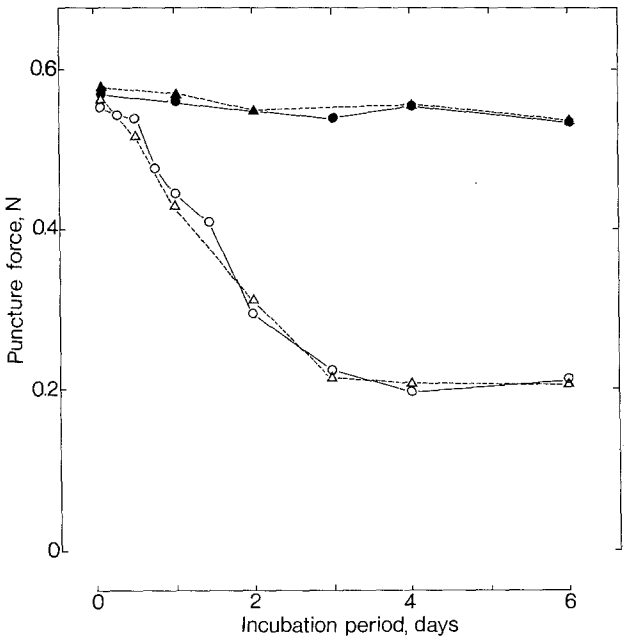

Fig. 5. Changes with time of the puncture force required in de-embryonated seed-halves to disrupt the layers that, in the intact state, opposed the tip of the radicle. Half-seeds of wild-type (△, ▲) and *ga-1* (○, ●) tomato were de-embryonated after 2 h of incubation in water and thereafter incubated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

the two genotypes resided only in the embryo. During incubation of embryoless seed-halves the weakening of the endosperm resistance, both in wild-type and *ga-1* seeds, depended absolutely on exogenous GA_{4+7} (Fig. 5), whereas in intact seeds this dependence 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 more slowly than in intact seeds: the puncture force decreased to the minimal value of 0.2 N in 72 h instead of 32 h (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. At a concentration of 100 μM fusicoccin the puncture force declined to 0.42 N in 48 h (data not shown).

The above experiment demonstrates that only wild-type embryos contain 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 embryonic 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 tomato, 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

A preliminary experiment was carried out to determine 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 depends absolutely 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 occuring 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. Gibberellin A_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, 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 seeds of the Grand Rapids cultivar

of lettuce 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 the result of 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 promoting 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 of tomato seed is directed to the weakening of the endosperm cells surrounding the radicle tip. Endosperm weakening never occurred in the *ga-1* mutant, either in intact or in de-embryonated seed-halves, without addition of GA_{4+7} (Figs. 3, 5). Since in the wild-type, weakening was only independent of exogenous GA in intact seeds, it is probable 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 naturally occurring factor in tomato seeds, the stimulative action of this compound on endosperm weakening certainly does not mimic an endogenous event.

Although the embryo probably contains a GA, growth of the embryo itself does not seem to be absolutely dependent on GA. When endosperm and testa were removed from the area opposite the radicle tip, embryo elongation occurred in *ga-1* seeds 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 the presence 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 habit for at least one year, while all plant parts were still sensitive to application of GA_{4+7} (data not shown).

Evidently, endogenous GAs are not requried 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. The transport of GAs from embryo tissues to aleurone cells in barley and other cereal seeds (e.g. Ashford and Gubler 1984) is well documented. In seeds of dicotyledons, e.g., celery, indirect evidence indicates 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 requried 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 for 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). This hydrolysis occurred exclusively as a post-germinative phenomenon (Bewley et al. 1983) and thus pointed to a function for GA 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 modifi-

cations in a restricted area of the endosperm opposite the radicle tip, prior to emergence of the radicle.

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