RELATIONSHIP BETWEEN GIBBERELLINS, HEIGHT AND STRESS TOLERANCE IN BARLEY (Hordeum vulgare L.) SEEDLINGS

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SUNITA SARKAR

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ABSTRACT

RELATIONSHIP BETWEEN GIBBERELLINS, HEIGHT, AND STRESS TOLERANCE IN BARLEY (*Hordeum vulgare* L.) SEEDLINGS

Sunita Sarkar

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Advisor:

Dr. R. A. Fletcher

Barley cv. Perth treated with two classes of plant growth regulators, a triazole (paclobutrazol) and two acylcyclohexanediones (trinexapac-ethyl and prohexadione-Ca) that inhibit gibberellin (GA) biosynthesis at different stages were exposed to either heat or paraquat stress. They exhibited a strong quadratic regression between height and tolerance to heat stress, with paclobutrazol-treated plants being the shortest and most tolerant. This suggested that lower GA levels enhance stress tolerance, since shoot-height is closely related to GA levels. In order to determine the role of height in stress tolerance, a comparison of stress tolerance in barley GA-responsive (*dwf1* and MC96) and GA-non-responsive (*Dwf2* and MC90) mutants was conducted. Both of the GA-non-responsive mutants, *Dwf2* and MC90, showed damage due to stress, although *Dwf2* was significantly shorter than MC90. The *dwf1* mutant had one-tenth the levels of total growth-active GAs compared to the two least tolerant genotypes, cv. Perth and *Dwf2*, and was the most stress tolerant. This confirmed that the endogenous GA level and its subsequent effect on height, was possibly a major factor in imparting stress tolerance in higher plants.

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I want to thank my family who has always kept me going. They have always been there my driving force, with their trust, care, and support every step of the way. Without them, I would not be here today. I want to thank my father for his support and "Dad, I promise to complete a Ph. D. next". Lastly, I want to thank my mother from the bottom of my heart for being my guardian angel, for giving me everything I ever needed, for being strong for me and just for being there when I needed her the most. I dedicate this thesis to Ma.

I want to share two simple quotes that I truly believe in.

"For what shall it profit a man, if he gains the whole world, but loses his own soul."

From the Holy Bible

"Happiness is where you find it, rarely where you seek it."

Anonymous

I hope that one day everyone will realize the importance of these ideas and in so doing come together to make this a better place for all of us, animals and plants included, to live in.

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EXPERIMENTAL SECTION

LIST OF ABBREVIATIONS

ABA	Abscisic acid
DAP	Days after planting
F_v/F_m	Variable to maximal chlorophyll fluorescence
GA	Gibberellin
Pac	Paclobutrazol
Pro	Prohexadione-Ca
SOD	Superoxide dismutase

LITERATURE REVIEW

1.0 Stress

Biological stress, as described by Levitt (1980), is an environmental factor capable of inducing potentially injurious strain on a iiving organism; strain being the reduced or changed physical, or chemical function. Organisms live in dynamic environments to which they must adapt. Animals are capable of moving from environments that are inhospitable to those that are less harsh, whereas plants must alter their physiology in an attempt to counter the imbalances created by the environmental fluctuation (Brodl, 1990). These plant responses (strains) to the severity of the environment (stress) have long been under investigation for their role in agriculture and in further understanding the functioning of the "normal" unstressed cell (Levitt, 1980).

Plant species vary in terms of their optimal environments and susceptibility to particular stresses (Jones and Jones, 1989). There are two ways plants can "resist" stress: avoidance and tolerance (Levitt, 1980). In the former, the plant excludes the stress either partially or completely using physical, chemical or metabolic barriers. Such is the case with most instances of "salt tolerance" which are actually salt avoidance. In contrast, a stress-tolerant plant either prevents, decreases or repairs the injurious strain induced by the stress, without active exclusion (Levitt, 1980). Common examples of this are freezing, drought and heat resistance, in which the plants are acclimated, or are better equipped to withstand damage due to the stress. A third method used by plants, known as

stress evasion, involves completing the vegetative stage of their life cycle prior to the imposition of a seasonal stress such as frost (Levitt, 1980).

Levitt also described two forms of strains, or responses to stress. The first is known as elastic biological strain, where the changes in an organism's function due to a biological stress return to optimal levels when the stress is removed. An example is the reduction in photosynthesis under low light intensities and the subsequent return to "normal" when light intensity reaches the optimum level. The second is a plastic biological strain, which is a permanent change in plant function such as, after exposure to extreme temperature (Salisbury and Ross, 1992). Several aspects of the responses of plants to the different stresses and of the stresses themselves indicate general principles (Jones and Jones, 1989). For instance, salinity, freezing, and water deficits may act, at least partly, through reduced water uptake in plants (McKersie and Leshem, 1994).

There is substantial evidence that plants respond to very different stresses in similar ways, for example altering cellular osmotica for adaptation to drought and salinity, as well as to confer protection from temperature extremes (Jones and Jones, 1989). The development of cross protection, whereby exposure to a certain stress causes a degree of hardening such that plants become more tolerant to other stresses, provides more evidence that general principles exist among different stresses and subsequent plant responses (Jones and Jones, 1989).

1.1 Types of Environmental Stresses

Stressful environments are often characterized by the occurrence of more than one stress simultaneously (Austin, 1989). Though there are similarities between the various stresses, the types of stresses that have been well described include those caused by low and high temperature, either a lack of, or excess water, free radicals, salt and environmental pollution (Levitt, 1980; McKersie and Leshem, 1994). Stress caused by high temperature (heat) and free radicals (oxidation) will be described in this thesis.

1.1.1 Heat stress

Heat stress commonly occurs during plant growth and development and can be responsible for major yield losses in crop plants (Kraus et al., 1995b). However, heat stress is difficult to evaluate in absolute terms since it is dependent on the optimal temperature range for normal growth and development of species, as well as on the duration the organism is exposed to the stress (Levitt, 1980). Furthermore, heat stress is usually closely associated with water stress, generally considered a secondary stress, caused by increased transpiration rates and eventual dehydration (Levitt, 1980). However, numerous studies conducted under conditions of optimal water supply have provided conclusive evidence that heat stress is an independent syndrome (McKersie and Leshem, 1994). The most extensively characterized stress response is that produced by a heat shock, i.e. exposure to supraoptimal temperatures (5 to 10°C above normal growth temperatures) for a period between 15 min and a few hours (Brodl, 1990).

Plants that can withstand high temperatures are termed thermotolerant, though most higher plants can tolerate up to 40°C (Levitt, 1980). There is hardly a physiological process that is not affected by heat stress, and the basic subcellular and molecular damage produced include enzyme denaturation, alteration of membrane phase and fluidity, and unfolding of nucleic acids (McKersie and Leshem, 1994). Optical and electron microscopy point to cellular membranes as the locus of injury, with the chloroplastic membrane being the most susceptible (Levitt, 1980).

Thermotolerant plants have developed mechanisms to counter these effects. These mechanisms include; modified photosynthetic systems to avoid photoinhibition and free radical damage (Sopher et al., 1999); altered membrane (Paliyath and Fletcher, 1995a) and protein structures (Brodl, 1990); synthesis and targeting of heat shock proteins (HSPs) in cells (reviewed by Viswanathan and Khanna-Chopra, 1996; Waters et al., 1996; Nover and Schraf, 1997) and increased evapotranspiration to reduce leaf temperature (Booker, 1991). A recent study by Gong et al. (1997) revealed that the acquisition of thermotolerance is also induced in part by extracellular Ca^{2+} that crosses the plasma membrane into the cell and mediates intracellular calmodulin.

1.1.2 Oxidative Stress

A common effect of many environmental stresses is to cause oxidative damage (Smirnoff, 1998). Oxidative stress results from the creation of active oxygen species and when this reaction is dependent on light, the resultant stress is termed photooxidation (Foyer et al., 1994). Many reactive oxygen species,

such as ozone, singlet oxygen, hydroxyl radicals, and organic oxyradicals, have been implicated in damage to plant organs and biopolymers such as chloroplasts, cell membranes, proteins, and DNA (Larson, 1995). However, even under optimal conditions, many metabolic processes, especially the photosynthetic electron transport system, produce active oxygen species (McKersie and Leshem, 1994). Furthermore, activated forms of oxygen are important in the biosynthesis of organic molecules, polymerization of cell wall constituents, detoxification of xenobiotics and in the defense against pathogens (McKersie and Leshem, 1994). Under non-stressful conditions the production and destruction of these active oxygen species is carefully regulated (Foyer et al., 1994). However both natural stresses such as heat and chilling, and anthropogenic stresses such as herbicides (paraquat) and air pollutants (ozone and sulphur dioxide) provoke increased production of toxic oxygen derivatives, which can ultimately lead to a loss of function and tissue destruction (Foyer et al., 1994). Oxygen radicals cause lipid peroxidation, protein cross-linking and inactivation, and mutations in DNA (McKersie and Leshem, 1994).

The principal defenses against these reactive molecules and free radicals in plants include detoxifying enzymes known also as antioxidants, such as catalase, peroxidase and SOD, and lower molecular weight secondary products, including phenolic compounds, carotenoids, nitrogenous and sulfur-containing materials with antioxidant activity (Larson, 1995). Numerous studies using transgenic plants that overexpress genes for antioxidant enzymes such as SOD (McKersie et al., 1999; and Van Breusegem et al., 1999) and ascorbate

peroxidase (Wang et al., 1999) report that these plants are more tolerant to a variety of stresses. Hence, the use of transgenic plants to probe the role of the antioxidant system continues to be an important approach in understanding and countering oxidative stress. The uncharted area of signal transduction in relation to oxidative stress is also beginning to attract attention (Smirnoff, 1998).

1.2 Role of hormones in stress

Various environmental stresses cause changes in the content of hormones in plants (Lehmann and Vlasov, 1988). Plant hormones are a group of naturally occurring, organic substances, which influence physiological processes at low concentrations either in distant tissues to which they are transported or in the tissue where synthesis occurred (Davies, 1995a). There are five classical plant hormones, which are all relatively simple molecules, and they include auxins (primarily indole-3-acetic acid - IAA), gibberellins (GA), cytokinin, ethylene and abscisic acid (ABA) (Kende and Zeevart, 1997). Due to their structural simplicity, plant hormones, unlike animal hormones, are not specific enough to match the variety of controlled reactions and it has been argued that there are other controllers involved in development (Canny, 1985). Contrary to this, it has been suggested that, in fact, hormones only provide a "turn on" or "turn off" signal and that the actual information is provided by the cell (Firn, 1985). This scenario is similar to that of calcium, which is now thought to be an intermediate in some hormonal responses (Davies, 1995b).

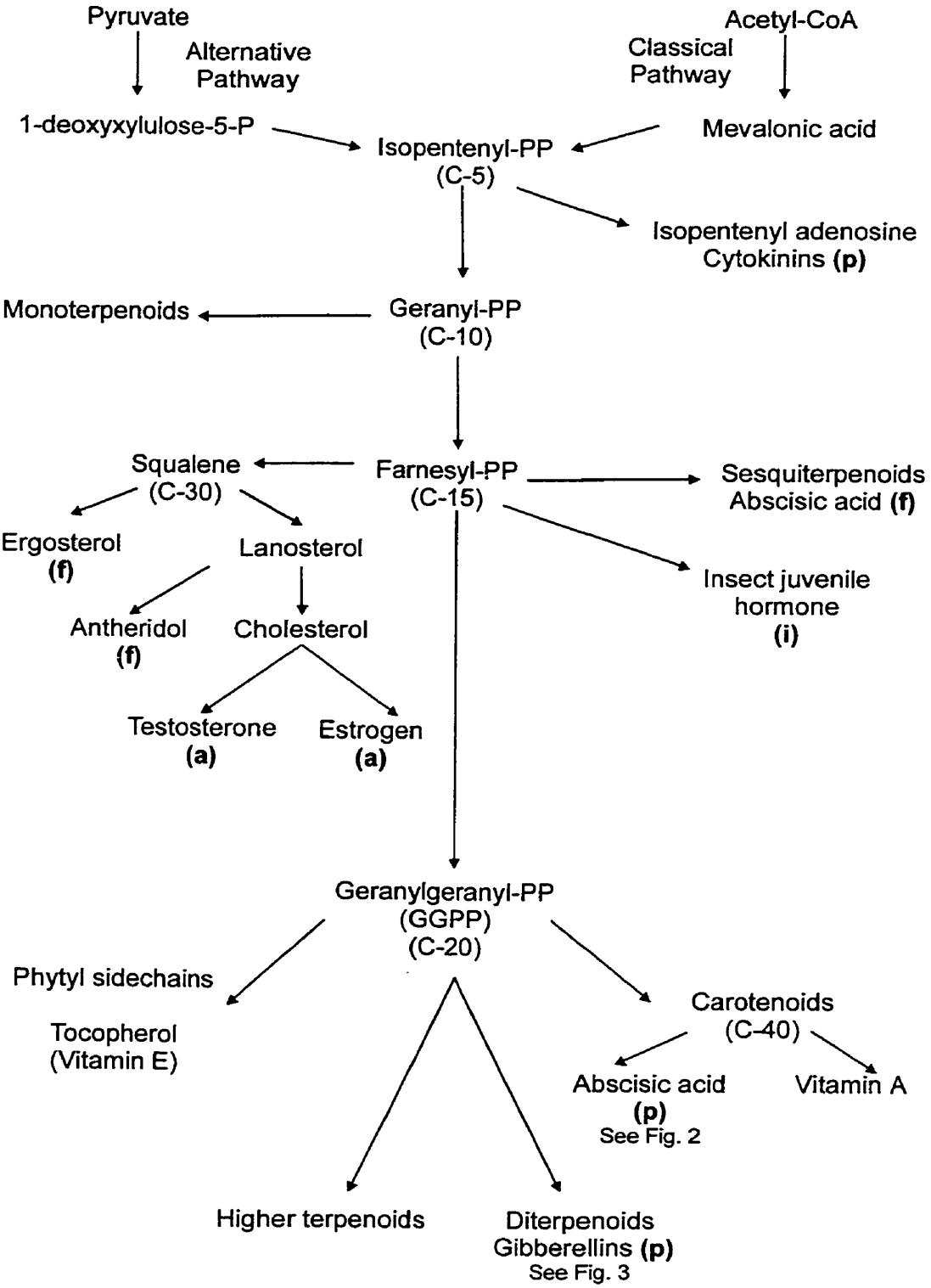
The two hormones for which there is consistent evidence for endogenous regulation in response to environmental stress are ABA and ethylene (Gianfagna

et al., 1992), however the other three are also implicated in stress response (Levitt, 1980). In 1979, a novel growth regulating steroidal substance called brassinolide was isolated from rape (*Brassica napus* L.) pollen, and since then a large number of related steroids have been isolated and identified from various plant sources including angiosperms, gymnosperms and alga (Moore, 1989). Brassinosteroids promote stem elongation, inhibit root growth and development, and promote ethylene biosynthesis and epinasty (Davies, 1995a). Furthermore, they have been implicated in stress tolerance (Clouse, 1996; Xu et al., 1996; and Khripach et al., 1999). A recent study by Dhaubhadel et al. (1999) provides evidence that brassinosteroids increase basic thermotolerance in *Brassica napus* and tomato seedlings. Brassinosteroids have now been added to the list of plant hormones (Khripach et al., 1999)

1.2.1 Abscisic acid

ABA was first described in 1965 when two independent investigations, one on the cause of leaf abscission (F. T. Addicott, University of California, Davis) and the other on bud dormancy in woody plants (P. F. Wareing, University College of Wales at Aberystwyth), converged (Moore, 1989). It has long been considered an inhibitor due to its role in abscission, dormancy and reducing shoot elongation, however evidence has accumulated to support a promoter role such as in inducing protein synthesis in seeds and to some degree in the defense against insect attacks (Davies, 1995a). ABA is transported in both the xylem and the phloem (Davies, 1995a).

Figure 1. The isoprenoid pathway, which generates animal (a), insect (i), plant (p) and fungal (f) products, is derived from five-carbon (C-5) units (Adapted from Fletcher et al., 2000). The classical acetate / mevalonate pathway for the production of isopentenyl pyrophosphate (PP) occurs in the cytosol. However, the alternative pathway occurs in the plastids of eubacteria, green algae and higher plants (Adapted from Lichtenthaler et al., 1997).


Pyruvate
Alternative Pathway
Acetyl-CoA
Classical Pathway
1-deoxyxylulose-5-P
Isopentenyl-PP (C-5)
Mevalonic acid
Isopentenyl adenosine Cytokinins (p)
Monoterpenoids
Geranyl-PP (C-10)
Squalene (C-30)
Farnesyl-PP (C-15)
Sesquiterpenoids Abscisic acid (f)
Ergosterol (f)
Lanosterol
Insect juvenile hormone (i)
Antheridol (f)
Cholesterol
Testosterone (a)
Estrogen (a)
Geranylgeranyl-PP (GGPP) (C-20)
Phytyl sidechains
Tocopherol (Vitamin E)
Carotenoids (C-40)
Abscisic acid (p)
See Fig. 2
Vitamin A
Higher terpenoids
Diterpenoids Gibberellins (p)
See Fig. 3

The integration of genetic, molecular and biochemical approaches has only recently elucidated the complex biosynthetic pathway for the simple ABA molecule (Kende and Zeevart, 1997). ABA is a sesquiterpene (C-15) i.e. it contains three isoprene (C-5) units and is a product of the isoprenoid pathway (Figure 1) as are other hormones, including GA (Moore, 1989). In higher plants, ABA is derived from the cleavage product of the C-40 isoprenoid, all-*trans*-violaxanthin (Walton and Li, 1995). A direct pathway from the C-15 isoprenoid, farnesyl pyrophosphate has been described in the fungal species *Cercospora*, however this pathway does not appear to function in higher plants (Walton and Li, 1995; and Cowan et al., 1999). Violaxanthin is cleaved to form one C-15 compound, 9'-*cis*-neoxanthin, which is then cleaved to form xanthoxin, which is then converted to ABA, and all these reactions are catalyzed by dioxygenases (Figure 2) (Kende and Zeevart, 1997; and Cowan et al., 1999). The site of xanthoxin synthesis is thought to be the chloroplasts of mature leaves and its final conversion to ABA occurs in the cytoplasm (Parry and Horgan, 1992). ABA is inactivated by conversion to phaseic acid (PA) by the cytochrome P450-mediated monooxygenase, ABA 8'-hydroxylase, located in the cytoplasm (Figure 2) (Walton and Li, 1995).

ABA levels rise and fall dramatically in several kinds of tissue in response to environmental and developmental changes (Walton and Li, 1995). Recently the focus of plant hormone research has shifted to the intracellular signaling processes that translate hormone perception into genetic, metabolic and developmental change (Jackson, 1997). ABA acts synergistically with Ca^{+2} in

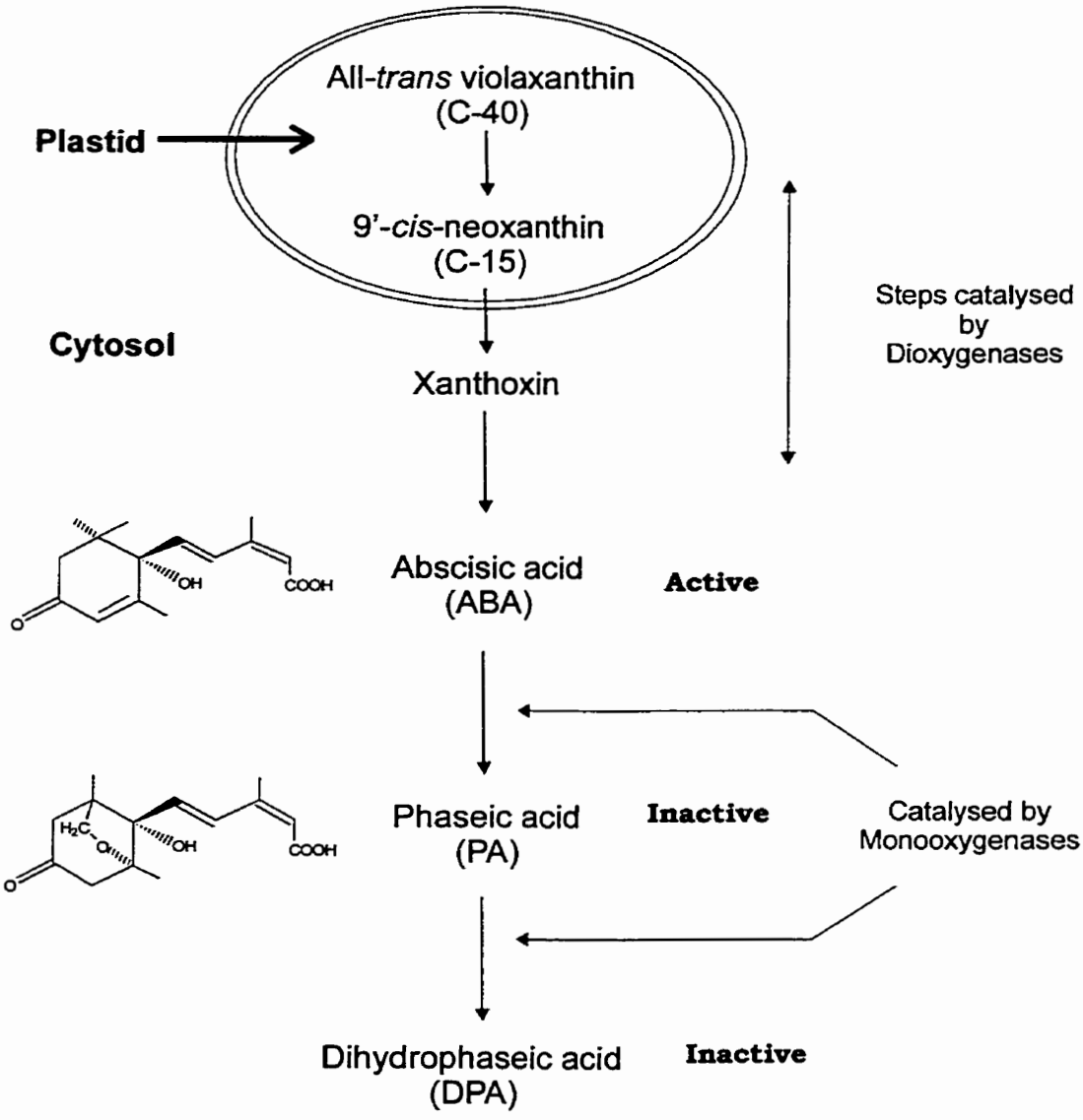


Figure 2. Putative biosynthetic pathway for abscisic acid (ABA), from C-40 all-*trans*- violaxanthin, showing the possible sub-cellular compartmentalization of the reactions. Adapted from Parry and Horgan (1992) and Walton and Li (1995)

inhibiting stomatal opening and is known to have a direct effect on membrane permeability (Owen, 1988). The increase in permeability allows more Ca^{+2} to enter the cell where it functions as a secondary messenger (Gong et al. 1997), hence indicating that ABA may have a wider physiological role in signal transduction (Owen, 1988). Furthermore, there is considerable evidence, which suggests that ABA acts as a 'stress hormone' involved in acclimation to a wide range of stresses (Jones and Jones, 1989). It is associated with tolerance to drought, chilling, freezing, salt and heat stress (LaRosa et al., 1985), all of which cause a water deficit in the plant (Levitt, 1980). However, the involvement of ABA in water stress goes beyond that of stomatal closure (Farquhar et al., 1989; and Slovik and Hartung, 1992). It is also associated with increased cellular levels of amino acids especially proline, an osmoticum, implicated in plant tolerance to cold, drought and salinity (LaRosa et al., 1987; and Churchill et al., 1998). In addition, ABA mediates changes in membrane stability and phospholipid composition, as well as in the modification of gene expression at the transcriptional level (Owen, 1988). Recent evidence suggests that ABA up regulates expression of the genes responsible for peroxidases in sweet potato (*Ipomoea batatas* L.) (Kim et al., 2000).

To utilize this effect on stress tolerance, ABA levels can be up regulated using either chemical analogues that are more persistent than natural ABA (Abrams et al., 1997), or inhibitors of the monooxygenases involved in ABA metabolism (Fletcher et al., 2000). Chemicals used to regulate the levels of cytokinin and GA also affect the levels of ABA, since ABA is antagonistic to both

of these hormones (Moore, 1989; and Cowan et al., 1999). The antagonistic characteristic of ABA to GA is well known in the regulation of α-amylase synthesis and secretion in barley (*Hordeum vulgare* L.) aleurone layers and it has recently been suggested that the balance between GA and ABA is important in stress tolerance (Vettakkorumakankav et al., 1999).

2.0 Gibberellins

Gibberellins (GAs) are a class of plant hormones that are involved through out the life cycle of plants, influencing germination, development, maturation, and environmental response (Hedden and Kamiya, 1997). Research on GAs originally began with the work of E. Kurosawa on the cause of "bakanae" disease in rice (*Oryza sativa* L.) (Tamura, 1991). The discovery that GAs are a natural product of higher plants was made independently in 1956 by C. A. West and B. O. Phinney, and Margaret Radley (Moore, 1989); since then over 121 GAs have been isolated from plants and fungi (Hedden, 1999). Numerous reviews on the various aspects of GAs have been published, including a comprehensive review by Hedden and Kamiya (1997) on the enzymes and genes involved in GA-biosynthesis and their regulation, which has since been updated by Hedden (1999) and Yamaguchi and Kamiya (2000).

2.1 Physiological roles of GA

The range of processes regulated by GAs covers all aspects of the life history of plants, from seed germination to vegetative growth and flowering (Ritchie and Gilroy, 1998). The effect of GA on the germination of barley has

been known since the 1960s, when it was demonstrated that application of GA_3 replaced the embryo signal required by barley endosperm to produce the hydrolytic enzyme α-amylase (Chandler, 1992). It is now known that the production of α-amylase in other cereals is also under hormonal regulation, with GA stimulating the synthesis and secretion of α-amylase and ABA reversing this effect (Jacobsen et al., 1995).

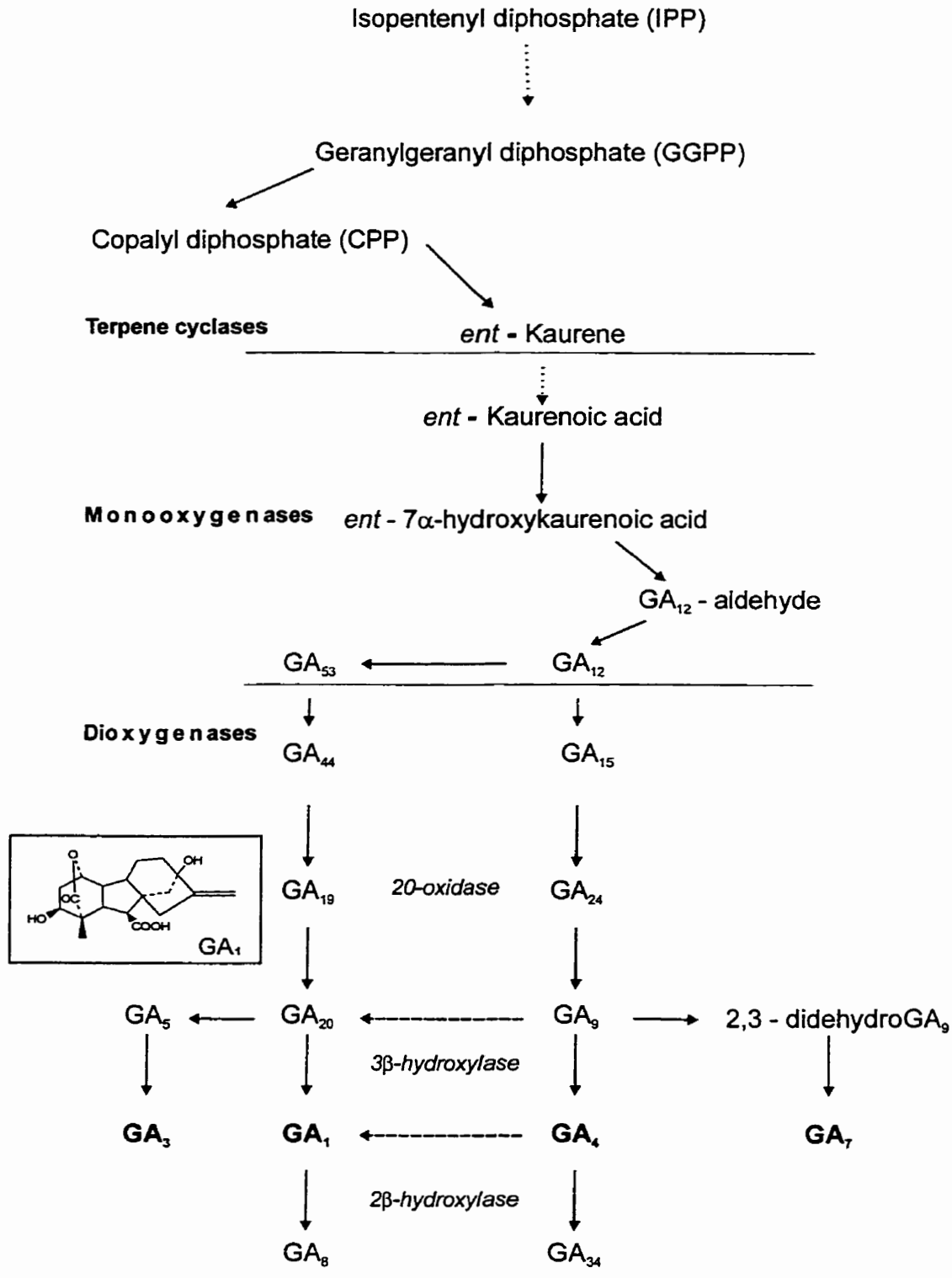
Evidence from barley caryopses suggests that the induction of α-amylase biosynthesis is caused by the physiologically active GAs, primarily GA_1 and GA_3, either derived from stored GA-precursors or from the conversion of a physiologically inactive GA (Grosselindemann et al., 1991). This is supported by the demonstration that inhibition of *de novo* synthesis of GA in the embryos, which occurs at least 24 h after the onset of imbibition, does not affect α-amylase production (Jacobsen et al., 1995). GA diffuses from the embryo, particularly the scutellum (Appleford and Lenton, 1997), to the aleurone cells where it interacts with yet to be identified external perception sites on the cell membrane (Ritchie and Gilroy, 1998). Once the receptors are activated, they trigger the GA signal transduction network that includes components such as calmodulin, GTP-binding proteins and protein kinases (Bethke and Jones, 1998). At this point, various genes are either suppressed or promoted, as well, there is a proliferation of ER and golgi membranes to assist in transportation of synthesized GA (Jacobsen et al., 1995; and Ritchie and Gilroy, 1998).

It has been suggested that the *de novo* GA-synthesis that occurs in the embryo after imbibition is directed towards shoot elongation (Grosselindeman et

al., 1991). The effect of GA on shoot elongation is clearly demonstrated by exogenous applications of GA_3 to plants that are responsive to GA (Chandler, 1992). The mechanism by which GA causes shoot elongation is by stimulating both, cell division and enlargement, the former being more common in younger cells, and the latter in older cells (Moore, 1989). In pea (*Pisum sativum* L.) shoots, the level of synthesizing activity of the first committed precursor of GA biosynthesis is directly correlated with elongation and this confirms results for other species (Moore and Coolbaugh, 1991). GA is now known to increase the level of activity of the enzyme, xyloglucan endo transglycosylase (XET), which is believed to be important in cell expansion and organ growth (Maheshwari, 1999). Biosynthesis of GA after germination occurs in various tissues, including the tips of shoots, as well as in the cytoplasm and chloroplasts of leaves (Moore, 1989; and Lichtenthaler et al., 1997).

GAs are also involved in the initiation of flowering, inducing male sex expression in flowers and parthenocarpy in some plant species (Takahashi et al., 1986). Application of GAs to long-day (LD) requiring plants and those LD plants that require vernalization induces flowering in the absence of either of the requirements (Takahashi et al., 1986). Induction of flowering by GA is not only due to the initiation of stem bolting that often follows floral initiation, but is also in the induction of the processes *per se* (Pharis, 1991). It is interesting that different GAs have differing effects on the various processes that they are implicated in, an example being that the GA involved in shoot elongation (GA_1) is not as effective as GA_4 and GA_7 at initiating flower development in certain

Figure 3. Gibberellin (GA) biosynthesis pathway from isopentenyl diphosphate to the inactive GAs, GA_8 and GA_{34}. The growth-active GAs are GA_1, GA_3, GA_4 and GA_7. The non-13-hydroxylation pathway consists of GA_{12} to GA_4, whereas GA_{53} to GA_1 constitute the early 13-hydroxylation pathway. The enzymes involved in each of the sections demarcated by horizontal lines are indicated on the left of the figure. Multiple steps in a reaction are indicated by a dotted line (———), whereas a dashed line indicates a proposed step (– – – –). Adapted from Hedden and Kamiya (1997) and Hedden (1999).


Isopentenyl diphosphate (IPP)
Geranylgeranyl diphosphate (GGPP)
Copalyl diphosphate (CPP)
Terpene cyclases
ent - Kaurene
ent - Kaurenoic acid
Monooxygenases
ent - 7α-hydroxykaurenoic acid
GA12 - aldehyde
GA53
GA12
Dioxygenases
GA44
GA15
GA19
20-oxidase
GA24
GA1
GA5
GA20
GA9
2,3 - didehydroGA9
3β-hydroxylase
GA3
GA1
GA4
GA7
2β-hydroxylase
GA8
GA34

species (Pharis, 1991).

2.2 GA biosynthesis and metabolism

GAs are tetracyclic diterpene carboxylic acids that possess an *ent*-gibberellane skeleton and are derived from the isoprenoid pathway (Figure 1) (Sponsel, 1995). There are a number of reviews on the biosynthesis (Hedden, 1999; and Hedden and Kamiya, 1997), regulation (Yamaguchi and Kamiya, 2000), signaling (Bethke and Jones, 1998) and molecular biology (Lange, 1998) of GA. The synthesis of GA can be divided into three stages depending on the enzymes that catalyze the reactions: the enzymes are terpene cyclases, cytochrome P450-dependent monooxygenases and 2-oxoglutarate-dependent dioxygenases (Yamaguchi and Kamiya, 2000). This division also reflects their subcellular compartmentalization (Lange, 1998).

Terpene cyclases are involved in the cyclization of the tetraterpene geranylgeranyl diphosphate (GGPP) to the first committed gibberellin precursor *ent*-kaurene (Figure 3) (Sponsel, 1995). GGPP is a direct product of isopentenyl diphosphate (IPP), which was initially considered a product of mevalonate, however it is now believed to be derived from glyceraldehyde-3-phosphate (GAP) and pyruvate via the 1-deoxyxylulose pathway (Figure 1) (Lichtenthaler et al., 1997). The conversion of GGPP to *ent*-kaurene is a two-step process: the conversion of GGPP to copalyl diphosphate (CPP), which is catalyzed by CPP synthase (formally *ent*-kaurene synthetase A), and the production of *ent*-kaurene from CPP, catalyzed by *ent*-kaurene synthase B (kaurene synthetase B) (Sponsel, 1995). These reactions occur in the proplastid of meristematic tissue

but not in mature chloroplasts of pea and wheat (*Triticum aestivum* L.) (Lange, 1998). The genes encoding CPP synthase have been cloned from *Arabidopsis thaliana*, *Pisum sativum* (pea), *Zea mays* (maize), and *Phacosphaeria sp.* and those for *ent*-kaurene synthase B have been cloned from *Cucurbita maxima* (cucumber) and *Phacosphaeria sp.* (Hedden and Kamiya, 1997; Lange, 1998; and Hedden and Proebsting, 1999).

The next stage in GA biosynthesis is the conversion of *ent*-kaurene to the first GA, GA_{12}-aldehyde (Figure 3). This is a multi-step process involving the oxidation of the methyl group on C-19 of *ent*-kaurene to form *ent*-kaurenol, *ent*-kaurenal, *ent*-kaurenoic acid, *ent*-7α-hydroxy-kaurenoic acid and finally GA_{12}-aldehyde (Sponsel, 1995). GA_{12}-aldehyde is first oxidized at C-7 to give the dicarboxylic acid GA_{12}, which is then oxidized at the C-20 methyl group giving the GA_{15} and GA_{53} open lactone forms (Sponsel, 1995; Hedden and Kamiya, 1997). The reactions up to the formation of GA_{15} and GA_{53} are catalyzed by microsomal monooxygenases located on the membranes of the ER (Figure 3) (Lange, 1998; and Hedden, 1999). The isolation of genes encoding monooxygenases has been hampered by the difficulty in functionally expressing their cDNAs in heterologous systems (Hedden, 1999).

The pathway branches after the formation of GA_{15} and GA_{53}, and which GAs are more predominant in a particular tissue is dependent on the genus and species. GA_{15} is oxidized to the C-20 aldehyde GA_{24} then to the physiologically inactive GA_9, which constitutes the non-13-hydroxylation pathway, giving rise to the growth active GA_4 (Hedden and Kamiya, 1997). GA_{53} is converted to GA_{44},

then on to the inactive GA_{20}, the precursor of the growth active GA_1 and GA_3; this pathway is known as the early 13-hydroxylation pathway (Sponsel, 1995). These reactions occur in the cytosol of plant cells actively producing GA and both pathways have been observed in cell-free systems from embryos / scutella of 2-day old germinating barley grain (Hedden and Kamiya, 1997; and Lange, 1998). The reactions up to the synthesis of growth active GAs are catalyzed by a single enzyme known as GA 20-oxidase, a multi-function 2-oxoglutarate dependent dioxygenase whose gene has been cloned in numerous plant species (Kamiya and Garcia-Martinez, 1999).

The synthesis of the growth active GA_1, GA_3, GA_4 and GA_7 from their respective precursors is catalyzed by soluble oxidases that also use 2-oxoglutarate as a co-substrate and are known as the 3β-hydroxylases (Figure 3) (Hedden and Kamiya, 1997). These growth-active GAs are deactivated by hydroxylation of C-2β, catalyzed by another set of dioxygenases, 2β-hydroxylases (Hedden, 1999). This is crucial for turnover of the physiologically active GAs. The genes encoding these dioxygenases have been cloned in cucumber (Lange, 1998).

2.3 GA regulation

Of the 121 GAs identified, only a few are thought to have intrinsic biological activity and these are as mentioned above, $GA_{1, 3, 4}$ and $_7$ (Hedden, 1999). GA_1 and GA_4 are thought to be involved in shoot elongation (Hedden, 1999), whereas flowering in woody angiosperms is promoted by GA_4 and not GA_1 (Pharis, 1991). Hence there is tight regulation of GA biosynthesis at the gene

level (Yamaguchi and Kamiya, 2000). Developmental control of GA levels occurs at the early steps of biosynthesis, the conversion of GGPP to *ent*-kaurene (Hedden, 1999). This has been extensively studied in *Arabidopsis*, wheat and pea, where the activity of the two enzymes involved are confined to the meristematic regions in nodes and shoot apices (Hedden, 1999; and Yamaguchi and Kamiya 2000). Regulation also occurs through feedback, whereby the action of the physiologically active GAs results in the production of a transcriptional repressor that limits the expression of GA biosynthetic enzymes (Hedden and Kamiya, 1997). Environmental signals, such as light, also exert fine control over the later steps in GA-biosynthesis (Hedden, 1999).

2.3.1 Light

Gibberellins (GA) can also control many of the light-regulated developmental processes mediated by phytochromes (Phy), such as seed germination and stem elongation, and can in fact mimic light in some cases (Kraepiel and Miginiac, 1997). Phy are regulatory photoreceptors in plants that measure both the quantity and quality of available light (Jordan et al., 1995). Phy occur in two interchangeable forms, the active form Pfr that absorbs far-red light and the biologically inactive red-light absorbing form, Pr (Kraepiel and Miginiac, 1997). Absorption of red light causes the conversion of Pr to Pfr, which then regulates both germination and shoot elongation (Jordan et al., 1995).

Evidence suggests that red light causes an increase in the endogenous content of GA_1 during germination, however it reduces the levels of GA_1 during de-etiolation (Kamiya and Garcia-Martinez, 1999). Phy controls the expression

of 3β-hydroxylase in germinating lettuce seeds, whereas its effect on dark-grown seedlings is primarily on 2β-hydroxylation (Yamaguchi and Kamiya, 2000). Jordan et al. (1995) have reported that overexpression of PhyA in transgenic tobacco (*Nicotiana tabacum* L.) results in dwarf plants that have reduced levels of GA.

2.3.2 Chemicals

One of the strategies for keeping abreast with the rising population and demand for food is to use chemicals that alter the biochemistry, physiology and morphology of crops (Lever, 1982). Chemicals responsible for such changes are referred to as plant growth regulators (PGRs) and they exert their effects by interfering with the various plant hormones (Graebe, 1987; and reviewed by Davis and Curry, 1991). PGRs have been used to increase yield by reducing lodging and increasing shoot to root ratios, improving quality, increasing value and decreasing costs of production in agriculture and horticulture (Lever, 1982; and Davis and Curry, 1991). PGRs have also played a crucial role in physiological research involving hormones and hormone action (Grossmann, 1990). There are three main classes of GA inhibitors based on their site of action (reviewed by Rademacher, 2000).

The first group of compounds, the onium-type, inhibit both CPP and *ent*-kaurene synthase, however the latter is inhibited to a lesser degree (Figure 4) (Davis and Curry, 1991). These chemicals possess either a positively charged ammonium, phosphonium or sulphonium group and include chlormequat chloride (CCC, Cyclocel), mepiquat chloride, AMO-1618 and Phosphon D (Grossmann,

1992). The next group of chemicals with a nitrogen-containing heterocycle, interfere with the steps catalyzed by monooxygenases (Figure 4) (Rademacher, 2000). The N-heterocycle can be in the form of either pyrimidines (e.g. in amcymidol), 4-pyridine (in inabenfide), 1, 2, 4-triazoles (in paclobutrazol, uniconazole) or norbornanodiazetines (in tetcyclasis) (Grossmann, 1992). Members of the last group contain the acylcyclohexanedione structure (e.g. trinexapac-ethyl) and they inhibit steps catalyzed by dioxygenases (Figure 4) (Rademacher et al, 1992). A recent development in chemical regulation of GA biosynthesis is the development of GA_5 derivatives known as 16, 17-dihydro-GAs, which are also dioxygenase-inhibitors most likely due to competition with GA precursors for the enzymatic site (Figure 4) (Rademacher, 1997). These compounds are found naturally in higher plants and GA–producing fungi, but they are most active in graminaceous plants (Rademacher, 2000). A recent study showed that these compounds are as effective as trinexapac-ethyl in reducing growth in cool-season turfgrasses (King et al., 1997).

3.0 Triazoles

The triazoles are a highly active group of growth-regulating compounds whose activity was discovered during fungicide screening programs in the 1970s (Davis and Curry, 1991). The triazole fungicides currently make up the largest and most important group of systemic compounds developed for the control of fungal diseases in plants and animals, however, certain azole compounds also interfere with GA biosynthesis (Fletcher et al., 2000). The fungicidal versus the plant-growth regulating properties of a triazole is dependent on the stereochemistry

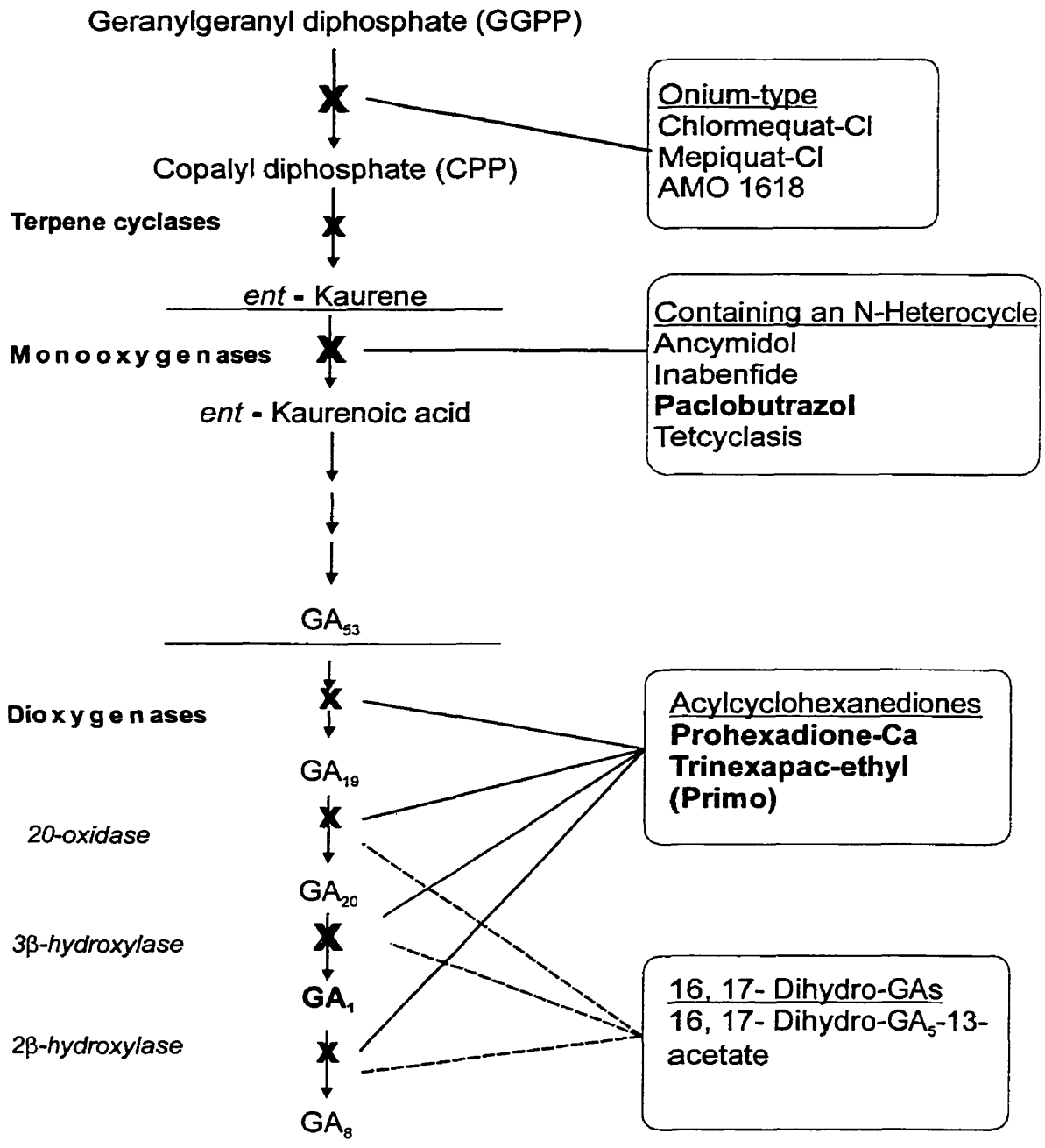
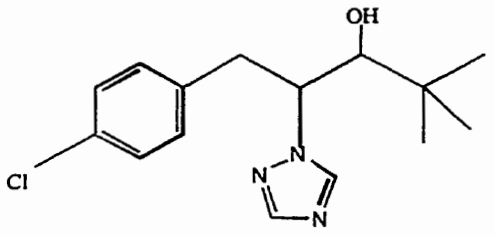


Figure 4. Simplified scheme showing the points of inhibition by plant growth regulators at the various stages of gibberellin (GA) biosynthesis. X, x indicating major and minor activity, respectively. Adapted from Rademacher (2000).

of the chiral carbon bearing the hydroxy group, with the *R* configuration favoring fungitoxic characteristics and the *S* configuration conferring plant growth regulating properties (Fletcher et al., 2000). The most potent of the plant growth regulating (GA-inhibiting) compounds in this group is uniconazole, followed by paclobutrazol (Figure 5) and these have been extensively studied for their effects in various plant species since around 1980 (Davis and Curry, 1991). Yokota et al. (1991) report that in pea cv. Holland there was a significant reduction in the levels of sterols and brassinosteroids in leaves treated with *R*-uniconazole than there was with *S*-uniconazole, which caused a greater reduction in height and GA levels. Studies on the efficacy of the two enantiomers of paclobutrazol on growth retardation have also been conducted, with similar results (Lenton et al., 1994).

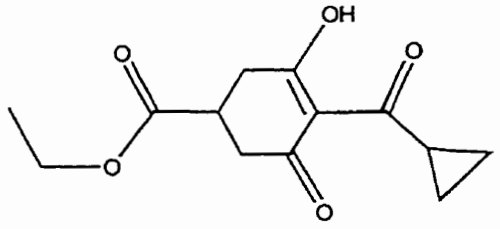
3.1 Translocation, methods of application and metabolism

Triazoles are primarily translocated acropetally in the xylem, however there are exceptions, such as in *Pistachia chinensis* Bunge and *Ricinus communis* L. (castor oil plant), where paclobutrazol has been detected in both the xylem and the phloem (Fletcher et al., 2000). Studies using the seedlings of *Malus x Domestica* (apple) show that there is no movement of paclobutrazol from the site of application on mature leaves and that foliar applied paclobutrazol is not transported to stems or roots (Davis et al., 1988). However, when added to the growth medium and taken up by the roots, paclobutrazol is more active (Wang et al., 1986).



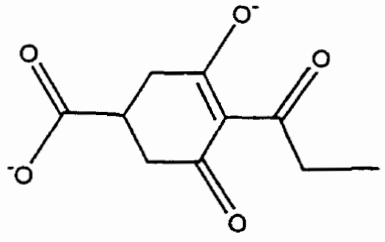
Paclobutrazol

(2RS, 3RS)-1-4(-4-Chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pent-1-en-3-ol



Trinexapac-ethyl

4-(cyclopropyl-α-hydroxy-methlyene)-3,5-dioxo-cyclohexanecarboxylic acid ethyl ester



Prohexadione-Ca

Calcium 3-oxido-4-propionyl-5-oxo-3-cyclohexenecarboxylate

Figure 5. Structures and chemical names of the triazole, paclobutrazol, and the acylcyclohexanediones, trinexapac-ethyl and Prohexadione-Ca.

The most common methods of application of growth retardants is foliar spray, however due to the poor uptake, root drenches are the preferred method for triazole application (Rademacher, 1997). The exception is in mango, which develops stunted new growth due to the accumulation of uniconazole in the trunk. Hence, in these trees application by foliar spray ensures that the activity is confined to where it is required, the developing leaves (Fletcher et al., 2000). Of the applied triazole, only a small amount actually reaches the target tissue, requiring high concentrations of the chemicals to be applied at one time (Davis et al., 1988).

Triazoles are generally metabolized slowly, though there is considerable variability in degradation by different plant parts (Davis and Curry, 1991). Furthermore, paclobutrazol can remain active in soil for several years, especially since they are immobile in soil and are, therefore, less accessible to microbes for degradation (Jackson et al., 1996). High persistence within the plant and in soil has hampered the wide spread use of these compounds (Rademacher, 1997). However, they have been extensively studied in both controlled growth environments and in the field, on various plant species including edible crops, trees and grasses (Fletcher et al., 2000).

Soil applications, therefore, pose a problem due to longevity of these compounds in soil. Hence, Fletcher and Hofstra (1990) developed a seed treatment technology that essentially programs the seeds to produce seedlings that exhibit to a high degree the characteristics of triazole-treatment. The treatment involves soaking seeds in treatment solutions containing the triazole

for a specified number of hours, depending on the tolerance of the seeds to imbibition, followed by a period of air-drying at room temperature (Fletcher et al, 2000). The addition of KCl to the treatment solution and a heat shock (acclimation) in the last hour of imbibition further increases the triazole-induced effects (Fletcher and Hofstra, 1990). This method of treatment has numerous benefits over the conventional procedures since it is simple, cost effective, reduces the concentration of the chemical used, has little or no persistence in the seed, and there is minimal spread to the environment (Fletcher et al., 2000).

3.2 Mode of action

The triazoles, like the other N-heterocycle containing compounds, interfere with the cytochrome P450 dependent monooxygenases (Figures 3 and 4) by interacting with the protoheme iron of cytochrome P450, preventing the oxygen required for catalytic reaction, from binding (Grossmann, 1992). Monooxygenases are involved in the synthesis of GA and sterols, as well as in the inactivation of ABA (Rademacher, 1997). The fungitoxic triazoles inhibit the 14α-methylsterol 14α-demethylase enzyme system involved in sterol biosynthesis in fungi, resulting in the accumulation of 14α-methylsterols and the depletion of the usual sterols, which can then lead to destabilization of membrane function and ultimately cell death (Yates et al, 1993). In plants however, obtusifoliol 14α-demethylase is inhibited and Yates et al. (1993) report that in celery (*Apium graveolens* L.) cell suspension cultures treated with four triazoles including paclobutrazol, there is an accumulation of 14α-methylsterols, a decline in 4-desmethylsterol and a decrease in the stigmasterol to sitosterol

ratio. Similar results have been obtained in barley treated with the fungicidal triazoles, triadimefon and triadimenol (Buchenauer and Röhner, 1981)

Triazoles inhibit the conversion of *ent*-kaurene to *ent*-kaurenoic acid in GA biosynthesis resulting in a reduction in the levels of all GAs (Figure 4) (Fletcher et al., 2000). Sheath segments of 8-day old barley cv. Herta seedlings treated with 10μmolar paclobutrazol applied as a seed drench have 6.6 and 25.6 % of the physiologically active GA_1 and GA_3, respectively, when compared to the control (Crocker et al., 1990). Furthermore, the levels of the inactive GA_{19}, GA_{20} and GA_8 are also reduced, elucidating the site of action of paclobutrazol. This reduction in GA levels is closely associated with the reduction in leaf sheath length. A similar trend is observed in pea seedlings, but the relationship is not as close due to the use of racemic mixtures of uniconazole (Yokota et al., 1991).

Apart from their affect on GA, triazoles change the levels of ABA, ethylene and cytokinin (Fletcher et al., 2000). Treatment of rice with uniconazole does not affect the levels of auxins (Izumi and Oshio, 1991), however in pea internodes, there is a reduction in IAA (Davis and Curry, 1991). Triadimefon increases the levels of cytokinins in the cotyledons and roots of cucumber seedlings by 42% and 84% when compared to the controls (Fletcher and Arnold, 1986). This confirms a previous study using *Amaranthus caudatus* seedlings (Buchenauer and Röhner, 1981). More recent evidence from various plant species using different triazoles has accumulated, further confirming that triazoles increase the levels of cytokinins (Izumi and Oshio, 1991; and Grossmann, 1992). This increase is thought to be a result of the effects of triazoles on the isoprenoid

pathway from which cytokinins are derived, but there is also a suggestion that the increase in root mass caused by triazole treatment is responsible for this increase (Fletcher et al., 2000). Cytokinins are synthesized in the roots and translocated to the shoot where they regulate mitosis and cell division, among other processes (Moore, 1989).

Treatment with triazoles is reported to decrease the levels of ethylene (Fletcher et al., 2000), the hormone considered to be responsible for, among other processes, ripening, abscission and epinasty (Moore, 1989). The enzyme involved in the conversion of the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) to ethylene is a monooxygenase known as ACC oxidase, and this enzyme is inhibited by triazoles (Kraus et al., 1992). The increase in the levels of ABA is a result of the inhibition of its conversion to the physiologically inactive phaseic acid (Rademacher, 1997). This is true in most plant systems tested (Hauser et al., 1990; and Mackay et al., 1990), however a reduction in ABA is observed in the fungi *Cercospora rosicola* with paclobutrazol inhibiting the synthesis of ABA at a step after farnesyl diphosphate (Norman et al., 1986).

3.3 Morphological effects

The most obvious effect on plant morphology, after treatment with triazoles, is the reduction in height and the shorter, broader, thicker, darker green foliage (Fletcher et al., 2000). However, the level of expression of these effects is dependent on the relative efficacy of the different triazoles and the plant species being used (Gilley and Fletcher, 1997). Reduced height is primarily caused by the reduction in the physiologically active GA_1, which has been

implicated in shoot elongation (Hedden, 1991), however in *Chrysanthemum* cv. Lillian Hoek, paclobutrazol also reduces shoot width by 50% (Burrows et al., 1992). The chlorenchyma was poorly developed or absent and the cortical and pith cells are shorter by 50 to 60%. Similarly, reduced internodal elongation, cell length and number further exemplify the reduction in height of treated safflower (*Carthamus tinctorius* L.) (Potter et al., 1993).

The changes observed in leaf thickness and size, are a combination of an increased thickness of palisade and spongy mesophyll, as well as a decrease in cell diameter and length (Gao et al., 1988; and Burrows et al., 1992). It has been suggested that the reason for increased leaf width in maize seedlings treated with paclobutrazol is increased interveinal distance (Sopher et al., 1999). In wheat treated with uniconazole, the increase in width is attributed to higher numbers of vascular bundles (Gao et al., 1988). The darker green color was previously thought to be a result of packing chloroplasts into smaller leaves (Fletcher et al., 2000). However, studies on chloroplast structure have revealed that the chloroplasts of triazole-treated wheat plants are larger, along both the long and short axes (Gao et al., 1988). Sopher et al. (1999) report that maize treated with paclobutrazol exhibit chloroplasts that have increased abundance of stromal lamellae and reduced numbers of larger grana stacks. These effects are thought to be associated with the increase in cytokinin levels since it is normally involved in chloroplast development (Fletcher and Arnold, 1986; and Fletcher et al., 2000).

An increase in epicuticular wax on the surfaces of treated leaves has also been observed and is thought to be the result of interference with lipid metabolism and the accumulation of free fatty acids and sterols (Gao et al., 1988). Triazole-treatment results in thicker roots that have few, if any lateral extensions and a higher root to shoot ratio (Fletcher and Hofstra, 1988). The increase in root diameter in *Chrysanthemum* and *Citrus* is due to an increase in rows of cells and cell diameter (Burrows et al., 1992). Plants treated with triazoles also exhibit a retardation of senescence, probably due to the reduction in ethylene levels (Grossmann, 1992). Furthermore, in cucumber the number of female flowers produced is increased by treatment with uniconazole (Izumi and Oshio, 1991) and there is an inhibition of floral bud initiation in *Rhodedendron simii* after treatment with paclobutrazol (Bodson and Thomas, 1995). These are probably a result of decreased GA levels. However, the extent to which all the above effects occur is dependent, not only on the type of triazole used, the concentration, application method, and age of the plant material, but also on the species of the plant (Burrows et al., 1992).

3.4 Tolerance to environmental stresses

Triazoles have been found to be highly effective in protecting plants from various environmental stresses (Fletcher et al., 2000). Paclobutrazol-treated wheat seedlings tolerate stress due to water logging and this is associated with the extensive rooting system that has the potential to access air and nutrients more effectively (Webb and Fletcher, 1996). Triazole-treated plants are also more tolerant to drought stress (Fletcher et al., 2000). It is thought that the

higher level of ABA causes stomatal closure, which increases diffusive resistance and water conservation (Asare-Boamah et al., 1986). The reduced leaf area of triazole-treated plants is not the main factor in increased drought tolerance since even the triazoles that do not decrease leaf area, increase tolerance (Gilley and Fletcher, 1997). The increased level of ABA is also implicated in increased cold hardiness by increasing the period of dormancy in *Actinidia arguta* (Tafazoli and Beyl, 1993).

Tolerance to heat stress has been partially attributed to a change in membrane structure. Paliyath and Fletcher (1995a) report that paclobutrazol treatment of corn coleoptiles stabilizes the cell membrane, which then repairs itself more effectively after heat stress. Furthermore, increases in the levels of both antioxidants (e.g. tocopherol and ascorbic acid) and antioxidant enzymes (e.g. SOD and peroxidases) in treated plants increase their ability to scavenge the free radicals produced during both heat and chilling stress (Upadhyaya et al., 1989; Sankhla et al., 1992; Kraus et al., 1995b). Booker et al. (1991) also suggest that thermotolerance in wheat seedlings treated with uniconazole is mediated by transpirational cooling.

There have been reports that the triazoles confer tolerance to the air pollutants SO_2 and ozone, as well as to UV-B radiation and salt stress (Fletcher et al., 2000). It has been suggested that stomatal closure could decrease the amounts of SO_2 entering the cells, however the exact mechanism is not yet known (Lee et al., 1985). Tolerance to ozone, however, has been related to the increased antioxidant potential of treated plants (Fletcher et al., 2000). Treated

plants are more tolerant to UV-B radiation probably due to the thicker layer of epicuticular wax on their leaves (Kraus et al., 1995a). Salt stress amelioration in peanut (*Arachis hypogaea* L.) seedlings treated with triadimefon is mediated by increased protein synthesis, osmoregulation and reduced energy requirements (Muthukumarasamy and Panneerselvam, 1997).

3.5 GA antagonism

Application of exogenous GA_3 to plants treated with triazoles causes a reversion of both the morphological and stress tolerant effects (Santakumari and Fletcher, 1987; Fletcher and Hofstra, 1988; and Gilley and Fletcher, 1998). This negation of stress protective effects has led to the suggestion that gibberellins are crucial in stress protection of plants (Vettakkorumakankav et al., 1999).

4.0 Acylcyclohexanediones

Prohexadione-Ca, trinexapac-ethyl (the active ingredient in primo and cimectacarb) and the experimental compound LAB 198 999 make up this group (Figure 5) (reviewed by Rademacher, 2000). These compounds were first developed in the early 1990s (Grossmann, 1992). Recently it was reported that their mode of action is similar to that of the plant growth retardant daminozide (trade names: Alar, Kylar, or B-Nine), which has been removed from the market due to toxicological concerns (Brown et al., 1997). All higher plants are responsive to the acylcyclohexanediones, however to varying degrees (Rademacher, 2000). They are currently being used in grasses such as the 'Diamond' zoysiagrass (*Zoysia matrella* (L.) Merr.) (Qian et al., 1998), fruit trees

[e.g. apples (Rademacher, 1999)], peanuts (Evans et al., 1996), and rice (Im et al., 1993).

These compounds are 2-oxoglutarate mimics; hence they interfere with the last steps in GA biosynthesis that involve the 2-oxoglutarate-dependent dioxygenases (Figures 3 and 4) (Rademacher, 2000). Using acylcyclohexanedione derivatives, Griggs et al. (1991) showed that these compounds inhibit the GA 2β-hydroxylases (Figure 3) by interacting with the 2-oxoglutarate binding site. However, the affinity of these compounds to the 3β-hydroxylases is higher, resulting in the growth retardation normally seen (Rademacher et al., 1992).

4.2 Translocation, methods of application and metabolism

The preferred method of application is via the leaf (Rademacher, 1993). Translocation occurs in the phloem, primarily acropetally and root absorption is negligible, however absorption of trinexapac-ethyl is higher from the plant base (collection of leaf sheaths surrounding the compressed stem and crown) of Kentucky bluegrass (*Poa pratensis* L.) than it is from the leaf blade (Fagerness and Penner, 1998). These compounds are rapidly degraded and prohexadione-Ca has a half-life of only a few hours (Rademacher, 1997).

4.3 Mode of action

The primary mode of action, as mentioned above, is the inhibition of GA biosynthesis, specifically GA_1, though the levels of its inactive metabolite GA_8 are also reduced, albeit to a lesser extent (Rademacher, 2000). Under practical

conditions, trinexapac-ethyl and prohexadione-Ca display similar degrees of activity when applied in appropriate formulation to graminaceous species; however in dicots, prohexadione-Ca usually has a greater effect than trinexapac-ethyl (Rademacher, 2000). Spray applications of LAB 236 734, an acylcyclohexanedione, resulted in the increase of GA_{20} and GA_{19} in wheat and a reduction in GA_1, GA_8 and GA_{44} (Figure 3) (Rademacher et al., 1992). These compounds affect the levels of the other phytohormones in a similar fashion to triazoles (Rademacher, 2000). Prohexadione-Ca, trinexapac-ethyl and LAB 198 999 reduce ethylene levels in sunflower (*Helianthus annuus* L.) cell suspensions and in leaf disks of wheat (Grossmann, 1992). These compounds also increase the levels of ABA and cytokinin, while there is no change in IAA levels (Rademacher, 2000). Prohexadione-Ca, and the related compounds, interfere with flavonoid metabolism and inhibit the formation of anthocyanin (Rademacher, 1999).

4.4 Morphological effects and stress tolerance

Acylcyclohexanediones rapidly reduce shoot elongation by decreasing the levels of GA_1 (Adams et al., 1992). Trinexapac-ethyl reduces excessive vertical growth and increases rhizome mass in 'Diamond' zoysiagrass (Qian et al., 1998). Similar results were seen in *Eucalyptus globulus* L. injected with trinexapac-ethyl, where fiber length and the number of cells in the cambial zone were reduced (Ridoutt et al., 1996). However, this reduction Is associated with a decrease in the levels of both GA_1 and GA_{20}, as well, at the highest concentration of trinexapac-ethyl applied, the levels of ABA and IAA are reduced.

Trinexapac-ethyl prevents the acceleration of primordium initiation in *Lolium temulentum* L. without inhibiting floral development (Evans and Blundell, 1996). Though the acylcyclohexanediones do not have fungitoxic effects, they appear to enhance the efficacy of other fungicides (Burpee et al., 1996). Golembiewski and Danneberger (1998) report that trinexapac-ethyl, in combination with N fertilizers, controls dollar spot (*Sclerotinia homoeocarpa* L.) in creeping bentgrass (*Agrostis stolonifera* L.).

These compounds induce tolerance to water stress in tall fescue (*Festuca arundinacea* cv. Kentucky 31) primarily by reducing water uptake and increasing water conservation (Marcum et al., 1997). However, in this study, trinexapac-ethyl reduced the total rooting depth, which can increase the plant's vulnerability to decreasing water tables. These compounds induce in plants, similar responses to the various environmental stresses as do the triazoles (Novartis Technical Bulletin, 1998). Zhang and Schmidt (2000) report that the improvement in stress tolerance of creeping bentgrass by trinexapac-ethyl appears to be associated partially with an increase of endogenous SOD activity.

5.0 GA mutants

Much of what we know today about the physiology of hormones was elucidated using mutants (reviewed by Reid, 1993; Ross et al., 1997). GA-deficient and GA-responsive mutants have been identified in many species including *Arabidopsis thaliana*, rice, maize, wheat, peas and barley (Ross, 1994; Chandler and Robertson, 1999). They have been crucial to understanding the role of GA on height, germination (Chandler, 1992) and more recently on stress

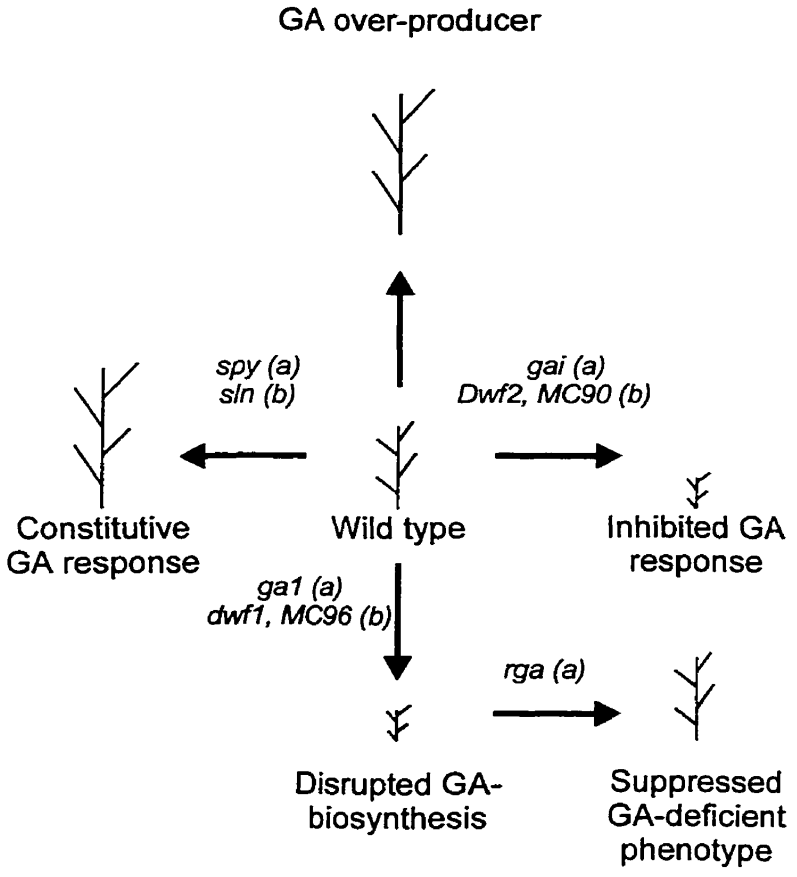


Figure 6. Characterization of the GA signal transduction pathway using mutants of *Arabidopsis thaliana* (a) and barley (b). The *spy* (spindly) and *sln* (slender) mutations are phenocopies of plants sprayed with GA. The *gai* (GA-insensitive), *Dwf2* and MC90 mutants do not respond to GA due to either a decrease in sensitivity to GA or a reduced growth response. The *ga1*, *dwf1* and MC96 mutants are GA-deficient, however the *rga* (repressor of GA1-3) mutation suppresses the phenotype associated with *gai* in *Arabidopsis*. GA producers are yet to be identified in species other than in Brassica (Rood et al., 1990). Adapted from Ogas (1998), and Chandler and Robertson (1999).

tolerance (Vettakkorumakankav et al., 1999). Using mutants from *Arabidopsis*, Ogas (1998) has recently developed a model, which can molecularly characterize the components of the GA signaling pathway (Figure 6). This pathway could enhance the understanding of the processes by which a simple molecule such as GA can regulate the many effects in which it is implicated. However, on a more practical basis, GA mutants have played an enormous role in modern agriculture.

5.1 Role of GA mutants in agriculture

In 1925, ten years prior to the discovery of GA, a semi-dwarf line of wheat was isolated in Japan and named Norin-10 (Hanson et al., 1982). This line of wheat contributed to the famed international agricultural development program in the 1960's, which was also known as the Green Revolution (Brown and Flavin, 1999). The genes controlling the dwarf characteristics of Norin-10, *Rht1* (*Rht-B1b)* and *Rht2* (*Rht-D1b*) were found to reduce plant height by decreasing the sensitivity of vegetative and reproductive tissue to endogenous and exogenous GA (Rebetzke and Richards, 2000). The discovery of this line of wheat led, in part, to the 13-fold increase in the number of people one U. S. farmer can feed today (Brown, 1999). Besides the *Rht1* and *Rht2* genes, there have been 20 major *Rht* wheat genes that have been identified, of which, 16 are sensitive to GA (Konzak, 1987). The most common, *Rht8* and *Rht9*, have been used in southern and Eastern Europe, Russia, China and Japan (Worland et al., 1998). Recently, Rebetzke and Richards (2000) reported that dwarf wheat lines that are sensitive to applied GA gave relatively consistent yield in all environments.

5.2 GA mutants of barley

There are two wide classifications of mutants, those with impaired synthesis and deactivation of GA, and the GA-non-responsive mutants (Ross et al, 1997). The first clearly identified phytohormone mutants were GA-deficient dwarfs of maize (Reid, 1993). So far, all the GA synthesis mutants identified have mutations in structural genes coding for enzymes involved in the biosynthetic pathway from the synthesis of *ent*-kaurene to 3β-hydroxylase-catalyzed reactions; no mutations in genes regulating the pathway have been isolated (Ross et al., 1997). The only example of a characterized GA deactivation mutant is *sln* in pea, and in this mutant there is a blockage in the conversion of GA_{20} to GA_{29}, and from GA_{29} to GA_{29}-catabolite (Reid, 1993). Hence, using gibberellin dose-response curves, Chandler and Robertson (1999) identified three classes of dwarf barley response mutants: *grd* (GA-responsive dwarf), *gse* (GA sensitivity), and *elo* (elongation). The *grd* mutants respond to GA_3 applications over the same concentration range as the wild type, and are proposed to have low levels of endogenous physiologically active GAs (Figure 6). A vegetative dwarf mutant, conditioned by a recessive allele *dwf1* that was obtained as a segregant in an M_2 progeny of gamma irradiated barley cv. Perth shows these characteristics (Falk and Kasha, 1982). When sprayed with exogenous GA_3, the younger basal internodes elongate rapidly, however as the plant "runs out" of GA, the rate of elongation decreases resulting in an obvious difference between the length of the lower and the apical internodes. Furthermore, there is a distinct difference between the internodes sprayed with

GA recently and those that had been sprayed earlier (Falk and Kasha, 1982), providing significant evidence that this mutant is GA deficient and probably a *grd* -type response mutant. Favret et al. (1975) described a mutant called *GA-less* (synonyms: *gal*, MC96 and Hv288), which was a light-insensitive dwarf that was very sensitive to gibberellin. This mutant was sensitive to concentrations as low as 10^{-8}, 100 times lower than the normal genotype and is probably an extreme case of a *grd* mutation (Favret et al., 1975). Börner et al., (1999) has mapped the gene coding for this mutation on the 2H chromosome of barley.

The GA non-responsive mutants, however, are a bit more complicated because a lack of response to GA in a mutant does not necessarily mean a deficiency in signaling, but rather could indicate deficiency in another factor that regulates growth, such as the brassinosteroids (Chandler and Robertson, 1999). The *gse* mutants are primarily characterized by an alteration in GA sensitivity and appear to have a defect in a component of GA signaling (Figure 6) (Chandler and Robertson, 1999). They respond to extremely high levels of GA. Such a mutation called *GA-ins* (synonyms: *gai*, MC90 and Hv287) has been described by Favret et al. (1975), and has been mapped to chromosome 2H by Börner et al. (1999). Another dominant dwarfing gene (*Dwf2*) in barley described by Falk (1995) is GA-insensitive and has characteristics that are similar to that of *gse* mutants. When the threshold for GA-response in these mutants is decreased by combining them with a normal GA-response wild type, this dwarf does respond to applied GA (Dr. D. E. Falk, personal communication) indicating that it has impaired GA-sensitivity. The *Dwf2* mutation occurred as a single dwarf tiller on a

spontaneously doubled haploid plant of H930-36 regenerated from anther culture. The gene for this mutation has been mapped to the short arm of chromosome 4H (Ivandic et al., 1999). The last class described by Chandler and Robertson (1999) are the *elo* and these do not respond to any concentration of GA, indicating that they have a defect in specific processes that are required for leaf elongation rather than in GA signaling.

An interesting mutant known as *slender* (*sln*) that occurred as a single-locus recessive mutation in barley cv. Herta (Lanahan and Ho, 1988), appears to have saturating concentrations of endogenous GA (Figure 6), even when the levels of the GA are reduced using GA-inhibitors (Crocker et al., 1990). Favret et al. (1975) was the first to describe this mutant under the name *gigas*. *Sln* is considered to be a constitutive GA-response mutant that is elongating at maximal rate even though its' endogenous GA levels are lower than in the wild type (Crocker et al., 1990). This indicates that the wild type product of this gene presumably functions as a negative regulator of GA signaling and it is defective in the mutant (Chandler and Robertson, 1999). It is also speculated that the GAI and RGA wild type genes in *Arabidopsis* act as direct negative regulators of GA signaling (Figure 6) (Ogas, 1998). Wild type plants can grow, presumably, because the endogenous GA levels are high enough to reduce the extent of negative regulation imposed by GAI, RGA and SLN (Chandler and Robertson, 1999). There are also other negative regulators such as SPY, which is predicted to be an *O*-GlcNAc transferase that catalyses *O*-linked glycosylation of serine and threonine residues with *N*-acetylglucosamine and is thought to activate GAI

and/or RGA (Ogas, 1998). Cloning of the GAI, RGA and SLN genes will provide further insight into GA signal transduction (Chandler and Robertson, 1999).

EXPERIMENTAL SECTION

INTRODUCTION

Plants treated with gibberellin (GA)-biosynthesis inhibitors are shorter and usually more tolerant to a range of environmental stresses (Rademacher, 1997; Vettakkorumakankav et al., 1999). Paclobutrazol, is one of a number of triazoles that enhance tolerance to various environmental stresses in several plant species (Fletcher et al., 2000). Triazoles act as GA-biosynthesis-inhibitors and fungicides by blocking cytochrome P450-mediated oxidation reactions (see reviews by Davis *et al.*, 1988 and Fletcher *et al.*, 2000). In higher plants the triazoles reduce growth by interfering with the conversion of *ent*-kaurene to *ent*-kaurenoic acid, an early step in GA biosynthesis (Rademacher, 2000). Triazole-application also triggers other hormonal changes including an increase in cytokinins, a transient rise in abscisic acid (ABA), and a reduction in ethylene levels (Fletcher and Hofstra, 1988). Furthermore, they increase the levels of antioxidants (α-tocopherol and ascorbic acid) and antioxidant enzymes (superoxide dismutase and peroxides) in treated plants (Kraus et al., 1995b). It has been proposed that the shift in the plants' endogenous hormonal balance coupled with the increase in antioxidant and antioxidant enzyme levels mediate the stress-protective effects of the triazoles (Fletcher et al., 2000; Kraus et al., 1995b).

Gilley and Fletcher (1998) reported that application of exogenous GA_3 counters both the growth inhibitory and stress-protective effects of the triazoles. Furthermore, a recent study using near-isogenic lines of tall normal and dwarf-

mutant (GA-responsive) barley (*Hordeum vulgare* L.) seedlings showed that tolerance to heat, chilling and drought could be enhanced in seedlings of the normal genotype by application of paclobutrazol and reduced in the GA-responsive dwarf seedlings by treatment with GA_3 (Vettakkorumakankav et al., 1999). The above studies thus suggested that modulation of endogenous GA levels was crucial for enhancing stress tolerance in plants.

In order to investigate this suggestion further and to determine whether specific GAs rather than the total GA content could be involved in stress tolerance, the triazoles were compared to a newer generation of GA-biosynthesis inhibitors, the acylcyclohexanediones. These compounds inhibit late-stage biosynthetic steps, particularly the conversion of the inactive 3-deoxy GA, GA_{20} to the growth-active 3β-hydroxylated GA, GA_1 (Rademacher et al., 2000). However, they have been reported to increase ABA levels (Adams et al., 1992), reduce ethylene levels (Rademacher, 2000) and increase stress tolerance in plants (Rademacher, 1995). However, at maximally effective doses the triazoles generally results in shorter plants than the acylcyclohexanediones (Yelverton and Isgrigg, 1997).

Interestingly, in the studies by Gilley and Fletcher (1998) and Vettakkorumakankav et al. (1999) the reversal of stress tolerance was also accompanied by an increase in height. Hence, the question arises as to whether a reduction in both endogenous GA levels and shoot height is essential for enhancing stress tolerance in plants? This question was addressed by examining tolerance to heat or paraquat stress in relation to endogenous GA

levels and shoot height of barley cv. Perth seedlings treated with either a triazole, or an acylcyclohexanedione, and in GA-responsive and GA-non-responsive dwarf mutant lines of barley.

MATERIALS AND METHODS

Plant Materials

Barley (*Hordeum vulgare* L.) cv. Perth was used as the normal / control. The other barley lines, GA-responsive *dwf1* and MC96, and GA-non-responsive *Dwf2* and MC90 used in this study, have been described by Falk (1995), Falk and Kasha (1982), Favret et al. (1975), and Börner et al. (1999).

Chemicals

Paclobutrazol ((2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazoyl)-pentan-3-ol) and paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) were obtained from ICI-Zeneca, Canada. Primo (4-(cyclopropyl-α-hydroxy-methlyene)-3,5-dioxo-cyclohexanecarboxylic acid methyl ester) was obtained from Novartis Inc., Canada, with prohexadione-Ca (calcium 3-oxido-4-propionyl-5-oxo-3-cyclohexenecarboxylate) being provided by BASF Corp., U. S. A.

Seed treatment and growing conditions

Seeds of barley cv. Perth were soaked in either distilled water (control), or aqueous solutions of 100 mg/l paclobutrazol, 150 mg/l primo, or 250 mg/l prohexadione-Ca, for 18 h at room temperature. These concentrations were determined from a preliminary dose-response study and they provided maximum growth inhibition without reducing germination (results not shown). After imbibition the seeds were rinsed with distilled water, air-dried for one day and planted in Promix BX (Plant Products, Canada) (method reviewed by Fletcher and Hofstra, 1990). The seedlings were grown in a greenhouse with day/night

temperatures of 24/18 ± 4 °C for 10 days, after which the growth regulatory and stress protective properties of the different GA-inhibitors were assessed.

The stress tolerance properties of the barley mutants MC90, *Dwf2*, MC96 and *dwf1* were also compared to those of the tall normal cv. Perth. Seeds of each genotype were planted in a mixture of soil : peat : Turface (3:2:1) and grown in a growth-room with 350 μmols light intensity, 60-70% RH, 20/15 ± 2°C day/night temperature and a 16-h photoperiod. Tall normal cv. Perth and MC90 seedlings were assessed 10 days after planting (DAP), whereas for the slower-growing dwarf-mutant lines, *Dwf2*, MC96, and *dwf1*, assessments were made 12 DAP.

All measurements for comparisons among the GA-inhibitors and of the mutants with cv. Perth were accomplished using seedlings at a similar morphological stage based on the appearance and development of the first and second leaf. Shoot height was measured as the total length from the surface of the soil to the tip of the longest leaf when extended. For both comparisons, one set of 24 seedlings was maintained in the greenhouse, and the other set was exposed to stress as described below.

Heat stress

Seedlings were exposed to 50 ± 1°C for 3 h at 60-70% RH under low light intensity (20 μmols), then returned to the greenhouse. Various parameters, described below, were analyzed according to the procedures described by Vettakkorumakankav et al. (1999).

Free-radical stress

The bi-pyridinium herbicide paraquat was applied as described in Gilley and Fletcher (1998). Leaf segments (1 cm) from the middle of the first leaf were pre-incubated in 0, 2, and 5 μmolar paraquat for 1 h in the dark, then exposed to 1000 μmols of light provided by 400-watt high-pressure sodium lamps (P. L. Light Systems, Canada) for 6 to 8 h. Total chlorophyll was measured as described below.

Stress parameters:

Ion leakage

The percentage of ions leaking from a sample of tissue is an indicator of membrane integrity of the cells and this was measured using the procedure described by Gilley and Fletcher (1998). The whole shoot from either a heat-stressed or an unstressed plant was immersed in 15 ml of distilled water and mixed in a vortex for 15 s. Conductivity (Co) was measured 1 h later using a Model-32 conductance meter (Yellow Springs Instrument Co., Inc. Ohio, U.S.A.). The tubes were then left for 24 h at room temperature and initial conductivity (Ci) measured. Finally, the tubes were placed in a boiling water bath for 30 min, cooled to room temperature and final conductivity (Cf) measured. The percent conductivity was calculated using the following equation:

$$\% \text{ Ion Leakage} = [(Ci-Co)/(Cf-Co)] \times 100\%$$

Chlorophyll fluorescence

This is a non-invasive method for rapid identification of photosynthetic injury to leaves and was estimated as described by Vettakkorumakankav et al.,

(1999). The ratio of variable to maximal fluorescence (F_v/F_m) was measured using a Hansatech fluorescence meter (Model-FMS 2, Hansatech, Ltd., U. K.) with a 30-min dark adaptation period. Measurements were made 24 h after heat stress on the first leaf of stressed and unstressed plants.

Photosynthetic pigments

Pigments were extracted from leaf tissue in 80% ethanol using a Polytron homogenizer (Brinkmann Instruments, Canada). The homogenates were clarified by centrifugation at 2500x*g* for 10 min, and absorbance read using a Beckman DU-65 Spectrophotometer (Beckman Inc., U. S. A.) at 663, 647 and 470 nm wavelengths. Total chlorophyll (a + b) and carotene levels were estimated as described in Gilley and Fletcher (1998).

Endogenous GA measurements

Control and primo-treated seedlings were harvested 10 DAP, while *dwf1* and *Dwf2* were harvested 12 DAP. All the seedlings were at the two-leaf stage. The inner-half of the second (younger) leaf was dissected and immediately frozen in liquid N_2 and freeze-dried. Approximately 1 g of the lyophilized tissue was pulverized in a mortar and pestle using liquid N_2. One set of tissue was analyzed for each of the tall normal / control and primo-treated cv. Perth seedlings, and the two dwarf genotypes, *dwf1* and *Dwf2*. Analyses of the endogenous GA contents of these samples were conducted using the following procedure under the supervision of Dr. R. P. Pharis at the University of Alberta, Calgary.

The ground samples were extracted with 80% methanol (MeOH) (Koshioka et al., 1983a), with 20 ng each of [17, 17- $^2H_2$] $GA_{1/3/4/5/7/8/9/15/19/20/24/44/53}$ added as internal standards. The 80% MeOH extract was purified with a C_{18} preparative column (C_{18}-PC) made of a syringe barrel (inside diameter (i. d.) 2 cm) filled with 3 g of C_{18} preparative reversed-phase material (Waters Associates) (Koshioka et al., 1983a). The 80% MeOH eluate was then loaded onto an ion-exchange column made of AG 1-X8 Resin (Bio-Rad Laboratories) and the GAs were washed off with 5% acetic acid in absolute MeOH. The GA-containing fraction was taken to dryness *in vacuo* at 35°C and then loaded onto a SiO_2 partition column (SiO_2-PC) (column i.d. = 1.5 cm) made of 5 g of Woelm SiO_2 (32-100 mesh) deactivated by equilibration with 20% water by weight (Koshioka et al., 1983a). The SiO_2-PC was eluted with ethyl acetate:hexane (95:5, v/v; saturated with 0.5 M formic acid).

The GA-containing fraction was taken to dryness *in vacuo* at 35°C and further purified by high performance liquid chromatography (HPLC) (Koshioka et al., 1983b; Pearce et al., 1994). The HPLC was a Waters Associates liquid chromatography apparatus with two model M-45 pumps, a model 680 automated gradient controller, and a model 7125 Rheodyne injector. The solvents were, pump A: 10% MeOH in 1% acetic acid (H_2O:MeOH:acetic acid = 89:10:1, (v/v/v)), and pump B: 100% MeOH. A Waters Associates reversed phase C_{18} Radial-PAK μ-Bondapak column (8 mm × 10 cm) was used with a 10-73% linear gradient program at a flow rate of 2 ml/min. The manually implemented 10-73% linear gradient program was 0-10 min (pump A, 100%; pump B, 0%), 10-40 min (pump

A, 100-30%; pump B, 0-70%), and 40-50 min (pump A, 30%; pump B, 70%). The HPLC fractions (1 min, 2ml fractions) were taken to dryness *in vacuo*. The C_{18} HPLC fractions were further purified with an Alltech Associates Nucleosil $N(CH_3)_2$ HPLC column (4.6 mm × 15 cm) eluted with 99.9% MeOH in 0.1% acetic acid (see Pearce et al., 1994), the 1 min, 1ml fractions being taken to dryness *in vacuo*.

The GA-containing fractions, based on retention times (Rts) of external standards, from the Nucleosil $N(CH_3)_2$ HPLC were methylated by ethereal CH_2N_2. The methylated sample was then silylated by BSTFA with 1% TMCS (Pierce Chemical Co.) (Hedden, 1987; Gaskin and MacMillan 1991). The identification and quantification of GAs was carried out by gas chromatography-mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode. The derivatized sample was injected onto a capillary column installed in a Hewlett-Packard 5890 GC with a capillary direct interface to a HP 5970 Mass Selective Detector (MSD). The capillary column was a 0.25 μm film thickness, 0.25 mm internal diameter, 15 m DB1-15 N column (J & W Scientific, Inc.). The capillary head pressure was 4 psi with a He carrier gas flow rate of 1.1 ml/min. The GC temperature program was as follows: 0.1 min at 60°C, climbing to 200°C at 20°C per min, then to 250°C at 4°C per min and finally to 300°C at 25°C per min, staying at 300°C for 5 min, then returning to 60°C. The interface temperature was maintained at 300°C and the MSD was operated with the electron multiplier at 1600 V. Three m/z ions for each deuterated and endogenous GA were monitored. The dwell time was 10 sec and the data were processed using Hewlett Packard's G1034C MS

ChemStation Software. Based on the capillary GC Rts and relative abundance of the characteristic m/z ions monitored, endogenous GAs were identified and amounts estimated based on the isotope dilution technique described by Fujioka et al. (1988).

Experimental design and statistical analyses

All experiments were randomized complete blocks replicated three times, with at least three sub-samples (plants per treatment) per block. However, depending on the parameter measured, more than three plants were sampled (see legends for figures for the number of plants used). All statistical analyses were conducted using SAS version 6.12 (SAS Institute, U. S. A.). Proc MIXED (mixed model) analyses with contrast statements and multiple-means comparisons were used to test the effect of treatment with GA-biosynthesis inhibitors and the inherent difference between the mutant lines. Regression in ANOVA with the variation due to treatment partitioned into linear, quadratic, and lack-of-fit components was conducted to determine the nature of the relationship between height and stress tolerance. Residuals were tested for randomness, homogeneity, normal distribution, a mean of zero and a common variance. All hypotheses were tested at a Type I error rate (p) of 0.05.

RESULTS AND DISCUSSION

Paclobutrazol and primo delayed germination, whereas prohexadione-Ca had no effect. This delay in germination was expected since *de novo* synthesis of GAs in barley is necessary for the mobilization of seed reserves and embryo growth (Jacobsen et al., 1995). Ten days after planting, the seedlings from all treatments including the control had emerged and had a similar number of leaves. However, treatment with paclobutrazol and primo had reduced seedling height by 67 and 28% respectively (Figure 7A), while prohexadione-Ca had no significant effect on height. In a similar study, Yelverton and Isgrigg (1997) had reported that primo was not as effective as paclobutrazol in retarding the growth of *Poa annua*. The leaves of paclobutrazol-treated plants appeared greener, though the amount of total chlorophyll per seedling was not significantly different (results not shown) and more twisted than the controls a morphological effect that has been reported in other species (Davis et al, 1988).

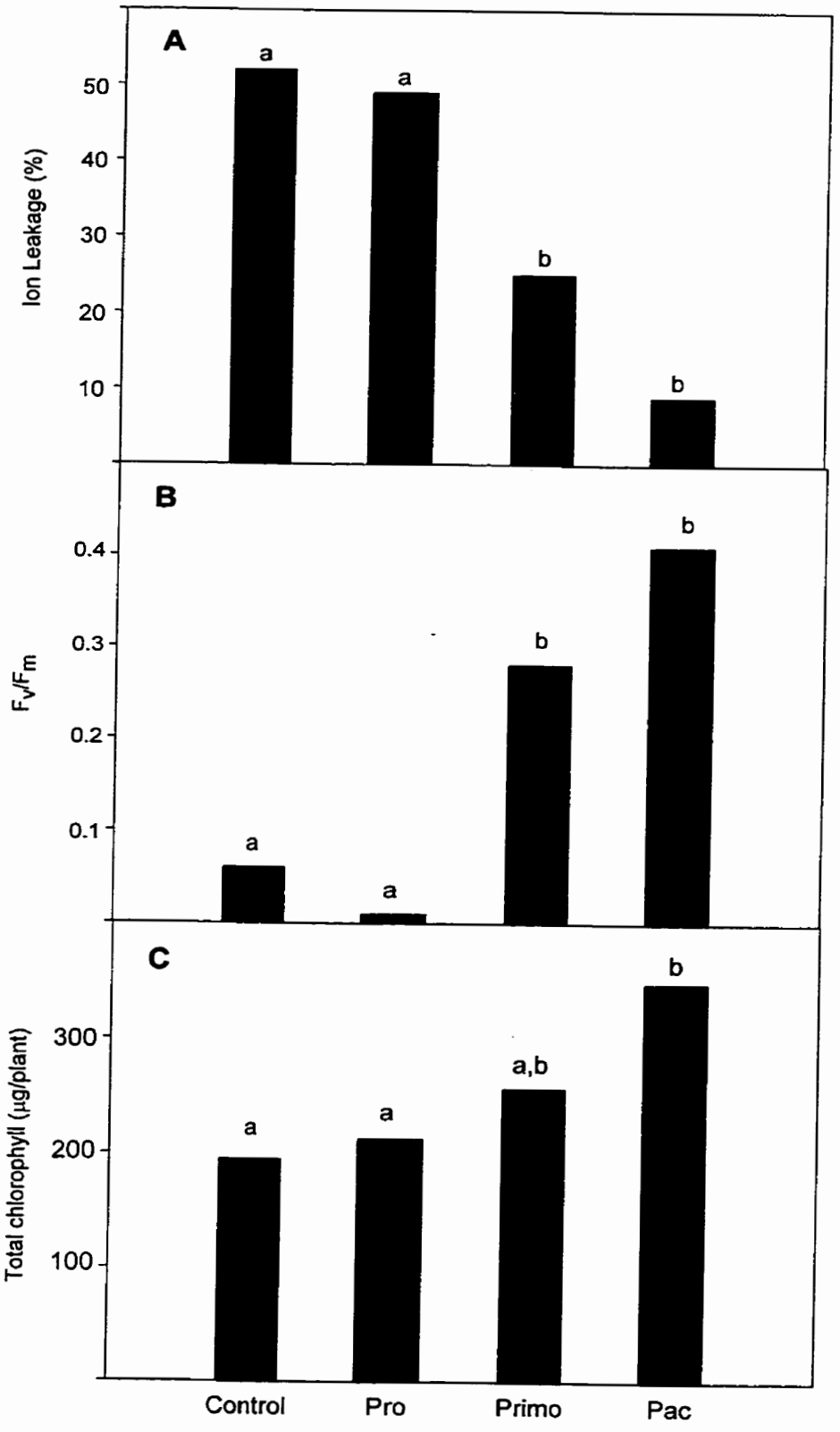
After exposure to 50°C for 3 h, the control and prohexadione-Ca-treated seedlings were severely damaged, showing typical symptoms of heat stress including flaccidity and leaf-tip burn (Figure 7B). These symptoms were prevented by treatment with paclobutrazol, but primo was only partially effective in this regard. Measurements of ion leakage, an indicator of membrane integrity, (Figure 8A) provided a quantitative assessment of the extent of heat damage. Before heat stress, measurements of percent ion leakage in both control and treated seedlings were similar (approx. 10%). However, after exposure to heat stress ion leakage values increased to 52, 49 and 25% in the controls,

Figure 7. Appearance of 10 day-old barley cv. Perth seedlings (A) before, and (B) 24-h after exposure to 50±1°C for 3 h. Seeds were imbibed in, from left to right (two pots per treatment) water (control), 250 mg/l prohexadione-Ca, 150 mg/l primo, and 100 mg/l paclobutrazol. Their mean heights were 115 (a), 114 (a), 84 (b), and 38 (c) mm respectively, with a standard error of 0.033, and n = 144. Means followed by the same letter are not significantly different from each other at a Type I error rate of 0.05.

A

B

Figure 8. Ion leakage (A), chlorophyll fluorescence (B), and total chlorophyll (C), for barley cv. Perth seedlings, from seeds imbibed in water (Control), 250 mg/l prohexadione-Ca (Pro), 150 mg/l primo, and 100 mg/l paclobutrazol (Pac), after exposure to 50±1°C for 3 h. The same letter indicates that the means are not significantly different from each other at a Type I error rate of 0.05. Standard error of the means for ion leakage = 5.6, chlorophyll fluorescence = 1.75, and total chlorophyll = 40, n = 36.


A
Ion Leakage (%)
50
40
30
20
10
a
a
b
b
B
F_v/F_m
0.4
0.3
0.2
0.1
a
a
b
b
C
Total chlorophyll (µg/plant)
300
200
100
a
a
a,b
b
Control
Pro
Primo
Pac

prohexadione-Ca- and primo-treated plants, respectively (Figure 8A). There was no significant difference between the values for heat-stressed and unstressed paclobutrazol-treated seedlings. These results show that paclobutrazol provided complete protection from heat stress, while primo was only partially effective.

Chlorophyll fluorescence is a non-destructive technique that can be performed quickly and with precision (DeEll et al., 1999). Measurement of variable to maximal chlorophyll fluorescence (F_v/F_m ratio) is an indicator of the efficiency of Photosystem II. The ratio for a normally functioning leaf varies between 0.75 and 0.85 and a decline in this ratio is indicative of photoinhibitory damage (DeEll et al., 1999). The F_v/F_m ratios measured 24 h after imposition of heat stress revealed extensive damage to control and prohexadione-Ca-treated seedlings with values of 0.06 and 0.01, respectively. However, paclobutrazol- and primo-treated plants maintained fluorescence ratios of 0.41 and 0.28, respectively (Figure 8B) indicating less damage to their photosynthetic apparatus.

One of the visible symptoms of heat stress is chlorosis and the control seedlings were the most chlorotic 5 days after heat stress. Chlorophyll retention was highest in paclobutrazol-, followed by primo- and prohexadione-Ca-treated seedlings (Figure 8C). Chlorophyll content in the paclobutrazol-treated plants after heat stress was not significantly different from that of unstressed plants, whereas in the controls heat stress reduced chlorophyll levels by 47%.

When leaf segments are soaked in paraquat solutions and exposed to light, the free radicals generated destroy chlorophyll molecules, producing a

bleaching effect (Kraus et al., 1995). In this study, when treated with a high concentration of paraquat (5 μM), stressed control tissues were severely damaged, with only 16% of the chlorophyll remaining after 6 h in light (Figure 9). However, primo- and paclobutrazol-treated tissues were not as severely damaged. Paclobutrazol-treated tissues retained twice as much chlorophyll as control tissues. Paliyath and Fletcher (1995b) found that paclobutrazol treatment prevented the decline in activity of the antioxidant enzymes, peroxidase and catalase, seen in the stressed controls, when corn coleoptiles were exposed to high temperature (1 h at 50°C). It has been suggested (Fletcher et al., 2000) that the stress-protective effects of triazoles, including uniconazole and paclobutrazol, are mediated by an increase in antioxidants (vitamins C and E) and antioxidant enzyme activity. This ability of the triazoles to alter the levels of antioxidants could be an important factor in maintaining cell integrity under free radical stress. Thus, a combination of changes induced by triazoles, such as in plant morphology, antioxidant levels and membrane properties, may be the key factors in increasing tolerance to various environmental stresses (Kraus et al., 1995).

Prohexadione-Ca was applied at a concentration 2.5 times higher than paclobutrazol, yet its growth regulatory effects were negligible. This apparent lack of activity in this study may be due to poor uptake or rapid metabolism of the chemical during or after the 18-h imbibition period. Prohexadione-Ca has a half-life of only 1 day in soil relative to several months for paclobutrazol and may

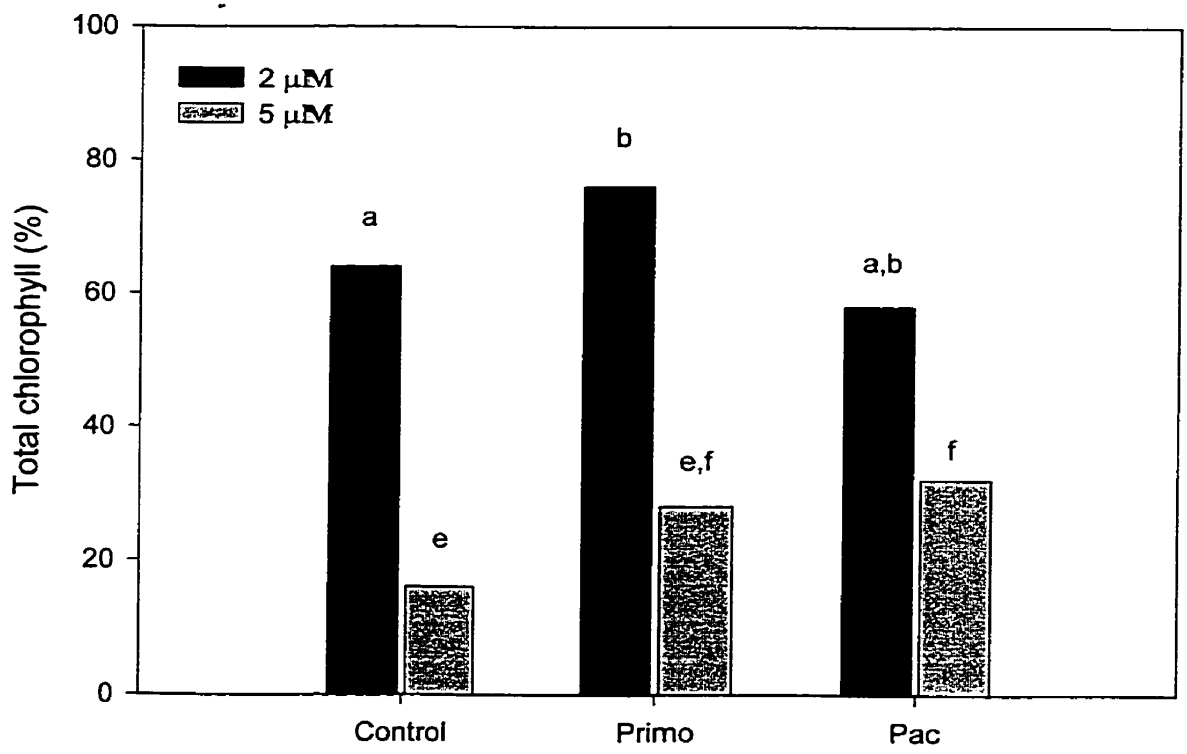


Figure 9. Total chlorophyll (a + b) in leaf-segments for barley cv. Perth seedlings, from seeds imbibed in water (Control), 150 mg/l primo, or 100 mg/l paclobutrazol (Pac), after exposure to two concentrations of paraquat under 1000 µmols of light, for 6 h. The values are expressed as percent of unstressed tissue. The same letter indicates that the means are not significantly different from each other at a Type I error rate of 0.05.

have degraded faster (Rademacher, 2000). When applied as a foliar spray prohexadione-Ca has been reported to have plant growth regulatory effects in cereals (Rademacher, 2000), although in preliminary studies for this thesis, using foliar sprays (0.2 kg/ha a.i. at 400 l/ha) on barley, these effects were not observed. Primo was also less effective than paclobutrazol at reducing height and enhancing stress tolerance at a concentration of 150 mg/l. However, when applied at 500 mg/l, primo yielded morphology changes and stress tolerance values similar to those of paclobutrazol-treated plants, though germination was severely impaired, with only 30 % of the seedlings emerging (data not shown).

The data thus far suggested that with increasing height there was a decrease in stress tolerance and since it has been reported that endogenous GA content and height are closely related (Hedden, 1999), decreased height may be indicative of lower GA levels. Hence, using the ion leakage measurements from control and treated cv. Perth seedlings as an indicator of stress tolerance (Figure 10), regression analysis showed a significant relationship between tolerance to heat stress and shoot height. The model:

$$\% \text{ ion leakage} = -6\ [\pm 26.3] + 0.3HT\ [\pm 0.69] + 0.002HT^2\ [\pm 0.0047],$$

where HT = height, and standard error of the coefficient is in brackets, was significant at a type I error rate of 0.05 ($p = 0.0066$, $R^2 = 0.97$). This model describes a quadratic relationship with a minimum height at which maximum stress tolerance can be achieved, which may in fact be indicative of a minimum GA level for maximum stress tolerance.

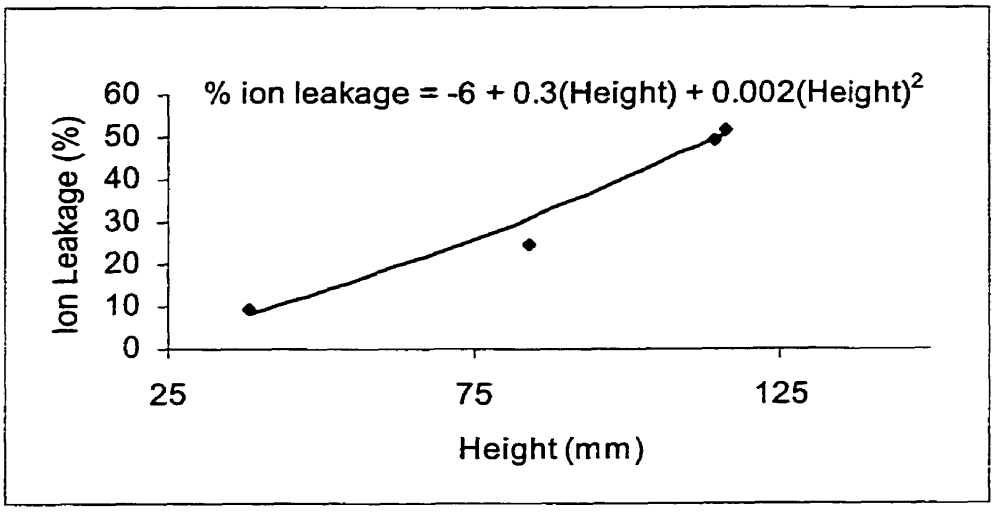


Figure 10. Relationship between shoot height and ion leakage after exposure to 50±1°C for 3 h (shown by the smooth line) of barley cv. Perth seedlings treated with water or the plant growth regulators prohexadione-Ca, primo and paclobutrazol. Each symbol (♦) represents mean heights and ion leakage for stressed seedlings. There was a significant regression at the Type I error rate of 0.05 ($p = 0.0066$, $R^2 = 0.97$).

To investigate this possibility and the role of specific GAs in stress tolerance, the endogenous GA content of primo-treated seedlings relative to control (cv. Perth) seedlings were analyzed (Table 1). These results were compared to published data (see Crocker et al., 1990) for paclobutrazol-treated barley seedlings. Analysis of the endogenous GA levels in the actively growing inner-portion of the second leaf indicated that both the early 13-hydroxylation and early non-13-hydroxylation pathways are functional in barley cv. Perth, (Table 1) which confirmed an earlier report (Grosselindemann et al., 1992). In primo-treated seedlings the reduction in shoot height was associated with increased levels of the 3-deoxy GA_{20}, but not of GA_9 (Table 1), which is also a 3-deoxy GA. In contrast, the growth-active 3β-hydroxylated GAs, especially GA_1, GA_4, and GA_3 were appreciably reduced (Table 1). However, the reduction in GA_4 levels did not result in an elevation of GA_9, implying perhaps a preference for the blockage of GA_{20}-3β-hydroxylase or a relatively low biosynthetic flow-through for the GA_{24} to GA_9 to GA_4 sequence at this stage of seedling ontogeny. Reduced levels of the 3-deoxy GA_{20} and increased levels of the growth-active 3β-hydroxylated GA_1 are consistent with the known mode of action of primo (Rademacher, 2000), which inhibits the 2-oxoglutarate-dependent dioxygenases that catalyses the conversion of GA_{20} to GA_1. Similar results have been obtained previously in barley with another acylcyclohexanedione, LAB 236735 (Hedden, 1991). Furthermore, the level of GA_8, the inactive 2β-hydroxylated metabolite of GA_1, was reduced by 78%, a finding that is also consistent with the mode of action of primo (Rademacher, 2000).

Table 1. Endogenous gibberellin (GA) levels (ng/g dry weight) in the inner portion of the second leaf of 10 day-old control and primo-treated barley cv. Perth seedlings and 12 day-old GA-responsive, *dwf1* and GA-non-responsive *Dwf2* barley mutants.

GA	Control	Primo	*dwf1*	*Dwf2*
GA_1*	6.47	3.63	ND	19.93
GA_3*	35.69	6.87	ND	22.96
GA_4*	8.55	1.67	5.80	9.22
GA_5	[a] ND	-	ND	-
GA_7*	ND	ND	ND	2.77
GA_8	36.79	8.14	ND	57.65
GA_9	13.43	8.00	41.19	6.53
GA_{15}	ND	ND	ND	ND
GA_{19}	[b] -	76.00	-	6.54
GA_{20}	3.68	44.06	35.56	10.95
GA_{24}	ND	ND	ND	ND
GA_{44}	22.30	31.20	7.84	6.20
GA_{53}	-	Trace	ND	ND

[a] ND, endogenous GA not detected; [$^2H_2$] GA detected
[b] [$^2H_2$] GA not detectable
* Growth-active GA

Table 2. Endogenous gibberellin (GA) levels (pg/g fresh weight) in sheath segments of 8 day-old control and paclobutrazol (10μM)-treated barley cv. Herta seedlings (Adapted from Crocker et al., 1990).

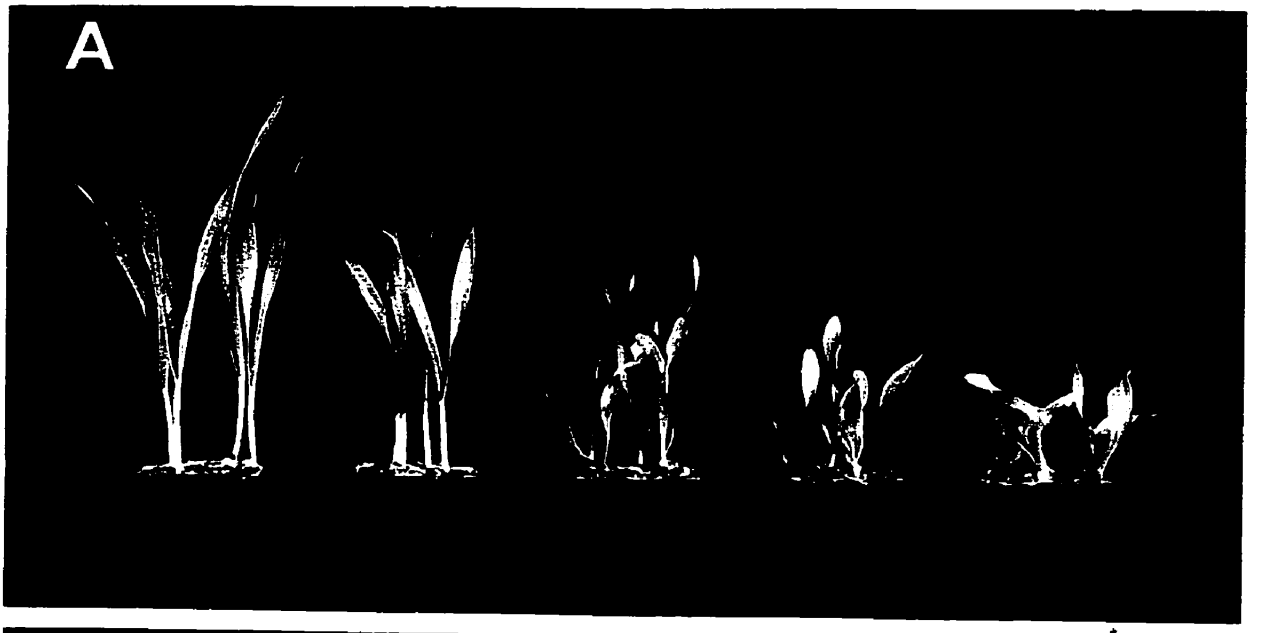
GA	Control	Paclobutrazol
GA_1*	226	15
GA_3*	250	64
GA_8	5607	1469
GA_{19}	3577	267
GA_{20}	719	92

* Growth-active GA

Crocker et al. (1990), in investigating the inhibitory effect of paclobutrazol on GA biosynthesis, found that levels of GA_1 and GA_3 were reduced by 93 and 73%, respectively, and that levels of GA_8, GA_{19} and GA_{20} were also decreased, but to a lesser extent (Table 2). Paclobutrazol acts at a very early stage in GA biosynthesis resulting in the reduced levels of GA_{19} and GA_{20}. Hence, its enhanced potency, relative to primo (compare Tables 1 and 2), is likely due to an overall reduction in all GAs, from 3-deoxy precursors to the growth-active 3β-hydroxylated products. In contrast, primo may allow more "leakage" of elevated levels of 3-deoxy GAs, such as GA_{20} through to its growth-active product, GA_1. The observation that paclobutrazol was more effective than primo at reducing GA levels and enhancing stress tolerance, confirms the suggestion that levels of endogenous GAs are crucial in stress tolerance (Vettakkorumakankav et al., 1999), however the specific GAs involved in stress tolerance cannot be determined at this point.

In order to further clarify the apparent relationship between GA levels, height of seedlings and stress tolerance, two GA-responsive (*dwf1* and MC96) and two GA-non-responsive (*Dwf2* and MC90) mutant lines of barley were studied. After 12 days of growth, the height and phenotype (dark green, twisted leaves) of the two GA-responsive mutant lines MC96 and *dwf1* were similar and comparable to cv. Perth seedlings treated with paclobutrazol (Figures 7A and 11A). This indicated that reduced levels of growth-active GAs, whether caused by mutation or application of GA-biosynthesis inhibitors, yield a similar

Figure 11. Appearance from left to right of barley seedlings, cv. Perth, of GA-non-responsive dwarf mutants MC90 and *Dwf2*, and of GA-responsive dwarf mutants MC96 and *dwf1* (A) before, and (B) 24-h after exposure to heat stress at 50±1°C for 3 h. Their mean heights were 133 (a), 98 (b), 56 (c), 51 (d), and 43 (e) mm respectively, with a standard error of 1.5, n = 36. Means followed by the same letter are not significantly different from each other at a Type I error rate of 0.05.


A

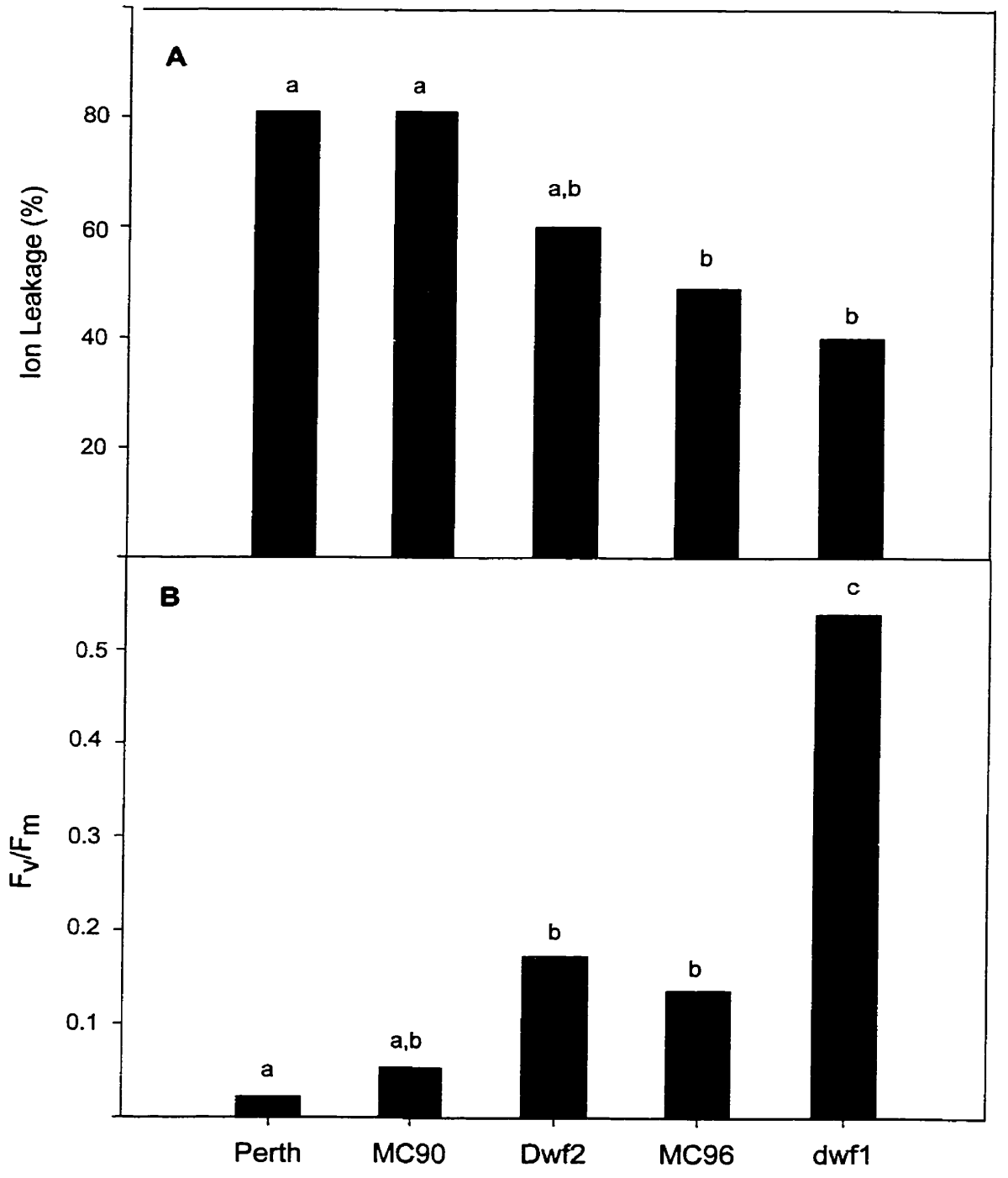

B

morphological state. Seedlings from the GA-non-responsive dwarf line MC90 were somewhat similar in size and phenotype to cv. Perth seedlings treated with primo. The other GA-non-responsive line, *Dwf2*, produced seedlings of intermediate size (Figure 11A).

After exposure to heat stress, cv. Perth and MC90 seedlings were severely damaged, while seedlings of *dwf1*, the shortest mutant, appeared unaffected, showing a high degree of stress tolerance (Figures 11B and 12). The inherent heat tolerance of *dwf1* allowed this mutant to maintain F_v/F_m ratios over 0.5, while cv. Perth and the other mutant seedlings had ratios lower than 0.2 after heat stress, which is indicative of severe damage. Measurement of ion leakage showed a similar trend (Figure 12A). Based on ion leakage and F_v/F_m values, the degree of tolerance to heat stress increased for the various genotypes as follows: tall normal cv. Perth = MC90 < *Dwf2* < MC96 < *dwf1* (Figure 12). It was evident that the GA-responsive mutants, *dwf1* and MC96, which were the shortest, were also more tolerant of heat stress than the GA-non-responsive *Dwf2* and MC90 mutant lines. However, although *Dwf2* seedlings were significantly shorter than MC90 seedlings, their tolerance to stress was not significantly different (Figure 12). Analysis showed no regression between height and stress tolerance among the genotypes and subsequently the regression model was not significant (R^2 = 0.84, p = 0.4552).

Paraquat response showed a similar trend. Leaf segments of the GA-responsive mutants *dwf1* and MC96 that were exposed to the lower concentration of 2 μmolar paraquat, retained 97 and 99% of their origin

Figure 12. Ion leakage (A) and chlorophyll fluorescence (B) for barley seedlings cv. Perth, for GA-non-responsive dwarf mutants MC90 and *Dwf2*, and for GA-responsive dwarf mutants MC96 and *dwf1*, after exposure to heat stress at 50±1°C for 3 h. The same letter indicates that the means are not significantly different from each other at a Type I error rate of 0.05. Standard error of the means for ion leakage = 0.14, and chlorophyll fluorescence = 0.099, n = 9.


A
Ion Leakage (%)
80
60
40
20
a
a
a,b
b
b
B
F_v/F_m
0.5
0.4
0.3
0.2
0.1
a
a,b
b
b
c
Perth
MC90
Dwf2
MC96
dwf1

chlorophyll levels, respectively (Figure 13). In contrast, the GA-non-responsive mutants, *Dwf2* and MC90, retained only 77 and 79% of their chlorophyll, respectively. These latter mutants were thus significantly more susceptible to damage from free radicals than the two GA-responsive dwarfs (Figure 13). However, at the highest concentration of paraquat (5 μmolar), MC96 was the only mutant that was significantly protected from free radical damage (Figure 13). Further studies on MC96 are thus warranted.

Analysis of the GA content in *Dwf2* and cv. Perth showed similar levels of growth-active GAs, i.e. the sum of GA_1, GA_3, GA_4 and GA_7 (Table 1), and both these genotypes had poor stress tolerance (see Figures 11B, 12A and 13). In contrast, *dwf1* had only one-tenth the level of growth-active, 3β-hydroxylated GAs (Table 1) and it exhibited a high degree of stress tolerance (Figures 11B, 12A, and 13). This was comparable to the stress tolerance of paclobutrazol-treated barley seedlings (Figures 7B and 8). The *dwf1* genotype likely gains its reduced titer of growth-active GAs by a severe blockage at the 3β-hydroxylation steps, which is evident by the elevated levels of GA_9 and GA_{20} and undetectable levels of GA_1, GA_3 and GA_7 in this genotype (Table 1). The level of GAs in *dwf1* and *Dwf2* (Table 1) was consistent with that found in *grd* (GA-responsive) and *gse* (GA-non-responsive) mutants, respectively, described by Chandler and Robertson (1999). They concluded that *grd* mutants were GA-deficient, whereas *gse* mutants had a reduced sensitivity to GA signaling that resulted in an accumulation of GA.

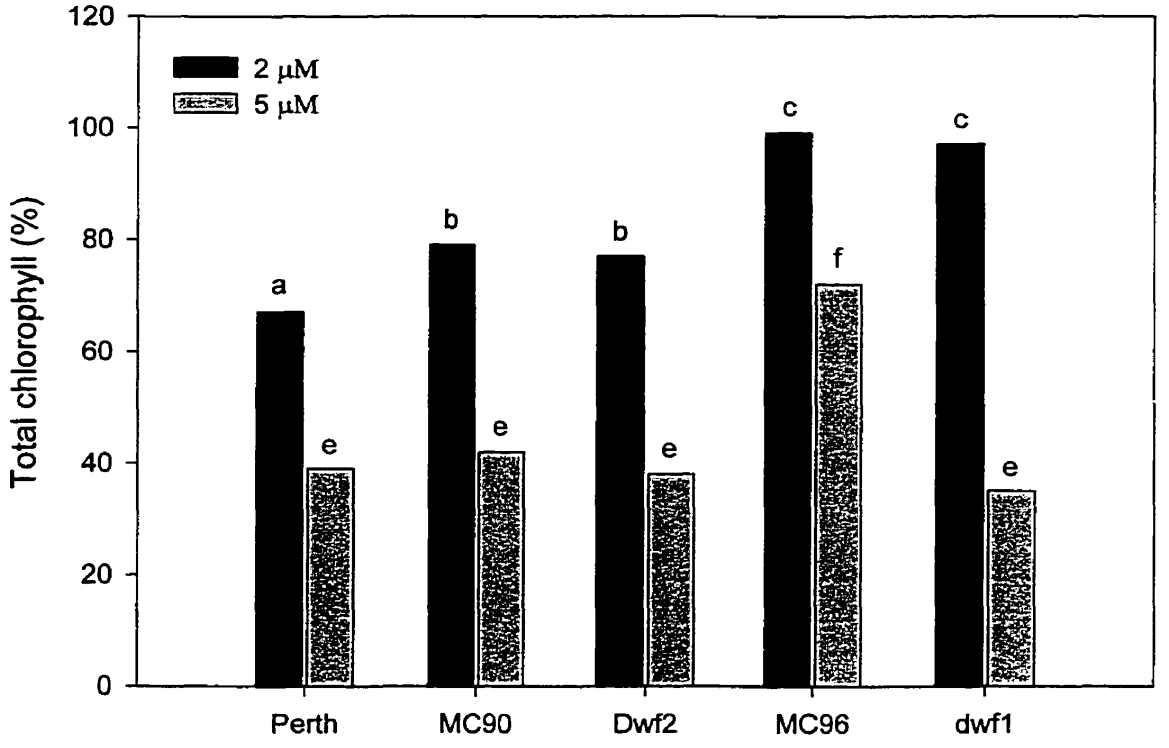


Figure 13. Total chlorophyll (a + b) in leaf segments for barley seedlings cv. Perth, for GA-non-responsive dwarf mutants MC90 and *Dwf2*, and for GA-responsive dwarf mutants MC96 and *dwf1*, expressed as a percent of unstressed tissue. The leaf segments were exposed to 2 and 5 µM of paraquat under 1000 µmols of light, for 8 h. The same letter indicates that the means are not significantly different from each other at a Type I error rate of 0.05.

MC90 may be similar to *Dwf2* in its response to GA, however with a lower threshold for response, which would indicate that it also has high GA levels. If this is in fact the case, then a reduction in height alone is not sufficient to provide stress tolerance. This is confirmed by the lack of a significant correlation between height and stress tolerance for the mutants. The *dwf1* mutant had lower levels of growth-active GAs and was more tolerant to stress than *Dwf2*, a comparison analogous to the observation that paclobutrazol, which effectively reduced levels of growth-active GAs, also produced plants that are much more tolerant to environmental stress. Furthermore, primo-treated plants, which had somewhat reduced levels of GA_1 and GA_4 and significantly reduced levels of GA_3 (Table 1), were more resistant to stress than controls but not as much as paclobutrazol-treated plants (Figures 8A, 8B and 9). Taken *in toto*, these results provide strong evidence that a significant reduction in endogenous GA levels and subsequently in height are causally associated with significant enhancement of stress tolerance.

Plants are subjected to various forms of stress during growth and development and these can lead to a significant reduction in crop yield and quality (Paliyath and Fletcher, 1995b). Results from this study suggest that in the short term, GA-inhibitors might be useful to protect plants from environmental stresses. For example, the acylcyclohexanedione, primo (half-life of 20 days) is much less persistent in soil than paclobutrazol (half-life of 6-12 months), making it a more desirable GA-inhibitor for use in agriculture (Rademacher, 2000). Hence, research into application methods that will enhance uptake in seedlings

of the acylcyclohexanedione class of GA-inhibitors is well worthwhile. It is also possible that the efficacy of acylcyclohexanediones in inducing stress tolerance can be improved by combining their application with other treatments that reduce growth-active GAs. Jordan et al. (1995) reported that overexpression of phytochrome A in transgenic tobacco (*Nicotiana tabacum*, cv. Xanthi) induced substantial dwarfing, increased pigmentation, delayed senescence, and reduced GA_1, which are all characteristics of GA-deficient plants. Hence, the efficacy of acylcyclohexanediones as stress protectants might, in theory, be improved by combining it with a red-light treatment during seed imbibition (see Fletcher et al., 2000).

A reduction in both endogenous growth-active GA content and height are likely to be important requirements for enhanced stress tolerance. The regression analysis used for the GA-inhibitor-trial suggested a quadratic relationship. This indicates that there may be an optimum combination of GA level (as indicated by reduced height) at which maximum tolerance can be achieved. Such a concept could form the basis for novel crop protection strategies using less persistent GA-inhibitors, with or without other adjunct treatments, and breeding using either classical or molecular genetics. That said, it is important to note that in natural systems there are many variables that influence the growth and development of plants and a reduced endogenous GA content for optimal stress tolerance may not always yield improved overall survival.

SUMMARY

The relationship between gibberellin (GA) levels, height and stress tolerance was investigated using barley (*Hordeum vulgare* L.), which were exposed to heat stress (50°C for 3 h) and a free radical generator (paraquat). The normal genotype cv. Perth and four mutants that were either, responsive (MC96 and *dwf1*) or non-responsive (MC90 and *Dwf2*) to applied growth-active GAs, were tested. Two classes of GA-biosynthesis inhibitors: a triazole paclobutrazol, and two acylcyclohexanediones, primo (trinexapac-ethyl) and prohexadione-Ca, that had different sites of action, were used to treat barley cv. Perth. These seedlings exhibited a strong quadratic regression between height and stress tolerance, which was indicative of their endogenous GA content. However, there was no relationship between height and stress tolerance for the barley mutants, indicating that a reduction in shoot height alone is not sufficient to enhance stress tolerance. The mutants, *dwf1* and MC96, were the shortest and the most stress tolerant, whereas *Dwf2* and MC90 responded similarly to both stresses, although *Dwf2* was significantly shorter than MC90.

Compared to the two least tolerant genotypes, cv. Perth and *Dwf2*, the most stress tolerant genotype *dwf1*, had one-tenth the levels of growth-active GAs: GA_1, GA_3, GA_4 and GA_7. Additionally, use of paclobutrazol, which reduced endogenous GAs (Crocker et al. (1990) *Plant Physiol.* 94:194-200) to levels comparable to *dwf1*, conferred a high degree of stress tolerance to cv. Perth. It was thus concluded that a reduction in endogenous levels of growth-active GAs,

and subsequently in shoot height, is an important factor to consider when generating stress tolerant plants.

GENERAL DISCUSSION

Coping with environmentally imposed adverse conditions by relatively rapid physiological changes is a frequent and essential manifestation of the adaptation of terrestrial plants to their often highly changeable physical environments (Moore, 1989). However, with the growing demand for food and the phenomenon of global warming, which could push agriculture into harsher environments, crop yield could be severely affected as a consequence of increased exposure to environmental stresses (Khanna-Chopra and Viswanathan, 1999). Currently, the main sources for plants that are tolerant to abiotic and biotic stresses include breeding (Hanson et al., 1982), biotechnology (Pauls, 1995) and the use of PGRs such as ethephon and ABA analogues (Gianfagna et al., 1992). These procedures can be either used simply as tools towards developing a stress-tolerant variety or in investigating the basis of the stress response. The latter is what is presently required in order to further our capacity to produce food for a rapidly increasing global population.

Plant breeding played an important role in defining modern agriculture (Hanson et al., 1982). However, it can take up to 15 years to develop a high-quality stable cultivar, hence in the past 20 years, plant biotechnology, which is a more rapid technique for identifying and introducing desirable traits into crop-plants is being incorporated into many breeding programs (Pauls, 1995). The fact that endogenous mechanisms of stress tolerance or avoidance are hormonally regulated is why some PGRs can successfully protect plants from environmental stresses (Gianfagna et al., 1992). Investigating the effects of

hormonal regulation on stress tolerance is an important aspect for understanding the mechanisms of stress and can provide a central theme for future crop protection strategies.

It has been established that triazoles protect plants from a variety of environmental stresses (Fletcher et al., 2000). However, their persistence in soil and resultant carry-over effects in the field is of major concern (Jackson et al., 1996). This has hampered the wide spread use of these chemicals, though their potency in both growth regulation and stress protection in various commercially important plants is well documented (Fletcher et al., 2000). The acylcyclohexanediones are less potent than the triazoles but have remarkably shorter half-lives making them attractive candidates in developing crop protection strategies. Both families of PGRs are GA-inhibitors, albeit at different stages of GA-biosynthesis. A study conducted by Gilley and Fletcher (1998) reported that the triazole-effect on both stress tolerance and growth are negated by application of GA. Using GA-responsive mutants of barley and normal tall barley treated with paclobutrazol, the role of GA in stress was recently exemplified by Vettakkorumakankav et al. (1999). They suggested that GA was crucial for stress tolerance. However, in their study the role of height was not clear.

The hypothesis of this study was that a reduction in both gibberellins and height was important for stress tolerance. One objective of the current research was to determine the role or specific GAs in inducing stress tolerance. The other objective was to delineate the effects of GA and height on stress tolerance. A practical outcome of this study was to determine the relative efficacy of the

acylcyclohexanediones, primo and prohexadione-Ca, to the triazole paclobutrazol. The results of the investigation showed that there is a near-perfect inverse correlation between the height of a shoot treated with one of the three GA-inhibitors and stress tolerance, and that the curve defined by regression has a minimum value. Paclobutrazol was more effective at both reducing height and increasing stress tolerance than primo and this appeared closely related to their endogenous GA levels. Hence, it was apparent that the greater the reduction in GA content, the shorter and the more stress tolerant the plant. When the concentration of primo applied to the seed was increased, the resultant seedling closely resembled paclobutrazol-treated seedlings, further confirming the above trend. However, it was not possible to determine whether a reduction in a specific GA or the total GA content was necessary for enhancing stress tolerance.

In order to clarify the role of height, the GA-responsive mutants, *dwf1* and MC96 were compared to the GA-non-responsive mutants *Dwf2* and MC90. The *dwf1* mutant was the shortest and most stress tolerant, whereas both the GA-non-responsive mutants were equally susceptible to stress in spite of MC90 being significantly taller than *Dwf2*. Hence, there was no significant correlation between height and stress tolerance in the mutants. This indicated that height alone was not responsible for the response to stress. Endogenous GA content of *dwf1* and *Dwf2* confirmed that the former was GA-deficient, whereas the latter, which had high levels of biologically active GAs, was defective in either GA-signaling or growth response. Based on the information provided by Falk

(personal communication), it is now presumed that *Dwf2* is defective in GA-signaling. Since MC90 is similar to *Dwf2* in its lack of response to applied GA, it can be assumed that the level of endogenous GA is near normal and hence, indicates that a reduction in GA content and subsequently in height, is important for enhancing stress tolerance. Further evidence to support this conclusion can be found in the studies conducted by Rebetzke and Richards (2000) using wheat dwarfs. They report that the GA-sensitive dwarfs (GA-deficient) had a consistent advantage in all environments, including those that were dry and hot, over the GA-insensitive dwarfs. However, the next step is to determine the probable mechanisms by which GA content and reduced height impact inherent stress tolerance.

On a cellular basis, studies have shown that paclobutrazol-treated plants have sun-type chloroplasts (Lichtenthaler, 1979), that are larger (Gao et al., 1988, Sopher et al., 1999), and modified membranes that are more efficient at discarding damaged regions and repairing themselves (Paliyath and Fletcher, 1995a). Furthermore, there is an increase in antioxidant enzymes such as SOD and peroxidases, as well as in antioxidants including ascorbic acid and tocopherol (Fletcher et al., 2000). Recently studies have shown that primo also causes a rise in the level of SOD in creeping bentgrass (Zhang and Schmidt, 2000). These results are expected since a change in hormone levels will ultimately change the physiology of a plant. However, it is apparent that plants with reduced GA have physiological alterations that increase tolerance to stress. Further evidence to support this comes from a study by Jordan et al. (1995),

where transgenic tobacco overexpressing Phy A were more tolerant to stress. These plants also had lower levels of the physiologically active GA_1 and this decrease was considered an effect of the phytochrome system.

In general, plants grown under high light intensity are shorter, probably due to interaction of the phytochrome system and GA biosynthesis, but they also have a higher compensation point at which the chloroplasts are damaged (Levitt, 1980). Hence, it appears that reduced GA levels induce the formation of chloroplasts that are more efficient at withstanding high light intensity and this is seen in plants treated with triazoles. In totality, it can be hypothesized that a reduction in GA levels induces the formation of resilient chloroplasts that can withstand damage due to both heat and free radicals, but also results in plants that are better equipped to deal with membrane damage caused by these and other stresses.

The reduction in height caused by increased light intensity is considered a result of reduced GA levels. Shorter plants are consistently more tolerant to stress (Levitt, 1980; Hanson et al., 1982; and Rebetzke and Richards, 2000). One of the reasons for this is simply stress avoidance since they are closer to the ground and the insulating layer of air. However, on a physiological basis, the requirement for reduced height is hard to explain and in fact, there may be more than one explanation for this phenomenon. A complication arises with the results from studies conducted using brassinosteroids. Dhaubhadel et al. (1999) reported that treatment with 2,4-epibrassinolide increased the thermotolerance of *Brassica napus*, however it is also known that brassinolides promote shoot

elongation (Davies, 1995a). This is an indication of the complexity involved in plant stress-response and hormone interactions.

Abscisic acid (ABA) is considered to be a stress hormone and is found to be an antagonist of GA in many systems including germination and shoot elonagation (Moore, 1989). For example, ABA causes a reduction in leaf elongation rates by decreasing the activity of xyloglucan endo transglycosylase (XET), whereas GA increases XET activity allowing for cell expansion and growth (Maheshwari, 1999). Triazoles inhibit the degradation of ABA to phaseic acid thereby leading to an increase in ABA levels (Rademacher, 2000). High ABA content has been associated with stress tolerance and it has been proposed that the stress protective effect of triazoles is a result of a change in the hormonal balance, specifically increased ABA levels.

Abscisic acid and GA are simple molecules that have profound physiological effects. They accomplish these by promoting calmodulin/Ca^{+2} mediated signal transduction pathways, similar to phytochrome (Ritchie and Gilroy, 1998). ABA increases the permeability of the membrane to Ca^{+2}, resulting in increased transcription of genes for enzymes involved in among other things, free radical scavenging (Owen, 1988; and Smirnoff, 1998). However, the plant responses that confer stress tolerance need not all be physiological. It is possible that some changes that confer stress tolerance are morphological in nature, such as reduced height and increased epicuticular wax (which, in triazole-treated plants has been implicated with avoidance of UV-B radiation

damage). It is also probable that these changes are what tip the balance in favor of stress tolerance versus susceptibility in the plant.

In summary, it is apparent that reducing GA levels, and subsequently height, is important for enhancing stress tolerance, however, since ABA is antagonistic to GA and has been dubbed the "stress hormone", its effects cannot be excluded. The balance between ABA and GA can be used as a central theme for developing future crop protection strategies around the world. However, it is important to remember that plants require a certain hormonal balance to sustain their physiological processes, without which they may respond by decreasing germination, growth and yield. For this reason, there may be cases where endemic species with naturally evolved stress resistance mechanisms are far more suitable for a particular environment. The stress-response system is complex and all the aspects have not have been considered here, hence there is a need for further studies that will elucidate the involvement of GA and ABA in signal transduction and the resultant effects at the cellular level which confer stress tolerance to the entire plant. In addition, the requirement for a reductions in height has to be confirmed and examined further.

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APPENDICES

STATISTICAL ANALYSES

Note:

Acycl - acylcyclohexadiones; C - control / normal; Cond - conductivity; Ht - height; NR - GA_non-reponsive mutant; P - Primo, Pac - Paclobutrazol; R - GA-responsive mutant; Rep - sub-sample; S - Stressed; Triaz - triazole; U-Unstressed

Each experiment was considered a block

All analyses were conducted in SAS version 6.12 using either general linear, or mixed models procedures.

APPENDIX 1: Comparison of GA-biosynthesis inhibitors

A1-1: Conductivity

General Linear Models Procedure

Source	DF	Sum of Squares	F Value	Pr > F
Model	43	33829.7977588	2.79	0.0030
TRT	7	20244.8575397	10.27	0.0001
BLOCK	2	2157.6390212	3.83	0.0343
REP	2	274.2223545	0.49	0.6199
TRT*BLOCK	14	1943.8115079	0.49	0.9169
BLOCK*REP	4	1324.3908730	1.18	0.3437
TRT*REP	14	5406.7519841	1.37	0.2332
Error	27	7605.4980159		
Corrected Total	70	41435.2957746		

$R^2 = 0.816449$

A1-2: Chlorophyll Fluorescence

Mixed models procedure

Source	NDF	DDF	Type III	F Pr > F
BLOCK	2	6	3.77	0.0870
TRT	7	80	47.07	0.0001

Source	NDF	DDF	Type III	F Pr > F
S vs U	1	80	294.97	0.0001
S C vs ACYC	1	80	1.94	0.1672
C vs TRIAZ	1	80	20.25	0.0001
S TRIAZ vs ACYC	1	80	14.46	0.0003
U C vs ACYC	1	80	0.01	0.9381
U C vs TRIAZ	1	80	0.00	0.9889
U TRIAZ vs ACYC	1	80	0.00	0.9509

Error	Estimate	Std Error	Z	Pr > |Z|
Residual	0.03634035	0.00554185	6.56	0.0001

A1-3: Regression Analysis

General Linear Models Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	4693.015336	670.430762	18.71	0.0066
BLOCK	2	325.283140	162.641570	4.54	0.0936
HT	1	3844.645105	3844.645105	107.29	0.0005
HT*HT	1	105.966753	105.966753	2.96	0.1606
LOF	3	417.120338	139.040113	3.88	0.1116
Error	4	143.335451	35.833863		
Corrected Total	11	4836.350787			

$R^2 = 0.970363$

A1-4: Chlorophyll content after paraquat stress

Mixed Models Procedure

Source	NDF	DDF	Type III F	Pr > F
TRT	8	92	29.38	0.0001
BLOCK	2	92	1.47	0.2343
LIGHT	1	92	12.84	0.0005

Source	NDF	DDF	F	Pr > F
0 C vs PandPAC	1	92	11.98	0.0008
0 PAC vs P	1	92	6.42	0.0130
2 C vs PAC	1	92	3.42	0.0677
2 C vs P	1	92	5.90	0.0171
2 PAC vs P	1	92	0.34	0.5630
5 C vs PAC	1	92	8.53	0.0044
5 C vs P	1	92	2.49	0.1181
5 PAC vs P	1	92	1.45	0.2308

Error	Estimate	Std Error	Z	Pr > |Z|
Residual	32.57415985	4.75142955	6.86	0.0001

APPENDIX 2: Comparison of GA mutants

A2-1: Height

Mixed models procedure

Source	NDF	DDF	Type III F	Pr > F
BLOCK	1	160	52.68	0.0001
TRT	4	160	812.23	0.0001
BLOCK*TRT	4	160	29.45	0.0001

Errors	Estimate	Std Error	Z	Pr > |Z|
REP(BLOCK)	0.02922953	0.06017637	0.49	0.6272
Residual	62.96954020	7.04392059	8.94	0.0001

A2-2: Conductivity

Source	NDF	DDF	Type III F	Pr > F
BLOCK	2	40	1.30	0.2848
TRT	9	40	79.73	0.0001
BLOCK*TRT	18	40	2.86	0.0028

Source	NDF	DDF	F	Pr > F
S C VS NR	1	40	11.14	0.0018
S C VS R	1	40	85.18	0.0001
S NR VS R	1	40	52.07	0.0001
U C vs NR	1	40	2.65	0.1112
U C vs R	1	40	3.21	0.0806
U NR vs R	1	40	0.01	0.9341
S D2 vs d1	1	40	20.44	0.0001
U D2 vs d1	1	40	0.20	0.6610

Errors	Estimate	Std Error	Z	Pr > |Z|
REP(BLOCK)	8.91731176	13.29482130	0.67	0.5024
Residual	89.56094964	20.01675261	4.47	0.0001

A2-3: Chlorophyll Fluorescence

Source	NDF	DDF	Type III F	Pr > F
TRT	9	93	37.15	0.0001

Source	NDF	DDF	F	Pr > F
S C vs R	1	93	20.14	0.0001
S C vs NR	1	93	3.41	0.0681
S R vs NR	1	93	8.57	0.0043
U C vs R	1	93	0.18	0.6710
U C vs NR	1	93	0.00	0.9935
U NR vs R	1	93	0.25	0.6159

Errors	Estimate	Std Error	Z	Pr > |Z|
BLOCK	0.00740574	0.00721282	1.03	0.3045
Residual	0.03411964	0.00500517	6.82	0.0001

A2-4: Regression analysis

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1684.172090	240.596013	1.51	0.4552
BLOCK	1	31.6840000	31.6840000	0.20	0.6995
HT	1	866.0655583	866.0655583	5.43	0.1452
HT*HT	1	367.7194347	367.7194347	2.30	0.2683
LOF	4	418.7030973	104.6757743	0.66	0.6779
Error	2	319.103910	159.551955		
Corrected Total	9	2003.276000			

$R^2 = 0.840709$

A2-5: Chlorophyll content after paraquat stress

Source	NDF	DDF	Type III F	Pr > F
TRT	14	27	6.68	0.0001

Source	NDF	DDF	F	Pr > F
0 C vs dwarfs	1	27	5.29	0.0294
0 NR vs R	1	27	3.39	0.0767
0 D2 vs d1	1	27	2.57	0.1203
2 C vs dwarfs	1	27	10.67	0.0030
2 NR vs R	1	27	11.30	0.0023
2 D2 vs d1	1	27	7.14	0.0126
5 C vs dwarfs	1	27	2.15	0.1544
5 NR vs R	1	27	3.28	0.0813
5 D2 vs d1	1	27	0.09	0.7636

Error	Estimate	Std Error	Z	Pr > |Z|
Residual	35.33977852	9.28068051	3.81	0.0001