

**STUDY ON GIBBERELIC ACID CONTROL OF
ALPHA-AMYLASE GENE EXPRESSION
IN ALEURONE TISSUES OF NORMAL AND DWARF WHEAT**

by

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**STUDY ON GA3 CONTROL OF α -AMYLASE GENE EXPRESSION IN
ALEURONE TISSUES OF NORMAL AND DWARF WHEAT**

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Study on gibberellic acid control of alpha-amylase
gene expression in aleurone tissues of
normal and dwarf wheat

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ABSTRACT

The expression of α -amylase genes in aleurone cells of a standard-height wheat Ramona 50 is known to be under the control of Gibberellic Acid (GA_3) while the production of α -amylase in aleurone layers of a dwarf wheat D6899 (carrying *Rht3* gene) is known to be insensitive to GA_3 . Northern blot experiment was carried out in which RNA samples isolated from GA_3 -treated or untreated aleurone layers of both Ramona 50 and D6899 were subjected to electrophoresis and then hybridized with barley cDNA clones of high-pI and low-pI α -amylase genes. It was shown that the blockage of GA_3 -induced expression of α -amylase genes in aleurone layers of D6899 was at the level of mRNA accumulation. Cold temperature treatment did not induce the GA_3 sensitivity of α -amylase production in D6899. A 417 bp promoter sequence of α -Amy2/54, one of the low pI α -amylase genes expressed in wheat aleurone cells, was synthesized using PCR and cloned into pUC19 plasmid. Gel retardation assays were performed to study DNA-protein interactions between this promoter sequence and percoll gradient purified aleurone proteins of Ramona 50 and D6899. Multiple aleurone proteins that bound to the promoter region were identified. Based on competitive binding studies, one DNA-protein interaction was defined as DNA-sequence-specific, while others appeared to be non-sequence-specific. Nuclear proteins from both GA_3 -treated and untreated aleurone cells of Ramona 50 or D6899 gave

similar retardation patterns. The cellular extracts from roots and leaves of the etiolated Ramona 50 did not contain aleurone nuclear proteins binding to α -Amy2/54 promoter.

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INTRODUCTION

The mechanisms by which gibberellins (GAs) mediate the regulation of α -amylase gene expression in plants are unclear. The GA-induced expression of α -amylase genes in isolated aleurone layers of cereals has been extensively studied in the past several years using barley, wheat and rice. In this study, the isolated aleurone layers of two wheat varieties, the standard-height cultivar Ramona 50 and a dwarf line D6899 which carries the *Rht3* gene and does not respond to GA₃ for production of α -amylase during seed germination, were used to investigate the role of GA₃ in α -amylase gene expression.

In Chapter I, the literature on GA-regulated gene expression in cereal aleurone system is reviewed. My studies on GA₃-induced α -amylase production and α -amylase gene transcription in aleurone layers of Ramona 50 and D6899 using α -amylase cDNA clones from barley, are described in Chapter II. The study on the interaction of aleurone nuclear proteins with the promoter region of a wheat α -amylase gene (α -Amy2/54) using gel retardation assays is reported in Chapter III. The results obtained are discussed in Chapters II and III.

CHAPTER I

LITERATURE REVIEW

A. Introduction:

Since their initial isolation and structural identification in the 1950s, gibberellins (GAs) have been recognized to be present throughout most of the plant kingdom and to be involved in regulation or control of a wide range of different biochemical and physiological processes during plant development (Jones, 1973; Stoddart and Venis, 1980). The most researched and best understood of these processes is that of the aleurone tissue of cereal grains. Using intact tissue or isolated protoplasts, it has been shown that exogenous application of GAs leads to a differential regulation of expression of several different genes (for reviews, see Ho, 1991), including α -amylase genes. The mRNA level of α -amylase in isolated aleurone cells may increase 50- to 100-fold following GA treatment. Recently, molecular techniques have been successfully used to isolate and characterize hormone-responsive genes. With mutagenesis and transformation studies, *cis*-acting sequences on α -amylase gene promoters have been identified (Gubler and Jacobsen, 1992; Huttly et al., 1992; Kim et al., 1992; Lanahan et al., 1992; Rogers and Rogers, 1992; Skriver et al., 1992). In addition, *trans*-acting elements, such as regulatory proteins which bind to the regions of α -amylase genes, were identified by gel retardation and DNaseI footprinting (Kim et al., 1992; Ou-Lee et al., 1988; Rushton et al., 1992; Sutliff et al., 1992). GA-insensitive mutants

offer a unique tool to study the mechanism of GA action (Scott, 1990). *Rht3* mutants in wheat, for example, in which the GA₃ induced production of α -amylase is blocked, are considered to be candidate materials to elucidate GA action.

In this chapter, studies on GA regulation of gene expression in cereal aleurone system, the use of GA mutants, and GA receptor work are discussed. A brief discussion of the mechanism of GA action is also presented.

B. Gibberellin (GA) structure and metabolism:

1. Chemistry of GA

The molecular structure of GAs is depicted by a diterpene hydrocarbon skeleton, *ent*-gibberellane, and substituents which include two hydroxyl-groups at carbons 3 and 13, respectively, a carboxyl group (C7), a methylene carbon (C17), a methyl group (C18), a carbonyl oxygen (C19) and a lactone ring with the ring oxygen linked through carbons 10 and 19 (Fig. 1; see also Witham et al., 1992). Gibberellin is a highly asymmetric molecule which possesses eight chiral centers, and theoretically has 256 enantiometric forms. GAs are defined as compounds which have an *ent*-gibberellane skeleton and biological activity by stimulating cell division or cell elongation, or both, or having such other biological activity consistent with this type of naturally occurring substance. Within the same basic *ent*-gibberellane ring system, there are two main types of GAs, namely the C20-GAs, which have a full complement of 20 carbon atoms, and the C19-GAs, in which the twentieth carbon atom has been lost by metabolism. GAs occur naturally in three chemical forms, two of which are chemically defined and the third of which is hypothetical: (1) "free GAs", (2) "conjugated GAs" and (3) other "water-soluble" or "bound GAs" (Moore, 1989). It is known that most GAs are metabolized to other biologically active GAs in bioassay

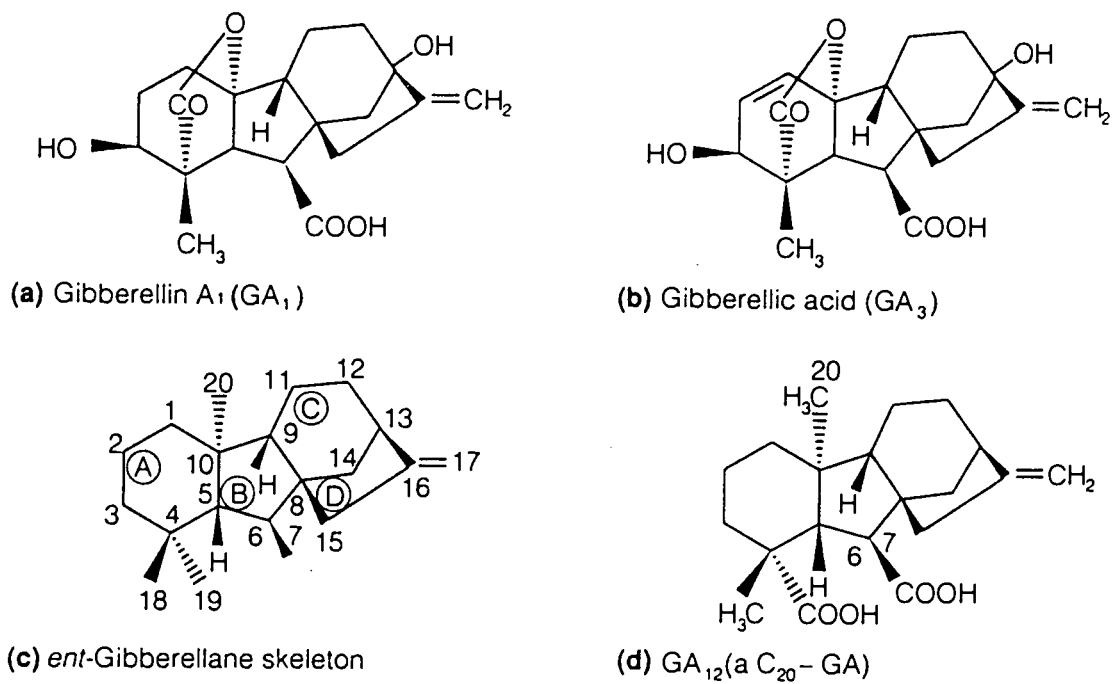


Fig. 1. *Ent*-gibberellane skeleton and structure of GA₁, GA₃ and GA₁₂ (Taiz and Zeiger, 1991).

systems. GA₁₂-aldehyde is the first GA formed during the biosynthesis in all plants (Mander, 1991). It is now thought that GA₁ is active in normal stem elongation (Phinney, 1985), and that all other GAs are precursors. GA₃, which is uncommon in higher plants, would probably also be active since it differs from GA₁ only in having one double bond (Fig. 1).

2. Range of GA regulated activities in plants

GAs affect every aspect of plant growth and development (see reviews by Jones, 1973 and Mander, 1991), but they typically enhance stem growth. Biologists now recognize that GAs were involved in the biogenetic differences between the tall and dwarf peas used by Mendel for his classical inheritance experiments. The phenomenon of bolting in rosette plants (i.e. the explosive growth which precedes flowering in plants like spinach) is caused by naturally occurring endogenous GAs, while dwarfism is due to a deficiency in natural GAs and may be reversed by applying exogenous GAs (Taiz and Zeiger, 1991). The vigorous shoot growth seen with maize hybrids has been shown to be due to the production of above normal levels of GAs (Rood et al., 1988). Flowering is also stimulated by GAs, although in some fruit trees, flowering may be reduced in the year following application. GAs may modify the sex expression of flowers, induce the parthenocarpic development of fruit and delay

senescence. They circumvent the need for exposure to red light in the germination of seeds and spores, and the need for vernalization (winter chilling) in the growth of bulbs and tubers. They are associated with the breaking of winter dormancy and stimulate the formation of hydrolytic enzymes in germinating cereal grain (Ho, 1991).

3. GA receptor

The primary mechanism of action of hormones in plants is believed to involve an interaction between the hormone molecules and some kind of receptor molecules. Unfortunately, the search for a GA receptor has not been successful. Earlier findings from the laboratories of Rappaport and Srivastava provide strong but putative evidence for the existence of GA receptors in a number of different plant species (see reviews: Srivastava and Sechley, 1991; Stoddart, 1986; Venis, 1985). Some structural selectivity of a soluble, cytoplasmic GA-binding site has been demonstrated (Yalpani and Srivastava, 1985) but there has been little progress in purification. From studies of [^3H]GA₄ binding by isolated nuclei from cucumber hypocotyls, the receptor protein was found to be present in the nuclei (Sechley and Srivastava, 1991). Binding of GA to a soluble site from maize leaf sheaths has also been reported, but binding was essentially nonreversible and selectivity between active and inactive GAs was poor (Keith and

Rappaport, 1987). Attempts to identify GA-binding proteins and GA receptors in aleurone protoplasts of wild oat (*Avena fatua* L.) by photo-affinity labeling with ^{125}I -labeled GA₄-derivative, [^{125}I]GA₄-O-ASA, have been reported (Beale et al., 1992). Photoaffinity labelling of [^{125}I]GA₄-O-ASA with gibberellin-specific monoclonal antibody was shown (Walker et al., 1992), suggesting that it can function as an effective and specific probe for a known GA-binding protein. A much more promising approach has been the development of anti-idiotypic antisera. A number of anti-GA monoclonal antibodies that show high-affinity recognition of specific epitopes of different GAs have been produced (Knox JP et al., 1987, 1988; Nakajima et al., 1991; Nester-Hudson et al., 1990). These antibodies have been used to localize the sites of putative GA receptor (Hooley et al., 1990) and could be used as an affinity matrix for purification of the receptor. Hopefully, this technique would reveal new insights into the primary GA action involving a GA receptor.

4. GA-insensitive mutants

A particularly interesting range of GA-response mutants is known (Table 1), which have been considered to provide a unique tool to study the mechanism of GA action. *Rht3* of wheat, *D8* and *Mpl1* of maize, and *Gai* of *Arabidopsis* are dominant mutations which cause GA-insensitive dwarfism. An entirely different type of GA-response mutants, which

Table 1. GA-insensitive mutant genes in plants.

Mutant Gene	Plant	Reference
Recessive:		
<i>lk</i>	pea	Ross and Reid, 1986
<i>lv</i>	<i>Pisum sativum</i>	Reid and Ross, 1988
<i>lw</i>		Jolly et al., 1987
<i>la&cry^S</i>		Potts et al., 1985
<i>sln</i>		Reid et al., 1992
<i>sln</i>	barley <i>Hordeum vulgare</i>	Lanahan and Ho, 1988
<i>d^X</i>	tomato	Nadzhimov et al., 1988
<i>pro</i>	<i>Lycopersicon esculentum</i>	Jupe et al., 1988
Dominant:		
<i>Rht3</i>	wheat <i>Triticum aestivum</i>	Gale et al., 1975
<i>D8 and Mpl1</i>	maize <i>Zea mays</i>	Harberd and Freeling, 1985
<i>Gai</i>	<i>Arabidopsis</i>	Koornneef et al., 1985

contain a single gene recessive mutant, is the slender (*sln*) genotype of barley and pea. These plants behave as if they are continually saturated with GA and appear to have no requirement for endogenous or exogenous GA during growth.

Rht3 mutant has a single gene dominant mutation at *Rht3* locus on chromosome 4BS (Gale and Youssefian, 1985) and the degree of its nonresponsiveness to exogenous GA₃ is dose dependent on the number of alleles present (Fick and Qualset, 1975; Gale and Marshall, 1975; Hoogendoorn et al., 1990; Pinthus et al., 1989). Wheat lines homozygous for *Rht3* are completely unresponsive to GA. It has been shown that there was little difference in the rate of metabolism of GA₁ in *rht3* (tall) and *Rht3* (dwarf) wheat lines (Stoddart, 1984) and in some other metabolic parameters (Ho et al., 1981), while there was a significant difference in α -amylase production between *rht3* and *Rht3* lines. GA does not induce α -amylase production in the *Rht3* mutant (Fick and Qualset, 1975; Gale and Marshall, 1975; Ho et al., 1981). It has been suggested that this phenomenon may be a consequence of blocked GA action. Recently, Hetherington and Laidman (1991) reported a contradictory finding in that after a lag phase of 20 h compared to GA-responsive *rht3* line (tall), the GA-insensitive *Rht3* line (dwarf) showed GA₃ induced α -amylase activity in wheat seeds of the variety April Bearded. Earlier, Singh and Paleg (1984a, b, c) reported that preincubation of deembryonated half seeds or isolated

aleurone layers of several varieties of wheat carrying *Rht* genes (*Rht1*, 2, 3) at low temperature (5°C) for 20 h restored the normal response to exogenous GA₃ with respect to α-amylase production. It is possible that the *Rht* gene codes for a protein at room temperature which either binds to the GA receptor or alternatively prevents the activated receptor from binding to HRE (Hormone Response Element) (Srivastava and Sechley, 1991).

The strongly dominant mutations *D8* and *Mpl1* in maize cause GA-insensitive dwarfism. Gene dosage studies imply that the products of these mutant genes show little sensitivity to the presence of wild-type gene product. Analysis of somatic clonal sectors indicated that, in certain parts of the maize plant, the effects of these mutant genes were confined to the cells containing them (Harberd and Freeling, 1989). The GA-non-responsive dwarf genotypes of both wheat (*Rht3*) and maize (*D8*) were thought to encode an active "inhibitory" product which interfered with the normal GA-response system (Harberd and Freeling, 1989). Scott (1990) reasoned that the "inhibitor" was a mutated regulatory protein that could not be inactivated by the normal inducer, GA. It has also been suggested that *D8* was a good candidate for a mutation in the GA receptor or signal transduction pathway (Fujioka et al., 1988).

Elongated mutants are rare compared to dwarf mutants, but have been isolated from a number of plant species (Scott, 1990). The enhanced elongation has been attributed to several causes, including GA over-production (Beall et al., 1991; Rood et al., 1990), GA hypersensitivity (Reid and Ross, 1988) and deficiency of one form of phytochrome (Koornneef et al., 1985; Lopez-Juez et al., 1992; Peters et al., 1991). In barley, slender (*sln*) is a single-locus recessive mutation which causes a plant to appear as if it had been grown in saturating concentrations of GA (Lanahan and Ho, 1988). The slender mutant can elongate in the presence of a GA biosynthetic inhibitor (ancymidol), while the elongation of the normal barley is severely retarded (Lanahan and Ho, 1988). In isolated normal aleurone layers, the synthesis and secretion of α -amylase, protease and nuclease were induced by exogenously applied GA₃. However, in the aleurone layers of the slender mutant, these enzymes were produced even in the absence of GA (Chandler, 1988; Lanahan and Ho, 1988). The endogenous levels of GA were nonetheless similar in normal and mutant half seeds (Lanahan and Ho, 1988). It was concluded that shoot elongation and hydrolytic-enzyme secretion in aleurone layers had a common regulatory protein which functions as the inhibitor for the appropriate GA-induced processes in normal barley (Lanahan and Ho, 1988). This inhibitor may have been lost in the slender mutant. In the garden pea (*Pisum sativum* L.), two mutants, *lv* (Reid and Ross, 1988) and the duplicate

combinations of *la cry*^S and *la cry*^C (Potts et al., 1985) which have elongated phenotypes, have been well-defined. Most recently, a new elongated mutant of garden pea was described and shown to be conferred by a recessive allele of a new gene, *sln* (Reid et al., 1992) similar to barley. The new *sln* mutant may have impaired the catabolism of GA₂₀ in maturing seeds, causing GA₂₀ accumulation and movement into seedling, where it is converted to GA₁ and promotes elongation growth. It is known that *sln* mutation has a different mode of action from the established *la cry*^C slender gene combination (Reid et al., 1992).

C. GA regulation of α -amylase gene expression in aleurone tissues of cereal grains:

1. Cereal aleurone tissues

Cereal seeds are divided into two parts, the embryo and the endosperm. The embryo region consists of the embryo itself and the scutellum, which is thought to absorb the nutrients from the endosperm (Taiz and Zeiger, 1991). The endosperm is composed of two tissues, the centrally located starchy endosperm which consists of starch grains, and the aleurone layer. The aleurone layer, which can vary from one or three cell layers thick in different plants, surrounds the starchy endosperm and is cytologically and biochemically quite distinct. Aleurone cells generally contain large numbers of organelles which store proteins called aleurone grains, or protein bodies, as well as lipid-storing spherosomes (Taiz and Zeiger, 1991). During seed germination, GAs which diffuse from the embryo into the endosperm and eventually to the aleurone cells, stimulate the synthesis of several hydrolytic enzymes (Ho, 1991), including α -amylase (EC 3.2.1.1), proteases, 1,3-1,4- β -glucanase (EC 3.2.1.73), xylanase and nuclease (EC 3.1.30.2). These enzymes are then secreted into the endosperm where they hydrolyze the starch and protein reserves into simple sugars and amino acids which are absorbed by the scutellum and transported to the embryo to

support the heterotrophic growth of young seedlings. In experiments carried out before the 1960s, the deembryonated half-seed was used to study the GA-induced synthesis of starch-degrading enzymes (Jones, 1973). Since the 1960s, investigators have utilized aleurone layers (Baulcombe and Buffard, 1983; Chandler et al., 1984; Deikman and Jones, 1986; Nolan and Ho, 1988) or even aleurone cell protoplasts (Arnalte et al., 1991; Huttly and Baulcombe, 1989; Jacobsen and Close, 1991). Nuclei isolated from protoplasts have also been used to study the control of transcription of α -amylase and rRNA genes by GA and ABA (Jacobsen and Beach, 1985; Zwar and Hooley, 1986). The aleurone layers are a convenient system for studying GA- and ABA-mediated gene expression and have several advantages (Ho, 1991): 1) This tissue consists of a homogeneous cell population which responds to both GA and ABA; 2) At least for GA, the source (embryo) and the target tissue (aleurone layers) of the hormone can be physically separated, thus the target tissue can be treated with known concentration of hormones; 3) Many enzymes and proteins are available which can serve as biochemical markers for studies of hormone action; 4) Protoplasts of aleurone cells that still respond to GA can be prepared; 5) Organelles such as nuclei can be more easily isolated from protoplasts than from the intact cells. Both GA and ABA alter the expression of several genes in aleurone layers of cereals, such as barley, wheat and rice. In the last few years, there have been numerous studies on GA-regulated

expression of genes, especially α -amylase gene expression, in aleurone tissues.

2. α -Amylase genes and their expression in aleurone tissues of plants

The breakdown of starch granules in the endosperm of germinating cereal grains is due mainly to the activity of two hydrolytic enzymes, α - and β -amylase. α -Amylase hydrolyzes starch chains internally to produce oligosaccharides or limit dextrins consisting of α -1,4-linked glucose residues. β -Amylase degrades starch from the ends of the molecules to produce maltose (Taiz and Zeiger, 1991). In cereal grains such as barley, wheat and rice, α -amylase is composed of two sets of isozymes of similar size (44 kDa) but with different net charge (Callis and Ho, 1983; Jacobsen and Higgins, 1982; Sargeant, 1980). These isozymes can be classified into two groups, the high and low pI α -amylases, based on their apparent isoelectric point (pI). It has been shown that total α -amylase activity is the result of the combined action of the isozymes encoded by a multigene family (Ho, 1991). GAs induce the synthesis of both α -amylase isozymes in aleurone tissues.

1) Barley and rice

Barley α -amylase genes have been genetically mapped to two chromosomes. From zymograms of α -amylases from wheat-barley chromosome addition lines, Brown and Jacobsen (1982) demonstrated that barley chromosome 6 contained the α -Amy1 locus, the site of genes coding for α -amylases with pIs in the range of 5.7-6.2 (high pI), and that chromosome 1 contained the α -Amy2 locus, the site of genes for α -amylases with pIs in the range of 4.4-5.2 (low pI). Several barley α -amylase cDNA clones have been isolated and sequenced in different laboratories (Table 2). On the basis of DNA sequence analysis, the existence of two types of clones is apparent. Also, a comparison of the amino acid sequence of the native proteins allows correlation of the two types of cDNA clones with the low and high pI groups of α -amylases (Chandler et al., 1984). A comparison of the nucleotide sequences of clone E and pHV19 showed about 80% nucleotide homology in the coding region for the mature polypeptide, but only 55% homology in the region coding for the signal peptide (Chandler et al., 1984). The sequence diversity among α -amylase cDNAs within the same sub-family of α -amylase genes is consistent with previous studies that demonstrated different tryptic peptides and cyanogen bromide cleavage fragments among individual α -amylase isozymes of the same pI group (Callis and Ho, 1983; Jacobsen and Higgins, 1982). By probing Southern blots of DNA from the same addition lines with two divergent α -amylase cDNA clones, it was found that α -amylase sequences on chromosome

6 were related to cDNA clone 103 (high pI) and that those on chromosome 1 were related to cDNA clone E (low pI) (Muthukrishnan et al., 1983).

Using cDNA clones of high and low pI α -amylases as probes, two groups of mRNAs have been distinguished (Deikman and Jones, 1986; Huang et al., 1984; Nolan and Ho, 1988; Rogers, 1985). The transcripts of the two groups of α -amylase genes display different dose and temporal responses to GA (Huang et al., 1984), which parallel the effects of GA measured at the enzyme level (Jacobsen and Higgins, 1982). These observations suggest that the regulation of GA₃ induction of α -amylase isozymes is mainly at the mRNA level. Ho (1991) summarized the results of the GA₃ induced expression of high and low pI α -amylase genes in barley aleurone layers. Before the addition of GA₃ to aleurone layers, the expression of either group of α -amylases was barely detectable. After 2 h of GA₃ treatment, the expression of high and low pI α -amylase was detectable. The expression of high pI α -amylase reached a maximum at around 20 h and then decreased afterwards. In contrast, the synthesis of low pI α -amylase continued to about 40 h after GA₃ treatment. This differential expression of α -amylase isozymes in GA₃-treated barley aleurone layers can be observed at the protein level by analyzing newly synthesized proteins with native gel electrophoresis (Nolan et al., 1987).

Primer extension techniques were recently used to study the regulation of α -amylase mRNA levels both within and between the high and low pI groups in barley. Different aleurone systems, namely isolated aleurone layers, aleurone protoplasts and aleurone from germinated grain, were used (Chandler and Huiet, 1991; Chandler and Jacobsen, 1991). In all aleurone systems the same set of α -amylase mRNAs was produced in response to either applied GA₃ or native gibberellins, indicating that the same set of genes was being expressed in each case. It was also found that there was a coordinate regulation of mRNA levels within the low pI group or within the high pI group, and there were differences between the three aleurone systems when regulation between groups was compared (Chandler and Jacobsen, 1991). The level of α -amylase mRNAs of high and low pI α -amylases in isolated aleurone layers in response to GA₃ was similar to that obtained in previous studies (Deikman and Jones, 1986; Huang et al., 1984; Nolan and Ho, 1988; Rogers, 1985).

From studies on the hybridization of coding sequence probes to blots of genomic DNA, which were digested with restriction enzymes that did not cut within cloned cDNAs, it has been suggested that a family of α -amylase genes exists in barley (Muthukrishnan et al., 1983; Rogers, 1985). Presently, ten α -amylase genomic clones have been isolated from barley and characterized by restriction mapping and

from barley and characterized by restriction mapping and sequence analysis (Table 3). Eight clones contained high pI isozyme sequences and two contained the low pI isozyme sequences. The number and position of introns and exons in both types of α -amylase genes are different. Although the nucleotide sequence of the promoter regions of high and low pI α -amylase genes showed little homology, both contained pairs of inverted repeat elements whose function is not yet clear (Knox CAP et al., 1987). Northern blots of RNA from GA₃-treated and control barley aleurone layers with probes corresponding to the 5' and 3' untranslated regions of genomic clones has indicated differential regulation of the genes (Khursheed and Rogers, 1988; Rogers and Milliman, 1984).

In rice (*Oryza sativa* L.), two full-length α -amylase cDNA clones (pOS103 and pOS137, O'Neill et al., 1990) have been isolated from gibberellic acid-treated embryoless half-seeds using barley α -amylase cDNA clones E (Rogers and Milliman, 1983) and clone I-28 (Deikman and Jones, 1986) as probes. Sequence analysis indicated that the clones encoded polypeptides of approximately 48 kDa, which possessed a signal peptide involved in directing secretion of the protein. A comparison of amino acid sequences of these two α -amylase showed that they were 76% similar to each other, and 85% to 90% similar to other cereal α -amylase genes (pM/C, clone E etc). During rice seed germination, mRNA

corresponding to pOS103 and pOS137 can be detected throughout the 48 h period of seed imbibition (O'Neil et al., 1990). RNA levels, however, were dramatically stimulated by treatment of embryoless half-seeds with exogenous GA₃. Rice α -amylases in germinating seed extracts consist of four distinct isozymes (Daussant et al., 1983). It is not yet known which isozymes correspond to pOS107 or pOS137.

The rice α -amylases are also encoded by multigene families. Thirty distinct genomic clones, representing eight genes, have been isolated and characterized from rice genomic libraries (Huang et al., 1990a). These genes have been classified into five groups on the basis of cross-hybridization and are expressed differentially in germinating seeds and rice callus tissue (Huang et al., 1990b; Simmons et al., 1991). Most of these genes belong to the rice RAmy1 and RAmy3 subfamilies which consist of groups of genes corresponding to the α -Amy1 and α -Amy3 classes in wheat (Lazarus et al., 1985) and barley (Knox CAP et al., 1987). All the α -amylase genes of rice have been mapped to five different chromosomes (chromosome 1, 2, 6, 8 and 9) (Ranjhan et al., 1991). DNA sequence and Southern blot analysis have identified three genes (RAmy1A, RAmy1B and RAmy1C) in Group 1 with DNA sequence identity of at least 90%. RAmy3D of Group 2 is identical to pOS137 cDNA clone (Huang et al., 1990b). RAmy2A, only representative of the α -

Amy2 subfamily, contains the largest intron of all the cereal α -amylase-encoding genes examined to date (Huang et al., 1992). A cluster of three Group 3 genes (RAmy3A, RAmy3B and RAmy3C) has also been characterized (Sutliff et al., 1991). These three genes, within a 28 kb of genomic DNA fragment, are separated from each other by about 5 kb and transcribed in the same direction. In rice, α -amylase genes display tissue-specific expression in which genes RAmy3B, RAmy3C, and RAmy3E are preferentially expressed in the aleurone layer, genes RAmy1A, RAmy1B and RAmy3D are expressed in both embryo and aleurone, and genes RAmy3A and RAmy2A are not expressed in either tissue (Karrer et al., 1991). It was indicated that the α -amylase mRNA detected in the scutellar epithelium was due primarily to RAmy3D gene expression while α -amylase mRNA in the aleurone was due to the expression of the RAmy1A gene (Ranjhan et al., 1992).

In addition, four α -amylase genomic clones, OSamy-a, OSamy-b, OSamy-c, and OSamy-d have been isolated from a rice variety (*Oryza sativa* cv. IR26), and characterized using restriction enzymes and hybridization analysis (Ou-Lee et al., 1988). The nucleotide sequence (Kim and Wu, 1992) of OSamy-c has been compared to several of the α -amylase genomic clones, including RAmy1A and RAmy1B (Huang et al., 1990a, b) from a different rice cultivar, M202. The sequences of OSamy-c and RAmy1A had 83% DNA sequence identity in the region of the first three exons and introns,

but only 68% identity in the fourth exon and no homology in 3' untranslated regions. OSamy-c also showed 90% DNA sequence identity to RAmy1A in the 5' upstream region up to -270 from the transcription start site. These results suggest that OSamy-c is a different gene from RAmy1A and RAmy1B, and they probably belong to the same α -amylase gene subfamily in rice (Kim and Wu, 1992).

2) Wheat

The α -Amy1 (high pI) isozymes in wheat are produced at a high concentration during germination and are controlled by the α -Amy1 gene family on group 6 chromosomes. The second group, present in developing grain as well as during germination, α -Amy2 (low pI), is encoded by the α -Amy2 genes on group 7 chromosomes (Ainsworth et al., 1987; Gale et al., 1983; Lazarus et al., 1985). A third group of α -amylase genes (α -Amy3) is located on the group 5 chromosomes (Baulcombe et al., 1987). α -Amylase cDNA clones (Table 2) of α -Amy1 and α -Amy2 have been isolated and distinguished by restriction enzyme mapping and by cross hybridization (Lazarus et al., 1985). It has been shown that different expression patterns of the two isozymes exist in wheat aleurone cells and developing grain. Studies on the levels of α -Amy1 and α -Amy2 mRNA indicated that the differences in isozyme expression are due to the pattern of mRNA accumulation (Lazarus et al., 1985). In aleurone tissues,

the α -Amy1 transcripts accumulated in parallel with other genes which are regulated by gibberellic acid, while the accumulation of α -Amy2 genes was sustained for 36 h longer (Lazarus et al., 1985). Using probes which were derived from the 3' ends of the cDNA clones and were specific for α -Amy1 and α -Amy2, it was shown that in half grains treated with GA₃ the accumulation of both types of α -amylase mRNAs commenced between 12 and 24 h of incubation, while in half grains incubated without GA₃, the mRNAs for both types of α -amylases could be detected only at very low levels. The α -Amy2 mRNA continued to accumulate for up to 96 h of incubation, but the α -Amy1 mRNA declined after 48 h of incubation (Lazarus et al., 1985). The sequences of α -Amy1 and α -Amy2 cDNA clones have not been published.

Hybridization of α -Amy1 and α -Amy2 cDNA probes to restriction enzyme digests of wheat nuclear DNA revealed that wheat has a multiple α -amylase gene family for both isozymes (Lazarus et al., 1985). Five genomic clones (Table 3) containing α -Amy2 genes have been characterized using DNA sequence analysis and Southern hybridization (Huttly et al., 1988), and found to be differentially expressed. α -Amy2/54 and α -Amy2/8 are expressed in both developing grain and in aleurone cells, while α -Amy2/34, α -Amy2/46 and α -Amy2/53 were only expressed in germinating grain. A comparison of the 5' upstream regions of all five genes showed high similarity, suggesting that regulatory elements responsible

for tissue specificity and gibberellin regulation may be located within these regions of similarity (Huttly et al., 1988). The nucleotide sequence up to -278 between wheat and barley α -Amy2 genes is highly conserved (Huttly et al., 1988). Between wheat α -Amy2/53 and barley Amy32b there was 90% similarity while between barley gKAmy155 and α -Amy2/34 there was 78% similarity. The transcriptional start site was also conserved between wheat and barley. However, comparisons between α -Amy2 genes and α -Amy1 genes of barley or wheat in the 5' upstream regions showed little to no homology despite similarities in their expression (Huttly et al., 1988). α -Amy3, different from α -Amy1 and α -Amy2, has a small multigene family. A genomic clone of α -Amy3, α -Amy3/33, has been identified (Baulcombe et al., 1987). α -Amy3/33 is expressed only in immature grains and unlike the α -Amy1 and α -Amy2 genes, not at all in germinating aleurones. It was suggested that α -Amy3 gene shared a common evolutionary ancestor with the α -Amy1 and α -Amy2 genes (Baulcombe et al., 1987). There is as yet no publication about wheat α -Amy1 genomic clone.

3. *Cis*-elements and *trans*-factors of α -amylase genes

It has been shown that GA increases the level of α -amylase mRNA. This was determined by *in vitro* translation experiment, the use of specific cDNA probes, and primer extension analysis. Based on run-on transcription

experiments using nuclei from barley (Jacobsen and Beach, 1985) and oat aleurone protoplasts (Zwar and Hooley, 1986), it is now clear that both GA and ABA exercise important control at the transcriptional level of α -amylase gene expression in cereal aleurone cells. Since GA and ABA receptors have not been identified, the explanation of how GA and ABA affect transcription of α -amylase genes through experimental approaches is not yet possible. An alternative approach would be first to identify *cis*-acting DNA sequences, which are responsible for mediating the effects of GA and ABA on transcription, within promoters of GA and ABA responsive genes. The approach would be to determine if these sequences interact with *trans*-acting factors which are regulated by GA and ABA. With this approach, it may be possible to provide a basis for working backward to identify the different pathways that could end in a common transcriptional response.

Cis-acting elements may be identified using a number of approaches including DNase I footprinting, *in vivo* footprinting, and by functional analysis of the promoter. Functional analyses of the promoters of α -amylase genes fused to reporter genes using transient expression assays have been initiated in several laboratories to identify gibberellin response elements (GARE) involved in hormone-regulated gene expression. The analyzed promoters (Table 4) are from α -amylase genes of barley, rice and wheat, and the

transient expression of the reporter genes is measured in aleurone protoplasts transformed by PEG method (Gopalakrishnan et al., 1991; Huttly and Baulcombe, 1989; Jacobsen and Close, 1991; Skriver et al., 1991) or by electroporation (Salmenkallio et al., 1990), or in aleurone cells transformed by biolistic method (Kim et al., 1992; Lanahan et al., 1992; see Table 4).

There is a considerable body of evidence which indicates that regulation of gene transcription is controlled by the interaction of *cis*-acting promoter sequences with *trans*-acting DNA-binding proteins (Mitchell and Tjian, 1989; Roeder, 1991). The protein which binds to the wheat ABA response element of the Em gene promoter has been identified as a leucine zipper protein (Guiltinan et al., 1990). The activity of the binding protein was increased by ABA. Also, DNA-binding proteins for α -amylase gene promoters have been detected in aleurones of barley, rice, and wheat, by gel retardation assay. Using the footprint technique, protein binding sites have been identified in promoters of barley Amy32b gene (Sutliff et al., 1992), rice OSamy-c gene (Kim et al., 1992), and wheat α -Amy2/54 gene (Rushton et al., 1992). The information on *cis*-acting elements and *trans*-acting factors of α -amylase genes in barley, rice, and wheat is summarized below.

1) Barley and rice

Skriver et al. (1991) were the first to demonstrate that ABA response element (ABRE) and GA response element (GARE) separately mediated the hormone regulated transcription of a barley high pI α -amylase gene (Amy6-4). A chimeric promoter containing six copies of the sequence from -148 to -128 of the Amy6-4 promoter fused to a minimal 35S promoter was shown to confer GA- and ABA-responsive expression on the reporter gene in barley aleurone protoplasts. The 21 bp sequence of GARE (GGCCGATAACAAACTCCGGCC) contained a conserved motif, TAACAAA, identified in sequence comparisons between α -amylase gene promoters of barley, wheat, and rice (Huang et al., 1990a). The effect on transcription from both ABRE and GARE was orientation-independent, indicating that they functioned as inducible enhancers in their native genes.

Lanahan et al. (1992) reported on the functional analysis of the promoter of an α -amylase gene. Using deletion and mutation analysis of a low pI α -amylase gene (Amy32b) promoter two separate but physically adjacent elements in the promoter, were defined to be essential for GA-induced transcription above a minimal level. Mutation or deletion of either element lowered the transcription to near baseline. One element, GTAACAGAGTCTGG, was very similar to the sequence of GARE defined by Skriver et al. (1991). The other element, called O2S, was very similar to a sequence identified as a binding site for the maize endosperm-

specific transcriptional factor, Opaque-2 (Lohmer et al., 1991). An additional element CCTTTT, which with the O2S forms part of an "endosperm box", was important in modulating the absolute level of expression of the Amy32b promoter similar to another separate, highly conserved element TATCCATGCAGTG. The DNA sequence containing GARE of Amy32b promoter has been shown to be capable of being bound by aleurone nuclear proteins (Sutliff et al., 1992). Footprint analysis indicated that functional *cis*-elements, TAACAGA and TATCCAT, are protein binding sites (Sutliff et al., 1992).

Rogers and Rogers (1992) demonstrated that O2S must be present to allow a single copy of either GARE or ABRE to mediate the hormonal effects in Amy32b α -amylase gene promoter. They considered O2S/endosperm box plus the GARE to be a GA response complex (GARC). O2S and GARE only function together in one direction with respect to each other and with respect to the TATA box. With increasing distance between these elements, the transcription from the promoter is drastically decreased.

Jacobsen and Close (1991) used another high pI α -amylase gene (AmypHV19, unpublished) promoter of barley, and found that the major GA- and ABA-responsive elements occurred between -174 and -41 bp upstream from the transcription initiation site. Furthermore, two boxes within

this region have been defined to play an important role in GA-regulated gene expression (Gubler and Jacobsen, 1992). It was proposed that the TATCCAC box acted cooperatively with the TAACAAA box to give a high level of GA-regulated expression, and that together these motifs formed important components of a GA response complex. The TAACAAA box also appeared to be the site of action of ABA. The above results confirm the proposal by Skriver et al. (1991) that the TAACAAA box played a central role in both GA and ABA regulation of α -amylase gene expression.

In addition, in barley, there have been some other reports (Salmenkallio et al., 1990; Gopalakrishnan et al., 1991) on the studies of transient expression of reporter genes linked to the promoters of high pI or low pI α -amylase genes. However, no additional functional elements have been defined.

Ou-Lee et al. (1988) were the first to report that GA₃ induces the production of a rice aleurone nuclear protein that binds to the upstream sequence of an α -amylase gene. Using gel retardation assay, it was indicated that a 500 bp sequence of a rice α -amylase gene (OSamy-a) specifically interacted with a GA-induced factor from rice aleurone tissues. A 80 bp fragment of the promoter could be protected from exonuclease III digestion by proteins in the aleurone extracts, revealing the approximate position of the protein-

DNA interaction. The binding protein existed only in GA-treated aleurone tissue, but not in leaves or roots of seedlings. Kim et al. (1992) analyzed the regulatory region of the promoter of a rice high pI α -amylase gene, OSamy-c, which was stimulated 20-fold by exogenous GA₃ in deembryonated half-seeds. A sequence which spanned position -231 to +29 of OSamy-c 5' flanking sequence was shown to be sufficient for GA-stimulated expression of the gene. Moreover, gel retardation assays were performed to study protein-DNA interactions between this putative regulatory promoter region and partially purified rice seed extracts. Multiple seed-specific proteins that bound to proximal regions of the OSamy-c promoter between position -231 and -162 were identified. Three protein binding regions were located by footprinting analysis. One of these, CCTTTT located between -211 to -206, was conserved in the upstream sequences of other GA-inducible genes. There was no evidence that these aleurone nuclear proteins binding to OSamy-c promoter required GA induction, a result that was different from the GA-inducible factor binding to OSamy-a promoter reported by Ou-Lee et al. (1988). Thus, it is believed that the mode of regulation of OSamy-a and OSamy-c genes are different (Kim et al., 1992).

2) Wheat

In 1989, Huttly and Baulcombe (1989) demonstrated that a 289 bp sequence of the promoter from a low pI α -amylase gene (α -Amy2/54) was sufficient to direct the synthesis of the reporter gene β -glucuronidase (GUS) in a GA-dependent manner. Furthermore, another detailed functional analysis of this promoter has been described (Huttly et al., 1992). Fusion of 1.8 kb of promoter sequence upstream from -117 bp to a minimal (-55 CaMV 35S) promoter gave rise to the hormone-independent expression, implying that the region 3' to -117 bp contained an element which repressed transcription in the absence of GA or ABA. Meanwhile, mutation analysis of the promoter, containing replacement or deletion, showed that three regions within the 289 bp upstream of the transcriptional initiation site, in addition to the TATA box, contained *cis* elements that were necessary for high-level GA-regulated transcription (Huttly et al., 1992). These regions were located between -68 and -117, -164 and -175, and -241 and -289 from the start of the gene transcription.

Rushton et al. (1992) have shown that the nuclear proteins from oat aleurone protoplasts bound to the α -Amy2/54 promoter regions. Five protected regions (boxes), -109 to -130 (Box 3), -136 to -157 (Box 2), -166 to -185 (Box 5), -242 to -264 (Box 4) and -332 to -349 (Box 1), have been defined by DNase I footprinting. Box 5 (-166 to -185) contained a *cis* element (-164 to -175) which was essential

for α -Amy2/54 transcription (see Huttly et al., 1992). Boxes 2, 3 and 4 are also located in regions which the functional data indicated to be required for α -Amy2/54 high-level GA-responsive expression (Huttly et al., 1992). It is likely that the nuclear proteins that bound to these boxes were involved in transcription of the gene. In the same paper, Rushton et al. (1992) also showed that the promoter of a high pI α -amylase gene (α -Amy1/18) contained the binding sites for oat aleurone nuclear proteins. Each promoter region of either α -Amy1/18 or α -Amy2/54 had at least one binding site with high homology to the cAMP response element and/or phorbol ester response element (Deutsch et al., 1988), suggesting the possible involvement of the bZIP types of transcription factors in the expression of α -amylase genes in aleurone cells. The conserved α -amylase promoter sequence TAACAGA in α -Amy2/54 promoter was also shown to be bound by the nuclear protein. The aleurone DNA-binding proteins above were reported to not be regulated by GA.

Table 2. Isolated α -amylase cDNA clones in plants.

Clone	Insert Size	Tissue	Reference
<u>barley high pI</u>			
clone 103	0.6 kb	aleurone	Huang et al., 1984
pHV19	1.5 kb	aleurone	Chandler et al., 1984
clone I-28	1.5 kb	aleurone	Deikman and Jones, 1985
pM/C	1.5 kb	aleurone	Rogers, 1985
<u>barley low pI</u>			
clone E	1.6 kb	aleurone	Rogers and Milliman, 1983
<u>wheat α-Amy1</u>			
clone 2128	1.5 kb	aleurone	Lazarus et al., 1985
<u>wheat α-Amy2</u>			
clone 4868	1.0 kb	aleurone	Lazarus et al., 1985
<u>rice α-amylase</u>			
pOS103	1.6 kb	seed	O'Neill et al., 1990
pOS137	1.6 kb	seed	O'Neill et al., 1990

Table 3. Isolated α -amylase genomic clones in plants.

α -Amylase Group	Clone	Reference
barley high pI	Amy6-4, Amy46	Khursheed and Rogers, 1988
	gKAmy101, gKAmy104 gKAmy109, gKAmy141	Knox et al., 1987
	gRAmy152, gKAmy56	Rahmatullah et al., 1989
barley low pI	Amy32b	Whittier et al., 1987
	gKAmy155	Knox et al., 1987
wheat α -Amy3	Amy3/33	Baulcombe et al., 1987
wheat α -Amy2	Amy2/54, Amy2/8 Amy2/53, Amy2/34 Amy2/46	Huttly et al., 1988
rice group 1	RAmy1A RAmy1B, RAmy1C	Huang et al., 1990a,b
group 4	RAmy2A	Huang et al., 1992
group 3	RAmy3A RAmy3B, RAmy3C	Sutliff et al., 1991
group 2	RAmy3D	Huang et al., 1990a,b
group 5	RAmy3E	Huang et al., 1990a,b
?	OSamy-a,b,c,d	Ou-Lee et al., 1988
	OSamy-c	Kim and Wu, 1992

Table 4. Studies on *cis*-acting elements of α -amylase genes of plants.

Promoter	Function Region	Reference
<u>barley</u>		
Amy6-4 (high pI)	GARE, ABRE	Skriver et al., 1991
*Amy32b (low pI)	GARE, O2S, CCTTTT and TATCCAT	Lanahan et al., 1992
Amy32b	GARC, ABRC	Rogers and Rogers, 1992
AmypHV19 (high pI)	TAACAAA and TATCCAC	Gubler and Jacobsen, 1992
<u>wheat</u>		
*Amy2/54 (low pI)	Region I,II,III	Huttly et al., 1992
<u>rice</u>		
*OSamy-c	Region I,II,III	Kim et al., 1992

*DNA-protein interactions have been identified in the promoter region.

D. Mechanism of GA action:

GA appears to be involved in regulation of a number of aspects of plant growth and development. Our understanding of GA action at the biochemical and molecular level has been advanced with research utilizing the cereal aleurone system, in which GA-mediated responses involve up- or down-ward regulation of gene expression (Ho, 1991). However, the manner in which GA is perceived by the target cells (aleurone cells) and the events that lead to activation of some genes and inactivation of others are still not known. To date, work on animal hormones has defined two general mechanisms of action: (1) regulation of transcriptional factors by steroid hormone binding (Beato, 1989), and (2) activation of regulatory factors via "second messenger" pathways (Deutsch et al., 1988). In contrast, models of GA action in plants are incomplete, in part because the information on GA receptors is very fragmentary (Srivastava and Sechley, 1991).

There is strong circumstantial evidence for the existence of GA receptors, which comes from the regulation of gene expression in aleurone tissue and in stem elongation, and the structural specificity of the GA molecule required for biological activity (Srivastava and Sechley, 1991). Hooley et al. (1991) demonstrated that GA receptors may be located at the plasma membrane of isolated

aleurone protoplasts of *Avena fatua*. It has been proposed that GA stimulation of α -amylase gene transcription in aleurone protoplasts may involve: 1) perception of GA at the plasma membrane, perhaps by integral GA-receptor proteins; 2) the propagation of this stimulus by an as yet undefined direct or indirect signal-transduction pathway; and 3) the subsequent induction or activation of specific *trans*-acting factors binding to *cis*-acting elements of the α -amylase gene promoter that are involved in the modulation of the gene transcription.

A direct interaction of GAs with DNA has also been reported (Witham and Hendry, 1992). Based on the computer molecular modeling, the GAs and related compounds have been shown to bind via an insertion mechanism to a specific double-stranded DNA dinucleotide between base pairs. This is consistent with standard physical and chemical parameters, and warrants further experimental investigation of the physical interactions of GAs with DNA and the influence of GAs at the transcriptional level, particularly in the aleurone cells of germinating cereal grains (Witham and Hendry, 1992).

An alternative approach to elucidate the mechanism of GA action would be to define the *cis*-acting elements and *trans*-acting factors which are involved in the expression of GA-regulated genes in cereal aleurone tissues. If *trans*-

acting factors that are common to all GA-regulated genes and are regulated by GA can be identified, one could eventually trace back to the early GA-induced events.

GA-insensitive mutants will undoubtedly play an important role in GA receptor research, and in the understanding of signal transduction pathway of GA action. The GA-receptor mutant, if available, would be a valuable tool in the purification of the receptor, in the cloning of the receptor gene, and in the study of the molecular interaction between GA and the receptor.

A thorough understanding of the regulation of α -amylase gene expression at the molecular level will expand our knowledge of GA action in plants. In the present work, aleurone tissues of the normal height wheat Ramona 50 and the dwarf mutant D6899 carrying the *Rht3* allele have been used to study the GA₃ regulation of α -amylase gene expression and the interaction of aleurone nuclear proteins with the promoter of a wheat α -amylase gene (α -Amy2/54).

CHAPTER II

GA₃ REGULATION OF α -AMYLASE GENE EXPRESSION
IN ALEURONE LAYERS OF
NORMAL (RAMONA 50) AND DWARF (D6899, *RHT3* MUTANT) WHEAT

A. Materials and methods:

1. Plant materials

Seeds of standard-height Ramona 50 wheat (*Triticum aestivum* L.) and dwarf D6899 (selection from the hybrid Tom Thumb-Sonora64xTacuari) carrying the *Rht3* gene were kindly provided by Dr. C.O. Qualset, Department of Agronomy, University of California, Davis, CA.

2. Preparation and treatment of aleurone layers

De-embryonated half-seeds of Ramona 50 were soaked in 20% Javex bleach (6% NaOCl) for 10 min and then thoroughly rinsed in sterile distilled water. The half-seeds were placed on two layers of filter paper (Whatman, Maidstone, England) in a 90x15 mm petri dish containing 15 ml of ddH₂O, and incubated at room temperature for 48 h. Aleurone layers were peeled from the half-seeds and incubated in Na-acetate buffer (2.0 mM NaAc, 20 mM CaCl₂, pH 5.5, 15 μM chloramphenicol), with or without GA₃ (Sigma, 1.0 μg/ml) for appropriate times. In some experiments, aleurone layers were imbibed in Na-acetate buffer and preincubated at 5°C or 25°C for 24 h before GA₃ treatment.

3. α-Amylase assay

At appropriate times, the aleurone layers were removed from the incubation buffer, and ground in the aleurone grinding solution (20 mM Tris.Cl pH 8.0, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride-PMSF). The homogenate was combined with the incubation medium, and the mixture was centrifuged at 2,000 x g for 10 min. The supernatant was heated to 70°C for 10 min, placed on ice for 15 min, and centrifuged again. α -Amylase was assayed in the supernatant. A certain volume of supernatant was pipetted to make a final volume of 1.0 ml with α -amylase assay buffer (0.2 M NaAc, 1.0 mM CaCl₂). Starch solution (0.05% potato starch, DIFCO Lab., in α -amylase assay buffer) was added at 1.0 ml, the solution was mixed, and incubated at 37°C for 10 min. The reaction was stopped by the addition of 5 ml I₂-KI solution (0.05% KI, 0.005% I₂, and 0.05 N HCl). α -Amylase activity was determined using a standard curve which was prepared using a commercial barley α -amylase (Sigma) with known activity. One enzyme unit is defined as the amount of enzyme which hydrolyzes 1 mg of maltose from starch in 3 min at 30°C. Results are expressed as units per half layers.

4. Extraction of total RNA from aleurone layers

Aleurone layers were removed from the incubation buffer, blotted dry, frozen in liquid N₂, and ground to a fine powder. The aleurone powder was transferred to a vial containing homogenization buffer (HB buffer=7.5 M guanidine-

HCl, 50 mM Tris.Cl pH7.5, 10 mM EDTA, and 100 mM β -mercaptoethanol) and mixed well. The homogenate was cleared by filtration through Miracloth and then by centrifugation at 10,000 x g for 10 min. Total RNA in the supernatant was precipitated with 10 M LiCl and collected by centrifugation (10,000 x g, 30 min). The pellet was dissolved in HB buffer. The supernatant was extracted with phenol and chloroform twice. Total RNA was precipitated with ethanol and dissolved in TES solution (10 mM Tris.Cl pH 7.5, 1 mM EDTA, and 0.5% SDS). RNA concentration was determined spectrophotometrically and verified by ethidium bromide staining of the agarose/formaldehyde gel.

5. Labelling of cDNAs of barley low-pI and high-pI α -amylase genes

The cDNA clones used, pM/C (Rogers, 1985) and clone E (Rogers and Milliman, 1983) containing 1.6 kb cDNA sequences of barley high-pI and low-pI α -amylase genes were obtained from Dr. J. Rogers at Washington University, School of Medicine, St. Louis, MO. Plasmid DNAs, extracted by the alkaline lysis mini-preparation method (Sambrook et al, 1989), were cut with BamHI and HindIII, and separated by 1.0% LMP (low melting point) agarose gel. The 1.6 kb DNA fragments of α -amylase genes were cut from the gel, extracted with phenol, and radiolabeled with $\alpha^{32}\text{P}$ -dCTP using

the oligolabeling kit from Pharmacia (#27-9250-01). The labeled probes were boiled for 10 min, put on ice for 5 min, and then used for hybridizations.

6. Northern blotting

Total RNA (5 $\mu\text{g}/\text{lane}$) was electrophoretically separated in formaldehyde-agarose gel and blotted onto GeneScreen membrane following the manufacturer's (Dupont) instructions. The baked membrane was prehybridized at 42°C for 3 h in 5x SSPE (1x SSPE=0.18 M NaCl, 10 mM NaH_2PO_4 , pH 7.7, 1 mM EDTA), 50% formamide (v/v), 5x Denhardt's solution (1x Denhardt's solution=0.02% each Ficoll, BSA, polyvinylpyrrolidone), 1% SDS (w/v), and 100 $\mu\text{g}/\text{ml}$ denatured sheared salmon sperm DNA (approximately 50 $\mu\text{l}/\text{cm}^2$). The hybridization was performed at 42°C for 6 h in the hybridization solution (prehybridization solution and 10^6 cpm/ml denatured labeled probe DNAs). The hybridized membrane was washed 3 times in washing buffer (1x SSPE, 2% SDS) at 65°C (30 min each time). The membrane was air dried, and exposed to Kodak X-Omat ARP-K X-ray film with a pair of cronex intensifying screens.

B. Results:

1. Production of α -amylases in isolated aleurone layers of Ramona 50 (normal) and D6899 (*Rht3* mutant)

The effect of GA₃ on α -amylase activity was initially determined in the isolated aleurone layers of normal wheat, Ramona 50. Results are shown in Fig. 2A. The level of α -amylase activity of Ramona 50 is clearly dependent on added GA₃ (1.0 μ g/ml). After 48 h incubation, the total α -amylase activity in the GA₃-treated aleurone layers and secreted into the incubation buffer was at least 20-fold higher than in the GA₃-untreated control. Total α -amylase activity increased up to 96 h of incubation. In the GA₃-treated aleurone layers of D6899 (*Rht3* dwarf mutant), however, the amount of α -amylase produced was about 10-fold lower than that in Ramona 50 after 48 h of incubation. In the absence of GA₃ the α -amylase activities in isolated aleurone layers of Ramona 50 or D6899 were barely detectable.

2. Effect of low temperature on GA₃-induced α -amylase activity in D6899

The aleurone layers of Ramona 50 and D6899 were preincubated in Na-acetate buffer at 5°C or 25°C for 24 h, and then treated with 1 μ g/ml GA₃ at room temperature for further periods of 24, 48, or 72 h. The production of α -

amylase in aleurone layers was determined after GA₃ treatment (Fig. 2B). The 5°C pretreatment of aleurone layers prior to the addition of GA₃ for 24 h produced a slight increase in GA₃-induced α -amylase activity in both Ramona 50 and D6899 (Fig. 2B). There was no significant difference in the α -amylase production when the aleurone layers were preincubated at 2°C or 5°C, and for 12, 24, or 36 h (data not shown). In no case, however, was the block to GA-induced α -amylase production removed in D6899 aleurone layers.

3. The transcription of α -amylase genes in aleurone layers of Ramona 50 and D6899

The levels of α -amylase mRNAs in total RNA extracted from GA₃-treated or untreated aleurone layers of Ramona 50 and D6899 after different incubation times were determined using the technique of RNA/gel blotting and a mixture of a high pI and a low pI α -amylase cDNA clones from barley (Fig. 3). In Ramona 50 aleurone layers, the α -amylase mRNA accumulation commenced within 4 h incubation with GA₃ and continued to increase up to 48 h of incubation (Fig. 3). No significant level of α -amylase mRNA was detectable at any time during incubation of D6899 aleurone layers. The aleurone layers without GA₃ treatment from both Ramona 50 and D6899 did not produce significant amounts of α -amylase mRNA at 48 h. These data indicate that in D6899 the low α -amylase activity in GA₃ treated aleurone layers at 48 h was

due to a low rate of α -amylase mRNA synthesis, rather than the inactivation of α -amylase or the mRNA translation.

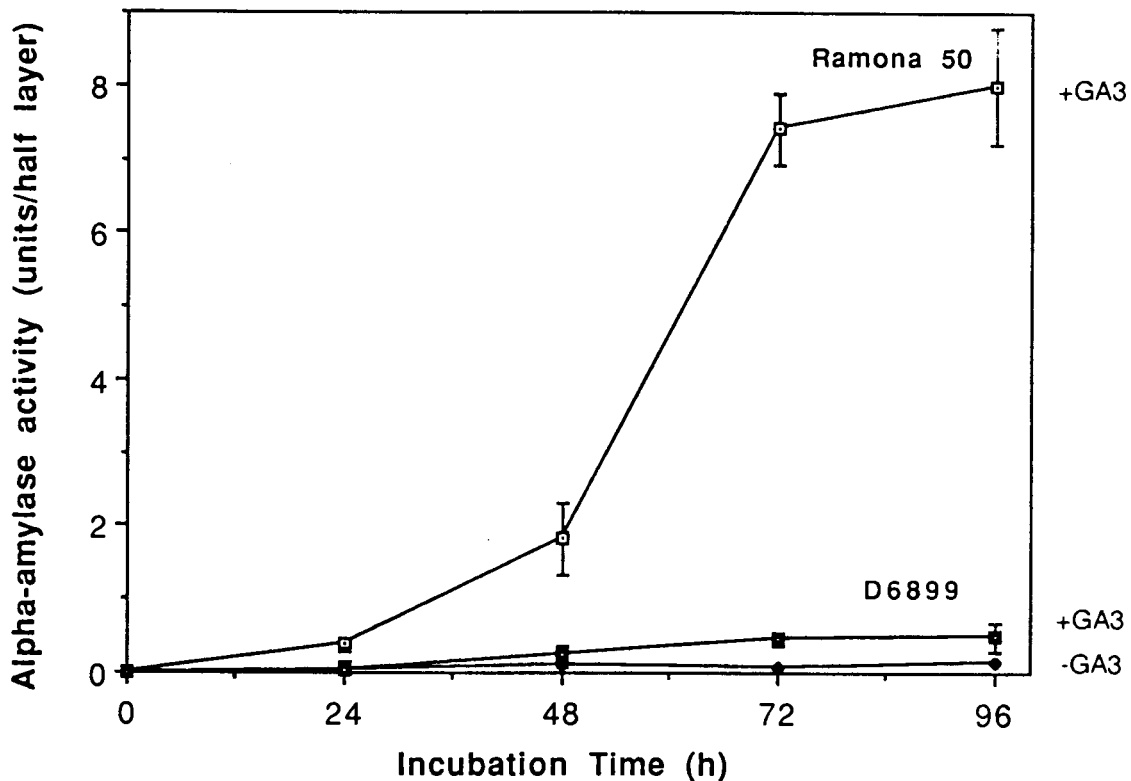


Fig. 2A. Time course of α -amylase production by isolated aleurone layers of Ramona 50 and D6899. α -Amylase was assayed at the end of incubation periods of different duration with (+) or without (-) 1.0 $\mu\text{g/ml}$ GA_3 at 25°C. Potato starch was the substrate, and the α -amylase produced was expressed relative to units of barley malt α -amylase. Each value represents a mean of three replicates.

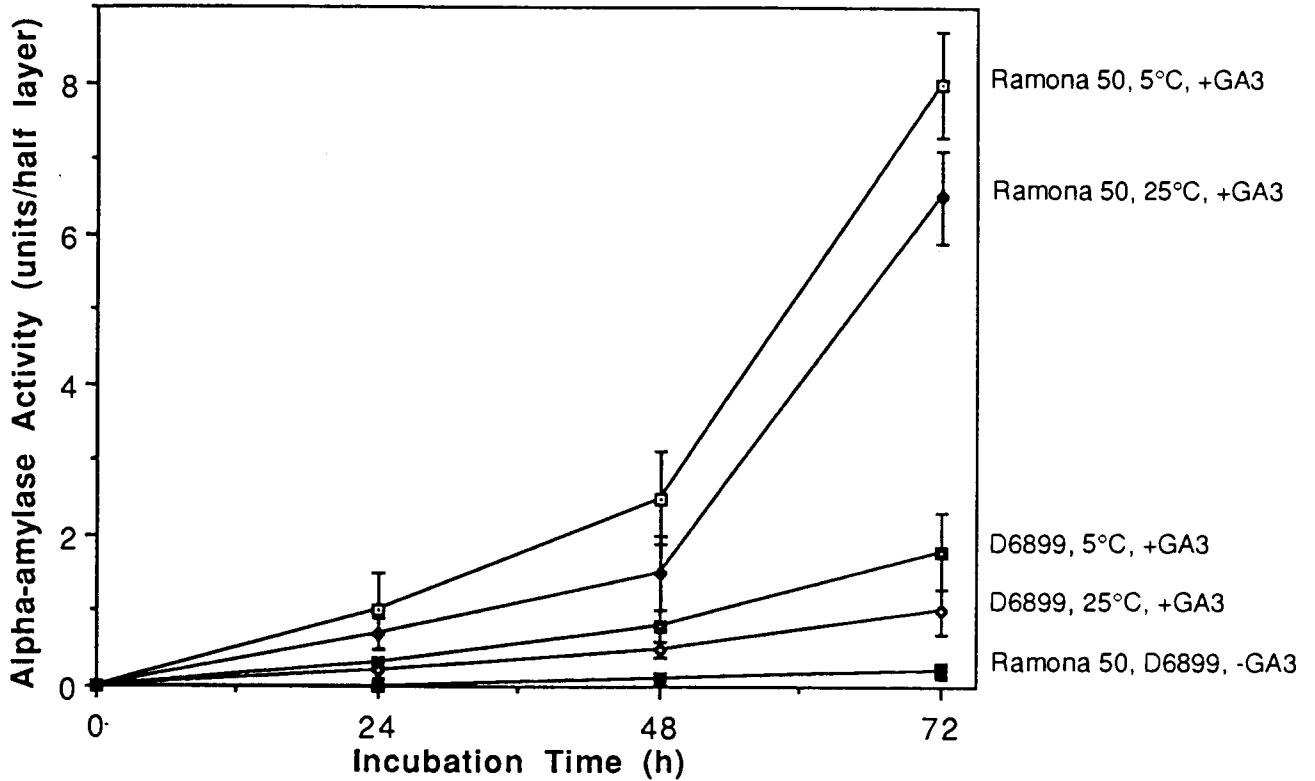


Fig. 2B. Comparison of the α -amylase production in isolated aleurone layers with the preincubation at 5°C and 25°C. Aleurone layers of Ramona 50 and D6899 were pretreated at 5°C or 25°C for 24 h and then treated with 1.0 $\mu\text{g/ml}$ GA₃ at room temperature. The α -amylase activity was determined at the end of incubation periods of 0, 24, 48, 72 h with (+) or without (-) GA₃.

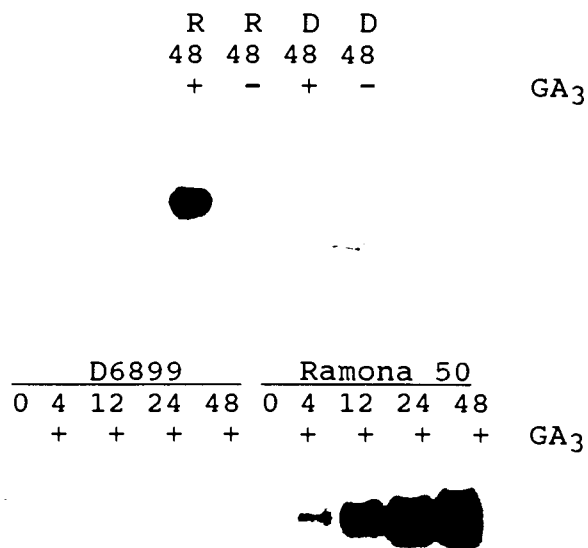


Fig. 3. Northern blots of aleurone RNA. The total RNA (5.0 μ g per lane) was isolated from Ramona 50 (R) and D6899 (D) aleurone layers incubated with (+) or without (-) GA₃ for different times (0-48 h). A mixture of barley cDNA fragments of high-pI and low-pI α -amylase genes was used as the probe.

C. Discussion:

There is considerable homology within the coding regions of both the high-pI and low-pI α -amylase cDNA sequences of barley and those of wheat (Huang et al., 1992; Sutliff et al., 1991). For this reason, barley cDNA clones could be used in this study to determine the transcriptional regulation of α -amylase genes in wheat aleurone layers. Using these probes, it was shown that the blockage to α -amylase production in *Rht3* wheat was at the α -amylase mRNA level. However, these hybridization results do not discriminate between the activity of individual high-pI or low-pI α -amylases. Sequence specific probes from wheat or barley need to be used for RNA/gel blotting, which were not available in this study.

Hetherington and Laidman (1991) reported that the aleurone tissue of the *Rht3* line (April Bearded variety) responded to GA₃ by producing α -amylase, after a lag period of 20 h compared to that for the *rht3* (tall) line. In the present study, however, no such lag period was observed for the *Rht3* line (D6899) compared to Ramona 50 (*rht3* line). D6899 aleurone layers produced very little α -amylase up to 96 h of GA₃ incubation. These data are similar to those published using the same *Rht3* line (Ho et al., 1981). The results on the effect of cold temperature pretreatment of aleurone layers in D6899 prior to the addition of GA₃ differ

from those of Singh and Paleg (1984a) using *Rht3* lines from a Cappelle Desprez/Minister cross. They reported that low temperature pretreatment could induce GA₃ sensitivity in aleurone tissues of *Rht3* lines (Tom Thumb and Tordo) in terms of α -amylase production. It is possible that genotypic differences in the *Rht3* lines could account for the difference in results from that of Hetherington and Laidman (1991) or that of Singh and Paleg (1984a), making it difficult to find the blockage site of GA-insensitivity in the *Rht3* dwarf wheat.

D. Conclusions:

1. GA₃ induced the production of α -amylase in the aleurone tissue of Ramona 50 (normal). The GA₃ induction of α -amylase was partly blocked in the aleurone tissue of D6899 (*Rht3*).

2. A 5°C pretreatment of aleurone layers before the GA₃ incubation increased slightly the GA₃-induced α -amylase activity in both Ramona 50 and D6899.

3. GA₃ induced the production/accumulation of α -amylase mRNA in the aleurone tissue of Ramona 50. However, in D6899 there was very little α -amylase mRNA after 48 h GA₃ treatment. The block appears to be at some step prior to gene transcription, but the possibility of α -amylase mRNA degradation can not be excluded.

CHAPTER III

INTERACTION OF ALEURONE NUCLEAR PROTEINS
WITH PROMOTER REGIONS OF
A WHEAT α -AMYLASE GENE (α -AMY2/54)

A. Materials and methods:

1. Plant materials

The source of Ramona 50 and D6899 seeds is given in Chapter II.

2. Purification of primers for PCR amplification and DNA sequencing

Two oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (Courtesy, Institute of Molecular Biology and Biochemistry, Simon Fraser University) and purified using C-18 Sep-Pak cartridges (#51910, Waters Associates, Boston, MA) following the manufacturer's instructions. One 25 base oligonucleotide primer (primer B) corresponds to nucleotides -318 to -294 of α -Amy2/54 5' upstream region. The second primer (primer A) is the complement of a 27 base sequence corresponding to nucleotides +72 to +98, relative to the start point of transcription (Fig. 5a).

3. PCR amplification and cloning of α -Amy2/54 promoter (R_{BA})

PCR amplification with total DNA isolated from Ramona 50 seedlings was performed in a TwinBlock Thermal Cycler (Ericomp). The amplification reaction of 50 μ l consisted of

0.2 μg DNA, 200 μM each dNTP, 10 mM Tris.Cl pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 0.5 μM primers, and 2.5 units Taq polymerase (BRL). The DNA denaturation was set at 95°C for 1 min, the primer annealing at 60°C for 1 min and the primer extension at 70°C for 1.5 min. The reaction was allowed to run for 35 cycles before a final 10 min primer extension at 70°C. The 417 bp PCR product was gel purified, and ligated to pUC19 T-vector. The cloning followed the procedures described by Marchuk et al. (1990).

4. DNA sequencing

The PCR amplified DNAs were gel-separated on low melting point agarose gel, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in TE buffer. The sequences were determined by double-strand dideoxynucleotide sequencing using two primers for the PCR. The sequencing protocol used was the Sequenase Version 2 (USB) modified by Casanova et al. (1990).

5. Extraction of nuclear proteins from aleurone layers

The preparation of nuclear extracts was carried out essentially according to the method of Staiger et al. (1990) with some modifications. The aleurone layers were collected at appropriate times after being incubated with or without GA_3 (1.0 $\mu\text{g}/\text{ml}$) and homogenized by tissue grinder in cold

Buffer A (25 mM Mes-NaOH pH 6.0, 0.25 M sucrose, 5 mM EDTA, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM spermidine) supplemented with 0.5% Triton X-100. The suspension was homogenized with a glass bar for 30 sec, filtered through 100 μ m and 40 μ m nylon meshes, and centrifuged for 10 min at 2,000 x g. The crude nuclear pellet was washed once in Buffer A, loaded on 80% percoll in Buffer A, and centrifuged for 5 min at 4,000 x g. The floating nuclei on 80% percoll were collected and diluted with 3 volumes of Buffer A. The nuclei were pelleted by centrifugation at 3,000 x g for 10 min, washed once, and resuspended in Buffer B (25 mM HEPES-NaOH pH 7.8, 5% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 420 mM KCl). The nuclear proteins were extracted by stirring on ice for 1 h. The lysate was clarified at 100,000 x g for 30 min. Ammonium sulfate was added to the supernatant to 70% concentration at 0°C, left for 30 min and the precipitated protein was collected by centrifugation at 10,000 x g for 10 min, and suspended in Buffer C (25 mM HEPES-NaOH pH 7.8, 20% glycerol, 0.1 mM EDTA, 50 mM KCl, 14 mM β -mercaptoethanol). The final protein concentration was determined using the Bio-Rad Protein Assay Kit (#500-0001) and bovine serum albumin as a standard. The extract was rapidly frozen in liquid nitrogen and stored at -80°C. The nuclear protein prepared in this manner was good for about 3 months. The yield of the protein ranged between 5-10 μ g/aleurone layer.

6. Preparation of whole cell extracts from roots and leaves of etiolated wheat

Roots and leaves were harvested from etiolated wheat (Ramona 50) seedlings grown for 7 days in complete darkness. The tissues were rinsed with cold distilled water, and then blended in cold Extraction Buffer (40 mM Tris.Cl pH 7.5, 5 mM MgCl₂, 0.5 M sucrose, 10 mM β -mercaptoethanol, 1.0 mM PMSF) in a Waring blender for 30 sec (10 g in 50 ml). The slurry was filtered through 100 μ m mesh filter. To the filtrate, 0.1 volume of 5 M NaCl was added and the mixture was gently agitated for 1 h on ice. The suspension was centrifuged at 100,000 x g for 1 h. Ammonium sulfate was added to the supernatant (0.3 g/ml) while 0.1 ml of 1 M NaOH/10 g ammonium sulfate was also added to keep the pH balanced. After stirring at 4°C for 30 min, the extract was precipitated by centrifugation at 10,000 x g for 10 min. The pellet was resuspended in Buffer C (as before). The concentration of proteins was determined in the same manner as described before. The extracts were aliquoted, quickly frozen in liquid nitrogen, and kept at -80°C. A total of 1.0 mg or 5.0 mg proteins could be obtained from 10 g roots or leaves, respectively.

7. Preparation of DNA probes for gel retardation studies

Three DNA probes, R_{BA} , R_U , and R_T , were used for gel retardation assays (Fig. 5c). R_{BA} , a 442 bp EcoRI-BamHI restriction DNA fragment from the plasmid, was gel purified, and labeled with $\alpha^{32}P$ -dATP (3000 Ci/mmol, Amersham) using the klenow fragment of DNA polymerase (Bandshift Kit, #27-9100-01, Pharmacia). The ^{32}P -labeled DNA was purified by Nick Spin Column (#17-0862-01, Pharmacia) according to the manufacturer's recommendation. R_U (146 bp) and R_T (291 bp) were generated from R_{BA} after digestion of labeled R_{BA} with NcoI (Fig. 5c). Labeled R_U and R_T were gel-separated and purified as described above.

8. Gel retardation assays

Binding reactions were carried out at 25°C for 30 min in a total volume of 25 μ l containing 40 mM Tris.Cl pH 7.5, 200 mM NaCl, 8% glycerol, 2 mM DTT, 4 mM MgCl₂, 1 mM EDTA, 1mM PMSF, 0.04% NP-40, nuclear protein, labeled DNA fragment, and 1 μ g of poly(dI-dC) or calf thymus (ct) DNA as a nonspecific competitor. The amounts of DNAs and proteins in the reactions are described in the Results. Competition reactions were conducted with various competitors added in the amounts described in the text. After incubation, 2.5 μ l of loading dye (250 mM Tris.Cl pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol) was added to each reaction. All samples were loaded on 5% polyacrylamide gels (30:0.8, acrylamide:bisacrylamide) in Tris-glycine

solution (40 mM Tris.Cl pH 8.5, 190 mM glycine and 1 mM EDTA), which had been pre-run at 5 V/cm for 30 min. Electrophoresis was performed at 10 V/cm for 4-6 h at 4°C. After electrophoresis, the gels were dried and exposed to X-ray film with a pair of intensifying screens at -80°C for 16-48 h.

II. Results:

1. α -Amy2/54 promoter sequences of Ramona 50 and D6899

In order to study the interaction of *cis*-acting promoter sequences with *trans*-acting DNA binding proteins, a 417 bp, -318-+98 fragment of the α -Amy2/54 promoter (Fig. 4) was synthesized using the PCR technique. The sequences of double-stranded PCR products using the total DNA from Ramona 50 and D6899 were determined with Sequenase Version 2.0, DNA Sequencing Kit (#70770, USB). The readable sequences of the PCR products of Ramona 50 and D6899 were exactly the same as reported for the α -Amy2/54 promoter from -280 to +60 (Fig. 4).

2. Binding of aleurone nuclear proteins to R_{BA}

After the 417 bp of α -Amy2/54 promoter DNA was cloned into pUC19 plasmid, a EcoRI-BamHI restriction DNA fragment (442 bp, R_{BA}) (Fig. 5) could be easily prepared, and labeled with $\alpha^{32}\text{P}$ -dATP by filling in with klenow enzyme. Since the accumulation of α -amylase mRNA in Ramona 50 aleurone layers reached the highest level after 48 h of GA₃ incubation (see Fig. 2), the nuclear proteins used for DNA-protein interactions were prepared from aleurone layers treated with GA₃ (1 $\mu\text{g}/\text{ml}$) for 48 h. The 0.5 ng of $\alpha^{32}\text{P}$ -labeled R_{BA} was incubated with 8.0 μg of the aleurone nuclear proteins and

was then subjected to electrophoresis in non-denaturing gel. When 1.0 μg of calf thymus (ct) DNA or poly(dI-dC) was included in the binding reaction as the non-specific competitor, different patterns of retarded DNA bands were obtained. Assuming that a band at the same retarded position was caused by the same DNA-binding protein(s), a total of eight retarded bands with different mobilities were detected (Fig. 6). Band B1 or B4a was the major DNA-protein complex when ct DNA or poly(dI-dC) was used as the non-specific competitor, respectively. Other retarded bands, B2, B3, B4b, B5, B6, and B7 were also observed. B2 and B3 could not be distinguished when poly(dI-dC) was used as the non-specific competitor. The intensities of the complexes increased in proportion to increases in the amount of the nuclear protein (Fig. 7). There was no binding activity with bovine serum albumin (data not shown). Treatment of the aleurone nuclear extract with proteinase K (50 $\mu\text{g}/\text{ml}$, 37°C for 15 min) or heating the extract (100°C, 5 min) resulted in the loss of DNA binding activities (data not shown), indicating that proteins were involved in the retarded bands.

3. Accumulation of DNA binding proteins during GA₃ incubation

Equal amounts (8.0 μg) of nuclear proteins from Ramona 50 aleurone layers treated with GA₃ for 0, 12, 24 and 48 h were incubated with the labelled R_{BA} (0.5 ng), together with

1.0 μg ct DNA or poly(dI-dC) as the non-specific competitor (Fig. 8). In both cases, very low levels of intensities of the complexes were obtained with nuclear proteins prepared from aleurone layers at 0 h or upto 24 h of GA₃ treatment. The intensities reached relatively high levels at 48 h after GA₃ treatment. However, the nuclear proteins prepared from aleurone layers which had been incubated for 48 h in the control without GA₃ also showed similar complex intensities (data not shown). This suggests that the increase in the intensities of the complexes may be a hydration effect.

4. Binding specificity of B4a complex

B4a was the strongest retarded band when poly(dI-dC) was used as the non-specific competitor (Fig. 6). A competition experiment using unlabeled R_{BA} DNA and unrelated DNA (sonicated calf thymus DNA, the size range varied from 200 bp to 6 kb) at increasing concentrations was carried out to investigate the specificity of the DNA-protein interaction in the B4a complex. As shown in Fig. 9, the B4a complex was partially competed for by 0.5 μg unlabeled R_{BA} (0.25 ng labeled R_{BA} in binding reactions), whereas only 0.075 μg of ct DNA could completely eliminate the formation of the B4a complex. Since the ct DNA could eliminate the formation of the B4a complex more efficiently than unlabeled R_{BA}, it was concluded that the protein(s) responsible for

forming the B4a complex showed non-sequence-specific binding to R_{BA}.

5. Specificities of the interactions between aleurone nuclear proteins and R_T or R_U

As shown in Fig. 6, when ct DNA was included in the DNA-protein binding reaction as the non-specific competitor, a total of seven retarded bands, B1, B2, B3, B4b, B5, B6, and B7, were obtained with the 442 bp R_{BA} probe. To obtain a better resolution of these DNA-protein complexes, shorter DNA fragments were used, which are likely to contain fewer protein binding sites and thus may generate a less complicated gel shift. To do so, the labeled R_{BA} (442 bp) was digested with NcoI restriction enzyme, and two one-end labeled DNA fragments, R_U (146 bp) and R_T (291 bp), were generated (Fig. 5c). R_U contains the α -Amy2/54 promoter sequences from -318 to -178, and R_T contains a 279 bp DNA fragment (-173 to +98) from the α -Amy2/54 promoter, including the TATA box (-31--24). Incubation of Ramona 50 aleurone nuclear proteins with labeled R_U resulted in the formation of one retarded band (Fig. 10II), whereas seven retarded bands were detected for R_T (Fig. 10III).

The specificity of DNA-protein binding interactions was assessed by including unlabeled R_{BA} DNA and unrelated DNA (440 bp DNA fragment of LPS1 gene of sea urchin, courtesy of

Dr. Brandhorst's lab., IMBB, SFU) in the binding reactions. As shown in Fig. 11, the intensities of the retarded DNA bands decreased in proportion to increasing amounts of the unlabeled competing DNAs. R_{BA} and LPS1 DNAs reduced the formation of the single complex of the aleurone nuclear proteins with R_U DNA with a similar competition ability (Fig. 11A). For the other probe R_T , most DNA-protein complexes were competed for by the addition of a 400-fold molar excess of unlabeled R_{BA} or LPS1 DNAs except for one complex as indicated by the arrow in Fig. 11B. This complex was completely eliminated by a 200-fold molar excess of the unlabeled R_{BA} , but a 400-fold molar excess of LPS1 still could not outcompete this complex. Thus, the protein(s) responsible for forming this complex may be specific for R_T DNA sequence. Other retarded DNA bands for R_U and R_T appear to be non-sequence-specific interactions between the aleurone nuclear proteins and the 442 bp segment of the α -Amy2/54 promoter DNA.

6. The binding of Ramona 50 nuclear proteins from GA_3 -treated and untreated aleurone layers to R_T

During cereal seed germination, endogenous GA_3 , believed to be synthesized by the embryo, stimulates α -amylase gene expression in aleurone tissue (Fincher, 1989). The data in Fig. 2 and 3 clearly demonstrate that α -amylase gene expression in isolated aleurone layers of Ramona 50 is

induced by exogenous GA₃. It has been suggested that in rice GA₃ may induce, or activate, the binding of a rice tissue-specific nuclear factor to an α -amylase gene promoter (Ou-Lee et al., 1988). Therefore, it was interesting to investigate the DNA-protein interactions of R_T with the nuclear proteins from GA₃-treated, and untreated aleurone layers. When equal amounts (4.0 μ g) of nuclear proteins from Ramona 50 GA₃-treated and untreated aleurone layers were used in gel retardation studies for labeled R_T probe, no apparent difference was observed (Fig. 12). This suggests that the proteins in the aleurone layers that interact with R_T DNA to form DNA-protein complexes do not require GA₃ induction.

7. Comparison of DNA-protein interactions between Ramona 50 and D6899

The GA₃-induced transcription of α -amylase genes was blocked in D6899. No difference was found in DNA sequences (-280 to +60) of the α -Amy2/54 promoter between PCR products of Ramona 50 and D6899. To determine whether or not D6899 contained the DNA binding proteins detected in Ramona 50, the nuclear proteins from D6899 aleurone layers treated with or without GA₃ were incubated with R_T. The DNA-protein interactions in Ramona 50 and D6899 were compared. When 4.0 μ g of nuclear proteins were included in the binding reactions, all the retarded bands obtained with Ramona 50

nuclear proteins were also present with D6899 proteins, and their intensities were similar (Fig. 12).

8. Binding activity of cellular extracts from roots and leaves of etiolated Ramona 50 seedlings to R_T

α -Amy2/54 gene is expressed in aleurone cells in germinating seeds. None of the α -amylase genes (α -Amy1 or α -Amy2) is believed to be expressed in roots or in leaves of young wheat plants (Huttly et al., 1988). Fig. 13 shows that cell extracts from roots and leaves of etiolated Ramona 50 did not bind to R_T as the aleurone nuclear proteins did. When 4.0 μ g or 20.0 μ g extract of roots was included in the binding reaction, no retarded R_T band was found. One retarded band showed up when 4.0 μ g of leaf extract was included in the binding reaction, and the intensity of that band was increased by increasing the amount of the extract (Lane L in Fig. 13). This extra band detected in Ramona 50 leaves could be the result of an interaction of R_T with another binding protein which does not exist in the aleurone cells.

-318
TCTTGCTCCATGTTGT

-250

TCAATTCATTTCTCCCATCTTTCTTCGCGCCCATGCTTATTTCCCATTGGAAAATCGG

-200

TTATCCAAAAGGCAAGGGCAACGCTGGGTTTCGATCCCAACTTGATGGTGCCATCTTT

-150

TTCCATGGAGTTTGTGCCACCCGGATTGACTTGACCGTCATCGGCTGCACCTTTTTTCG

-100

TAACAGAGTCTGGTATCCATGCAGTGCCTCCAAGCAACACACTCTACGGTACGTAGCTCG

-50

CGTTAAATACTGCCATGCCATCCACTGCCTTATAAATACCAAGCACGCGGGACACTTGTGG

0 +50

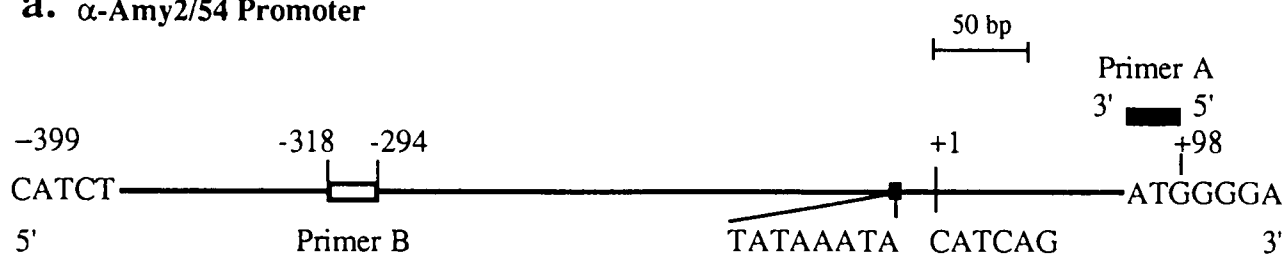
CCATCAGTCGATCAGCCAGTCAGCCAATCATCCATCCATCCGGAGAAGAAGAAGAGTCTA

CTGTGCTGTGGGATCGACACTAECGTAGCGTACGCGATG

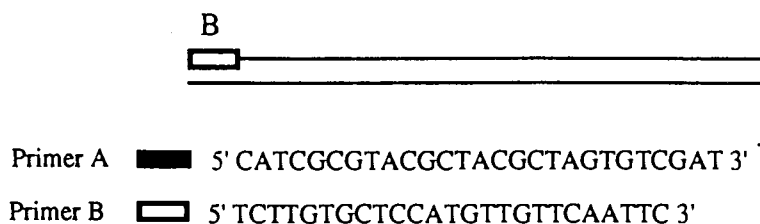
+98

Fig. 4. The 417 bp nucleotide sequence of the 5' upstream region of α -Amy2/54 from -318 to +98 (Huttly et al., 1988). The nucleotide sequence is numbered from the start of transcription at position 0. A potential TATA box motif and the start (ATG) of translation are underlined.

a. α -Amy2/54 Promoter

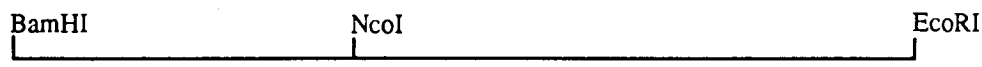


b. PCR Product



c. RBA

(442 bp)



RU

(146 bp)



RT

(291 bp)

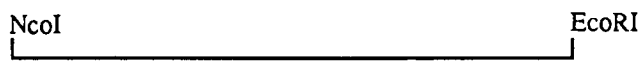


Fig. 5. DNA probes for gel retardation assays.

a. Schematic representation of the 5' upstream region of α -Amy2/54 gene. The construct containing the DNA fragment of 399 bp (-399-0) as transcriptional fusions to GUS retained in the presence of GA₃ 50% of the expression from the full-length (~2 kb) construct in transformed oat aleurone protoplasts (Huttly et al., 1989). Numbers above DNA indicate the positions relative to the transcription initiation (+1). The potential TATA box is indicated. Bars represent the primers (closed-A, open-B) for PCR amplification.

b. PCR product, 417 bp (-318+98 of α -Amy2/54), obtained from wheat genomic DNA using primer A and primer B.

c. R_{BA}, 442 bp of the EcoRI-BamHI restriction DNA fragment from the plasmid, contains α -Amy2/54 5' upstream region from -318 to +98. R_U (146 bp) and R_T (291 bp) were obtained by NcoI digestion of R_{BA}.

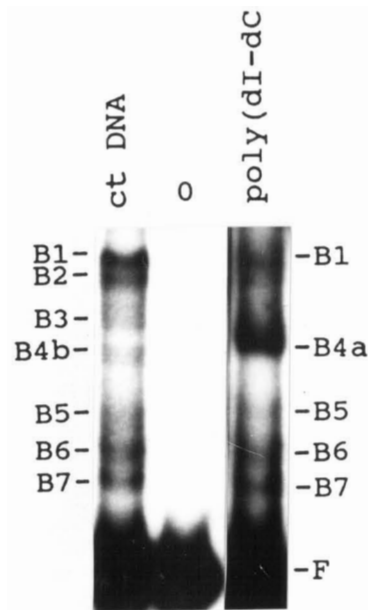


Fig. 6. Binding patterns of the aleurone nuclear proteins to labeled R_{BA} DNA. The binding reaction included $8.0 \mu\text{g}$ protein, 0.5 ng labeled R_{BA} DNA, and $1.0 \mu\text{g}$ of ct DNA or poly(dI-dC). 0, control without proteins. F, free labeled R_{BA} DNA. The retarded bands were denoted B1, B2, B3, B4a, B4b, B5, B6, and B7.

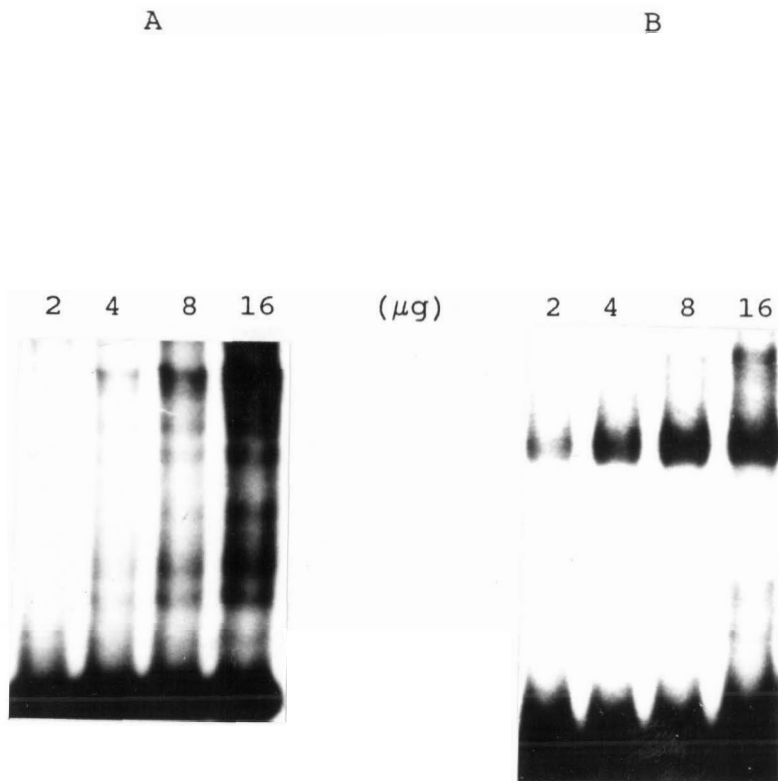


Fig. 7. Binding of nuclear proteins from Ramona 50 aleurone layers to labeled RBA. The labeled RBA (0.5 ng, 30,000 cpm) was incubated with increasing amounts (4, 8, 16 μg) of nuclear proteins prepared from aleurone layers after 48 h of GA₃ treatment. A, ct DNA as non-specific competitor. B, poly(dI-dC) as non-specific competitor.

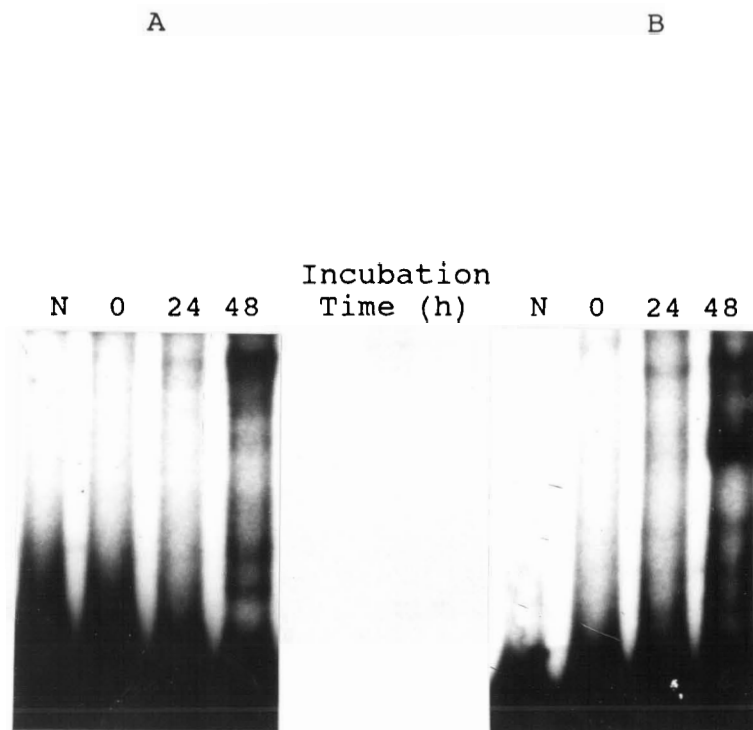


Fig. 8. Accumulation of R_{BA} binding proteins during GA₃ incubation. Nuclear proteins were isolated from aleurone layers treated with GA₃ for various time (0, 24, 48 h). 8.0 μg of the protein was incubated with 0.5 ng of labeled R_{BA} DNA except for lane N where no protein was added. A, ct DNA as non-specific competitor. B, poly(dI-dC) as non-specific competitor.

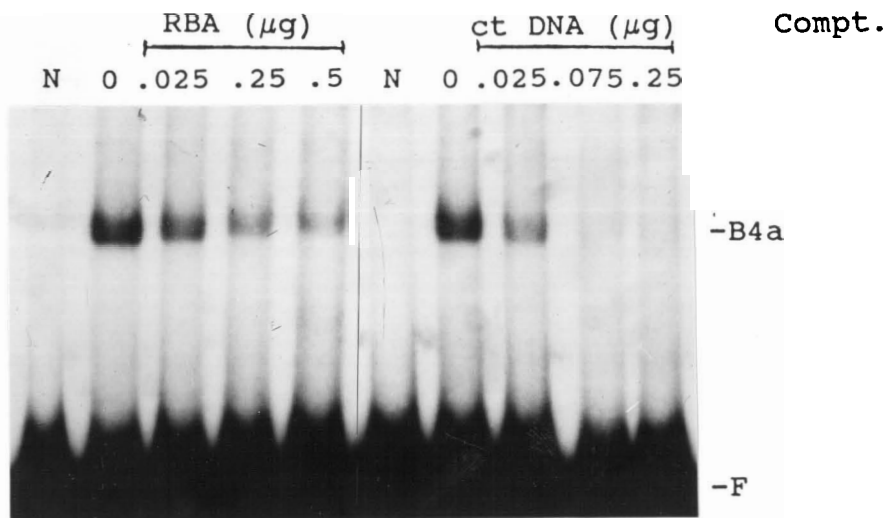


Fig. 9. Competition analysis of B4a complex. In each binding reaction, 0.25 ng (15,000 cpm) of ^{32}P -labeled RBA fragment was incubated with 4.0 μg of nuclear proteins from Ramona 50 aleurone layers treated with GA_3 for 48 h, and 1.0 μg poly(dI-dC) was included. Unlabeled RBA and ct DNA (unrelated DNA) were included in the binding reactions as competing DNA (Compt.), respectively. The amounts (μg) of competitors are shown above the lanes. N, no nuclear proteins added. 0, no competitor added. F, free labeled RBA DNA. B4a complex is indicated.

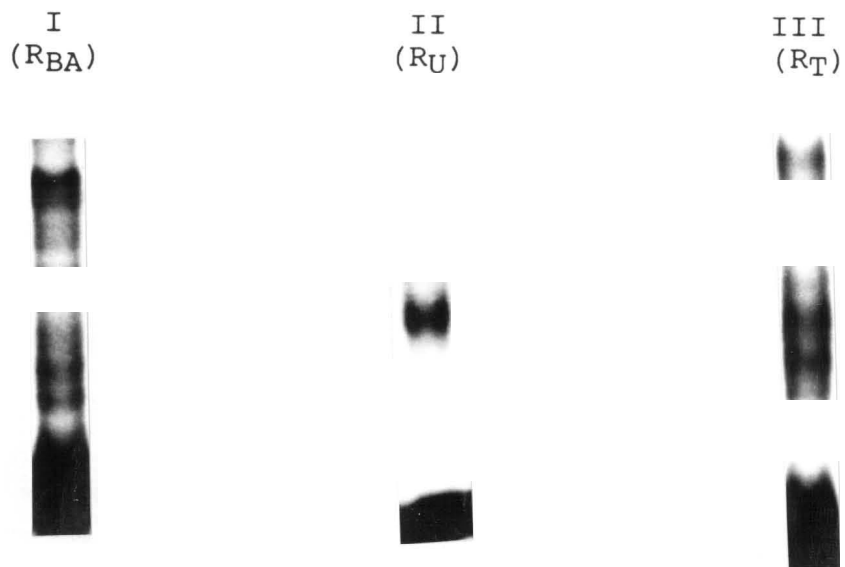


Fig. 10. Bindings of aleurone nuclear proteins to labeled R_{BA} (I), R_U (II), and R_T (III). Labeled DNAs (15,000 cpm) were incubated with 8.0 μg nuclear proteins and 1.0 μg ct DNA was used as non-specific competitor in each binding reaction.

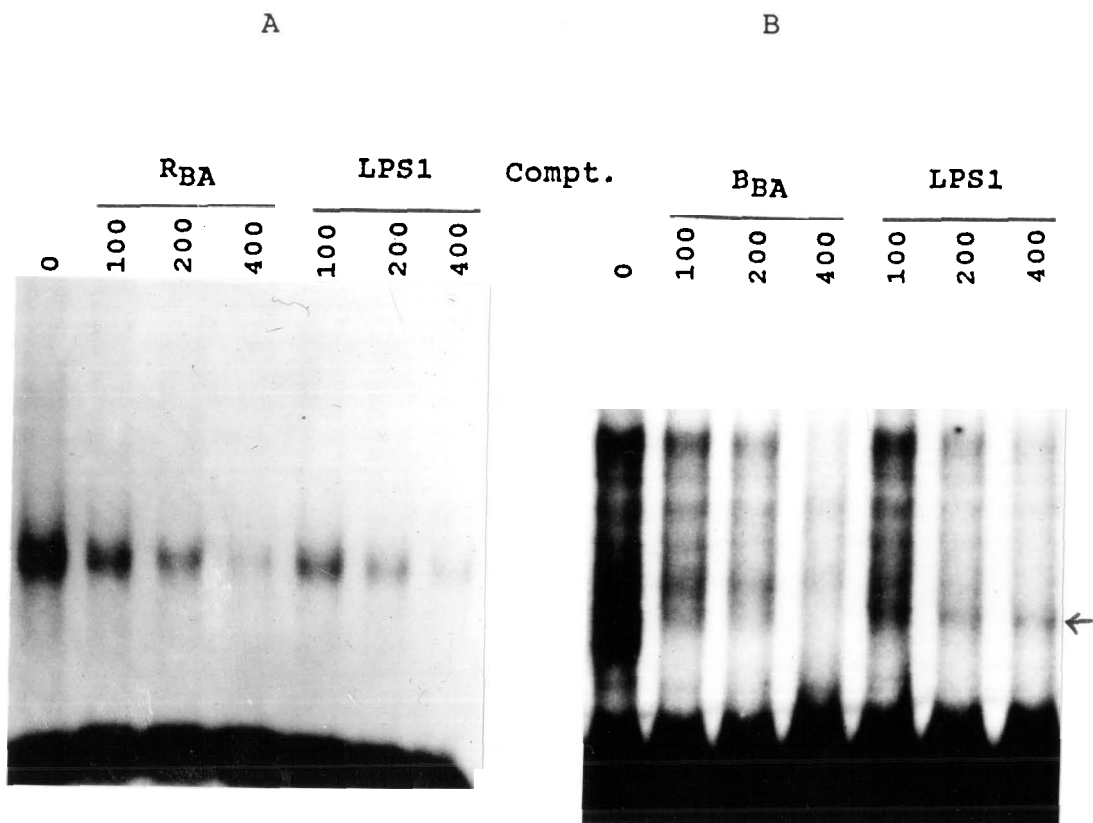


Fig. 11. Competition analysis for DNA binding proteins.

A. Labeled R_U (146 bp) as probe.

B. Labeled R_T (291 bp) as probe.

The labeled probes (0.5 ng) were incubated with nuclear proteins (4.0 μ g) from aleurone layers treated with GA₃ for 48 h. All the lanes contained 0.5 μ g of ct DNA as a non-specific competitor. Unlabeled R_{BA} and LPS1 (unrelated DNA) were included in the binding reactions as competing DNA (Compt.), respectively. The folds molar excesses of competitors are shown above the lanes. 0, no competitor added. F, free labeled DNA. The arrow indicates one retarded band which could be outcompeted by R_{BA} at 100-fold molar excess, but could not be outcompeted by LPS1 (unrelated DNA) at 400-fold molar excess.

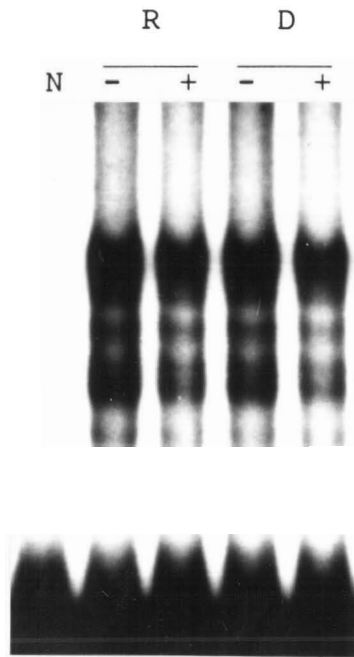


Fig. 12. Comparing bindings of nuclear proteins from GA₃-treated (+) and untreated (-) aleurone layers of Ramona 50 (R) and D6899 (D) to R_T. Each binding reaction included 4.0 μg of nuclear proteins and 0.5 ng of labeled R_T except for lane N which was in the absence of nuclear proteins.

N	L	L	R	R	A	
0	4	20	4	20	4	μg

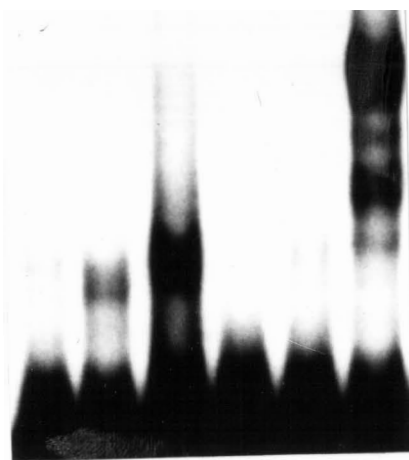


Fig. 13. R_T binding activity of cellular extracts from tissues of Ramona 50. The binding assay was performed under the same condition as described in Fig. 11 except different amounts of tissue extracts that were used. Lane A, extract from aleurone layers with 48 h of imbibition with GA_3 . Lane R, extract from roots of the etiolated wheat. Lane L, extract from leaves of the etiolated wheat. Lane N, no extract added in the binding assay. The amounts of proteins used for binding reactions are shown above lanes.

C. Discussion:

1. α -Amy2/54 promoter binding proteins from aleurone cells of Ramona 50 and D6899

It has been shown that exogenously added GA₃ stimulates the accumulation of mRNA for α -Amy2 (Lazarus et al., 1985), and furthermore that the α -Amy2/54 gene is expressed in wheat aleurone tissues (Huttly et al., 1988). Analysis of the promoter of α -Amy2/54 in a transient expression system using oat aleurone protoplasts revealed that elements involved in directing GA₃-regulated expression lie within 300 bp upstream of the start of transcription (Huttly and Baulcombe, 1989). More recently, Rushton et al. (1992), using DNase I footprinting, showed that the α -Amy2/54 promoter region had several sites which bound aleurone nuclear proteins from oat.

In this study, nuclear proteins from wheat aleurone tissues were found to bind to the α -Amy2/54 promoter. Although the accumulation of α -amylase mRNAs was induced by GA₃ in Ramona 50 aleurone layers and was blocked in D6899 (Fig. 3), similar binding patterns were obtained for the α -Amy2/54 promoter (R_T) when nuclear proteins from GA₃-treated or untreated aleurone layers from Ramona 50 or D6899 were used in the binding reactions. The crude cellular extracts from roots and leaves of etiolated Ramona 50 seedlings, in

which α -amylase gene is not transcribed, did not contain the aleurone nuclear proteins that bound to the α -Amy2/54 promoter (Fig. 13). In studies of hormone regulation, it is important to identify and then isolate the intermediate(s) between the hormone and the target sites (induced gene). The steroid receptors modulate transcriptional efficiency through the functional interactions among the receptor molecules as well as interactions with other essential transcription factors (Beato, 1989). Ou-Lee et al. (1988) reported that after GA treatment a tissue-specific nuclear factor was produced which showed binding to the promoter region of a rice α -amylase gene. The aleurone nuclear proteins that bind to the α -Amy2/54 promoter fragment in my study do not need GA induction, and also exist in the GA-insensitive *Rht3* line, D6899. It is possible that some aleurone nuclear factors present in small amounts were GA-induced and bound to the α -Amy2/54 promoter, however, they could not be detected or resolved in the gel retardation studies.

2. Interactions of aleurone nuclear proteins and α -Amy2/54 promoter

The use of crude nuclear extracts for the study of the DNA-protein interaction was complicated because the extract contained both sequence-specific and non-sequence-specific DNA-binding proteins. To discriminate between the two, a

large excess of heterologous DNA or RNA (non-specific competitor) was added to the incubation mixture to eliminate or reduce background interaction between the DNA probe and proteins that nonspecifically bind DNA. Many different types of nucleic acids can be used. These include DNA from heterologous sources (e.g., salmon sperm, calf thymus, *E. coli*), and alternating copolymers, such as poly(dI-dC), and tRNA. Selection of an appropriate non-specific competitor may enhance the binding of a specific protein to a DNA probe. Rushton et al. (1992) reported that incubation of aleurone nuclear proteins from wild oat protoplasts that had been incubated for 4 days with 0.1 μ M GA₁, with a 424 bp fragment (-1 to -424) from the promoter of the α -Amy2/54 gene, resulted in the formation of one major DNA-protein complex. This complex was considered sequence specific in that the binding was eliminated by competition with non-radioactive probe, but not by the control competitor. These authors reported no non-specific DNA binding proteins in their study. In my study of the DNA-protein interactions, different binding patterns were observed when calf thymus DNA (ct DNA) or poly(dI-dC) was included in the binding reaction (Fig. 6). One major binding complex (B4a) obtained when poly(dI-dC) was used as the non-specific competitor could be almost completely eliminated by ct DNA, but not by the nonradioactive R_{BA}, suggesting that the complex B4a was a nonspecific interaction (Fig. 9). Among the other DNA-protein complexes, obtained when shorter DNA probe R_U and R_T

were used, one band was shown to be sequence specific. Higher concentrations of the nonradioactive probe (R_{BA}, the -318 to +98 fragment of the α -Amy2/54 gene) eliminated the binding completely, whereas a 400 fold excess of sea urchin DNA (LPS1) did not eliminate the binding (Fig. 11). By increasing the amount of ct DNA, poly(dI-dC), or their mixture in the binding reactions, the intensity of the non-specific bands was reduced, but could not be removed completely without losing the signal of the specific band on the gel (data not shown). This suggests that the proportion of the non-specific DNA binding proteins to the specific protein(s) in my aleurone nuclear extract was too high to be eliminated efficiently by the non-specific DNA competitors. The putative sequence-specific nuclear protein-DNA interaction observed in my study needs to be studied in greater detail. Partial purification of the nuclear fragments as well as use of shorter promoter fragments together with DNase I footprinting may resolve the specific DNA sequences where this specific protein binds.

Control of transcription involves the interaction of protein factors with specific DNA sequence elements, including promoter and enhancer, and the arrangement of various *cis* elements within the promoter of a gene dictates its transcriptional pattern (Mitchell and Tjian, 1989; Roeder, 1991). In the present study, the one sequence specific band that was formed between the aleurone nuclear

protein and R_T DNA (Fig. 11) may be one of these transcriptionally significant DNA-protein complexes. In order for the gene to be transcribed, however, a transcriptionally poised chromatin structure must be at hand before the sequence specific DNA-protein complexes can be formed (Felsenfeld, 1992; Gross and Garrard, 1987; Spiker, 1988). Two groups of proteins, the high mobility group (HMG) non-histone chromatin protein and histone variants, must become available for the successful assembly of an initiation complex. These two groups of chromosomal proteins most likely recognize a specific configuration of DNA, rather than recognizing a specific nucleotide sequence (Jacobsen et al., 1990; Solomon et al., 1986). The proteins binding to the α -Amy2/54 promoter in a non-sequence-specific manner probably were these chromosomal proteins. The questions concerning the function of the multiple binding interactions and the *cis*-DNA sequences bound by the proteins remain open.

D. Conclusions:

1. Ramona 50 and D6899 had the same DNA sequences of α -Amy2/54 promoter.
2. There were several DNA-protein interactions between aleurone nuclear proteins and R_{BA} .
3. B4a, one major interaction when poly(dI-dC) was used as non-specific competitor, was a non-sequence-specific interaction of aleurone nuclear proteins with R_{BA} .
4. One interaction between aleurone nuclear protein(s) to R_T showed DNA-sequence-specificity while others were non-sequence-specific interactions (R_U and R_T).
5. No difference was detected in the binding of the nuclear proteins from GA_3 -treated and untreated aleurone layers to R_T .
6. Nuclear proteins isolated from D6899 incubated with R_T probe gave the same sequence-specific and non-sequence-specific interactions as the nuclear proteins isolated from Ramona 50.

7. Protein extracts from roots and leaves of etiolated Ramona 50 seedlings did not contain the aleurone nuclear proteins binding to R_T.

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