Acta Bot. Neerl. **41**(1), March 1992, p. 1–23

REVIEW

The new plant physiology—molecular approaches to studying hormonal regulation of plant development

L. C. VAN LOON and J. BRUINSMA

Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

CONTENTS

Introduction	1
Molecular-biological and genetic approaches	2
Developmental programs	2
Seed development and its regulation by abscisic acid	4
Analysis of plant gene expression	5
Functional analysis of plant genes	7
Hormonal regulation of gene expression	10
Actions of gibberellin and abscisic acid in the cereal aleurone	10
Regulation of tomato fruit ripening by ethylene	12
Induction and significance of stress proteins	14
Signals for gene regulation	16
Conclusions	19

Key-words: gene expression, hormonal regulation, molecular biology, molecular genetics, morphogenesis, mutants.

INTRODUCTION

In early botany, the study of morphogenesis in higher plants started at the level of the whole plant and its organs, describing their life cycle and the formation of the vegetative and generative organs during plant development. This stage of morphology was followed by that of physiology, in which experimental interference with developmental processes aimed at the analysis of causal relationships in plant morphogenesis. The past century offers beautiful examples of sophisticated experimental analysis of such developmental processes as, e.g., apical dominance and flower induction. It became apparent that plant metabolism, growth and development are genetically determined, and that the expression of the genetic information is dependent upon and modulated by internal and environmental factors such as, e.g., hormones and light. Neither the nature of the genes and the extent of their expression in particular developmental processes, nor the mechanism of action of the endogenous growth-regulating substances mediating internal and external signals were understood, however, although it seemed reasonable to assume that hormones influence developmental processes by bringing about changes in gene expression.

Abbreviations: ABA: abscisic acid, GA: gibberellin, GUS: β-glucuronidase, IAA: indoleacetic acid, JA: jasmonic acid, P_{fr}: phytochrome far-red-absorbing form, PG: polygalacturonase, PRs: pathogenesis-related proteins, SA: salicylic acid.

Application of biochemical knowledge and methodology allowed for extension of experimentation at the cellular and subcellular levels, leading in several cases to the unravelling of hormonal or light-induced effects on enzyme activities in metabolic pathways. However, the significance of research at the protein level for the explanation of morphogenetic effects at the organ or whole-plant level has been severely limited by the correlative nature of the argumentation. The evidence is largely derived from simultaneously occurring variations at both levels, but apart from such correlations a firm causal argument cannot be obtained from combining physiological and biochemical data alone. Moreover, cell division, extension and differentiation, as well as the formation of organs and the spatial and temporal integration of various meristematic activities all depend on the interplay between various hormonal signals. Different combinations of hormones may act independently, synergistically, or antagonistically, and can influence each other's metabolism, recognition by cellular receptors, or signal-transduction pathways. These complex interactions stem in part from the stochastic nature of hormonal regulation in plants itself (Trewavas 1991) and give rise to the plasticity of plant development, in which the pattern of growth and development is open to modulation by internal and external constraints and growth of new organs is invariably linked to loss of function of old organs (Woolhouse 1978).

Although each class of plant hormones is synthesized preferentially in certain organs, all hormones can be produced in all vegetative plant parts and also be active there. The amount of hormones at each particular site is the balance between synthesis and degradation, import and export, and their effect will, in addition, depend critically on their perception through hormone receptors, the signal-transduction chains and the physiological process involved. However, new approaches make it possible to supersede the correlative evidence obtained so far and to establish causal relationships between developmental processes and gene expression at the molecular level.

MOLECULAR-BIOLOGICAL AND GENETIC APPROACHES

Developmental programs

As growth and development are genetically programmed, it is commonly assumed that developmental regulation occurs through selective gene expression, i.e. genes that determine the structure or metabolism of a particular cell type or organ are expressed only in that cell type, c.q. organ. Analysis of the hybridization kinetics of polysomal mRNAs has been employed to gain insight into the number of single or low-copy genes that are transcribed in different organs of the tobacco plant (Kamalay & Goldberg 1980). Thus it was established that during its life cycle 70 000 different structural genes are expressed. In leaves, stem, roots, petals, anthers and ovary between 24 000 and 27 000 different mRNAs are present. Each organ has a large gene set that is not detectably expressed at the mRNA level in the other organs. For example, the stem has approximately 6 000 diverse mRNA species—representing 25% of the stem RNA complexity—that are not detectable in the polysomes of other vegetative and floral organs (Fig. 1). Similarly, cultured suspension cells of soybean were estimated to contain about 60 000 diverse mRNAs, about two-thirds of which only were shared with hypocotyls (Ulrich & Key 1988).

These numbers contrast sharply with the differences that one observes when comparing protein patterns of different organs by, for instance, two-dimensional polyacrylamide gel electrophoresis. Apparently, protein analysis detects no more than in the order of 1% of all translation products present in a particular tissue, due to insufficient resolution and to

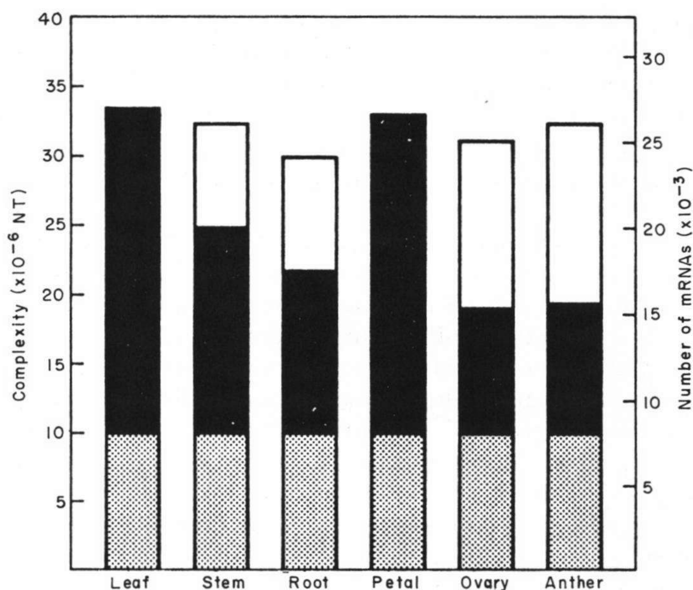

Fig. 1. Sets of diverse mRNAs in organs of the tobacco plant. Bar length represents the sequence complexity of total polysomal mRNA of each organ, expressed in total numbers of nucleotides (complexity) or in numbers of gene transcripts with an average length of the mRNA of 1240 nucleotides. Stippled portions depict a common set of mRNAs present in all organs and dark portions the set of mRNAs that are present in or shared with the leaf. The open areas represent the set of mRNAs that are unique for each organ. (From Kamalay & Goldberg 1980).

amounts of individual proteins too low to be detected by staining (Klerk & Van Loon 1991). Moreover, the proteins that are detected mostly show few differences between different organs. Probably, the common proteins are present in all organs in relatively high concentrations and have house-keeping functions. However, when differences are found, at either the mRNA or the protein level, these techniques do not provide information on the function of these products. It has been established that differences occur between different organs but at this stage their significance cannot but remain unclear.

Another approach to obtain evidence for the involvement of specific genes in plant morphogenesis is the use of mutants in which a particular developmental process has been genetically altered. Thus, in *Arabidopsis thaliana* and in snapdragon (*Antirrhinum majus*) a number of homeotic mutants are known in which the fate of flower organs is altered (Bowman *et al.* 1989; Coen 1991). For instance, in the *Arabidopsis* mutant *apetala* petals are lacking and sepals are converted into leafy structures. In *agamous* sepals and petals are formed but further development of the flower is not completed: the anthers become a further whorl of petals and at the site of the stamen a new flower of the same type is formed repeatedly. Such morphogenetic alterations each depend on a single, recessive mutation and demonstrate that flower development depends on a small number of genes determining the induction of specific organs, whereas presumably a much larger number of genes is involved in the expression of their differentiated states.

Arabidopsis is ideally suited for molecular-biological and genetic studies because of its short life-cycle, extensive seed production, and the fact that it has the smallest genome known for any higher plant (Meyerowitz 1989). Single-gene mutants that are either deficient in or insensitive to a particular regulating factor are magnificent tools in the

study of the morphogenetic action of such factors as light and hormones (Karssen *et al.* 1990; Reid 1990; Scott 1990; Koornneef *et al.* 1992). In 'long hypocotyl' mutants of *Arabidopsis* hypocotyl elongation is not inhibited in the light, either because hardly any phytochrome is present, or due to unresponsiveness of the tissue to P_{fr}. The observations directly establish the role of phytochrome in the transduction of the light signal and offer possibilities for the molecular dissection of the signal-transduction pathway (Koornneef *et al.* 1991).

Hormone-deficient mutants have been used extensively to analyse hormone biosynthetic pathways and to study the physiological role of plant hormones. Hormone response mutants have provided new information on the way hormones act, and interact, to regulate development. Ethylene-insensitive mutants lack responses to this hormone such as inhibition of cell elongation, promotion of seed germination, enhancement of peroxidase activity, acceleration of leaf senescence, and feedback suppression of ethylene synthesis by ethylene, clearly demonstrating that these pleiotropic responses originate from the presence of a single gene product, probably the receptor for ethylene (Bleecker *et al.* 1988). Germinated seedlings are unable to emerge through sand and lack the thickening below the soil surface occurring in hypocotyls of wild-type plants. This indicates that increased ethylene production in response to mechanical impedance is required for emergence of seeds from soil to occur (Harpham *et al.* 1991).

Seed development and its regulation by abscisic acid

The insights gained from the use of abscisic acid (ABA)-deficient and -insensitive mutants in studies of seed development are of particular interest. Contrary to generally accepted views, ABA does not affect assimilate partitioning to developing seeds: seeds developing on ABA-deficient pea and *Arabidopsis* plants attained similar weights and also in other respects were indistinguishable from seeds developing on wild-type plants (S. de Bruijn, D. Vreugdenhil and C. M. Karssen, unpublished observations). However, these seeds had reduced dormancy. As exogenous ABA inhibits seed germination, it is held that endogenous ABA has a similar function in preventing precocious germination. Indeed, comparison of the seed from a selfed ABA-deficient *Arabidopsis* mutant (*aba*) with the seed from a wild-type pollinated with that mutant, showed that the mutant seed, containing hardly any ABA throughout its development, had practically no dormancy at maturity (Karssen *et al.* 1983). In contrast, the heterozygotic seed, with up to 0·6 $\mu g\ g^{-1}$ ABA 10 days after pollination, was completely dormant at maturity (Table 1). At that stage, the endogenous ABA content had dropped to about the level of the mutant. Thus, dormancy was apparently imposed by ABA during seed development and retained in its absence upon seed maturation.

When a mutant flower is pollinated with wild-type pollen, only the embryo is able to produce ABA. The level of ABA therefore remains much lower, but dormancy is nevertheless complete. Thus, it must be the embryonic ABA that imposes the dormancy (Table 1). That it is this embryonal ABA only, can be concluded from the pollination of the heterozygote with the mutant. Under these conditions the seeds are a mixture of 50% ABA-producing and 50% ABA-deficient embryos in a background of ABA-containing maternal seed coats. Half of the seeds germinated precociously in spite of this ABA-producing background (Karssen *et al.* 1983) (Table 1). Thus, ABA from the maternal tissues is unable to impose dormancy.

Three different ABA-insensitive mutants, *abi1*, *abi2* and *abi3*, have a similar phenotype. The same holds for the double mutants *aba,abi1* and *aba,abi2*. However, the double

Table 1. Percentage germination and ABA content (ng g^{-1}; between brackets) of immature (10 and 16 days after pollination) and mature (26 days after pollination) F_1 seeds of crosses between wild type (*Aba/Aba*) and ABA-deficient mutant (*aba/aba*) plants of *Arabidopsis thaliana.* (After Karssen *et al.* 1983)

		Genotype			
	♀:	*Aba/Aba*	*aba/aba*	*Aba/aba*	*aba/aba*
	♂:	*aba/aba*	*Aba/Aba*	*aba/aba*	*aba/aba*
Days after pollination	F_1:	*Aba/aba*	*Aba/aba*	50% *Aba/aba*, 50% *aba/aba*	*aba/aba*
10		39 (596)	44 (35)	42 (259)	64 (8)
16		29 (59)	49 (36)	55 (38)	99 (1)
26		0 (0)	0 (0)	55 (0)	95 (0)

mutant *aba,abi3* revealed a novel and very highly ABA-sensitive process in seed development. The seeds obtained from the recombinants of the deficient and the insensitive *abi3* mutant fail to desiccate at the normal time, remain green until ripe, and lose viability upon drying (Koornneef *et al.* 1989). Accumulation of seed storage proteins is reduced but germination-specific proteins become apparent, suggesting that in this double mutant seed development is not completed and the program for seed germination is initiated prematurely (Meurs *et al.* 1992). Normal development, but not dormancy, could be fully induced by either maternal or exogenous ABA. Apparently, the minute amounts of ABA perceived are insufficient for the induction of dormancy but suffice to completely saturate the requirement for seed maturation and for desiccation tolerance to develop.

In vegetative organs the lack of ABA in the deficient mutant and the insensitivity to ABA in the *abi1* and *abi2* mutants is expressed by an inability to close the stomata, leading to severe wilting. The *abi3* mutant lacks this phenotype, indicating that the gene concerned is expressed mainly or exclusively in the developing seed. In contrast, the *abi1* and *abi2* mutations do not impair desiccation tolerance of the seed, suggesting that they are expressed mainly in the vegetative parts. The two classes of mutants, the *abi1, abi2* class and the *abi3* class, affect different but overlapping sets of responses (Finkelstein & Somerville 1990) and the differing physiological abnormalities illustrate the complexity of the responsiveness of plant cells to ABA. The three loci may be related to different receptor molecules or to different transduction pathways. These responses can only be fully understood when the receptor(s) have been identified and the signal-transduction pathway(s) have been characterized at the molecular level. Progress in this area is expected to be rapid, now that genetic and molecular-biological techniques can be combined.

ANALYSIS OF PLANT GENE EXPRESSION

The development of recombinant-DNA and plant transformation and regeneration techniques has allowed an enormous leap in our abilities to identify, isolate and analyse plant genes of interest (Kuhlemaier *et al.* 1987; Verma & Goldberg 1988; Marcus 1989).

By differential hybridization it is possible to identify individual mRNA species that are expressed exclusively in one type of organ, at a specific stage of development, or in response to particular external manipulation. Mutant genes and their corresponding wild

types can be isolated as a result of genetic fine mapping and/or tracking of restriction fragment length polymorphisms, discernable by the different cleavage pattern of a mutant and its corresponding wild-type gene by particular restriction enzymes, followed by construction of genomic libraries containing large DNA fragments and 'chromosome walking' (Koornneef *et al.* 1983; Chang *et al.* 1988; Nam *et al.* 1989). Alternatively, genes can be cloned by transposon tagging, in which the gene of interest is inactivated as a result of the insertion of a transposable element of known sequence (Chandlee 1991; Dooner *et al.* 1991).

As the number of developmental mutants available in plants thus far is limited, a common approach is to seek individual mRNAs whose presence is linked to the developmental process under study. Technically, this involves the isolation of the mRNA fraction from the organ concerned and its use as a template for the synthesis of complementary DNA (cDNA) with the help of the enzyme reverse transcriptase. The various cDNAs, each corresponding to a separate mRNA molecule present in the original extract, are then inserted into a suitable vector by cutting the vector DNA with the appropriate restriction enzyme and linking a cDNA molecule between the cut ends. The vector can be a bacterial plasmid or a bacteriophage that is multiplied in a suitable host, mostly a crippled form of the bacterium *Escherichia coli.* Together the population of the recombinant DNA-containing bacteria forms a cDNA library of sequences corresponding to the mRNAs that were originally present. By differential hybridization of the recombinant DNA from each of the bacterial clones with cDNA corresponding to the mRNA from other organs or from the same organ at a different developmental stage, individual clones can be selected that contain a cDNA of an mRNA that is expressed exclusively in a single organ or at a specific developmental stage. Such organ- or stage-specific clones can then be used to investigate the kinetics of the expression of that particular mRNA in the organ under study by hybridization with total mRNA isolated from that organ at various stages throughout its development or after exposure to varying conditions.

In the past decade almost every developmental process in plants has been probed for the occurrence of specific gene products, both at the mRNA and the protein level. The two may be easily equated by in-vitro translation, whereby the mRNAs are translated into protein by using cell-free protein-synthesizing systems and translation products are analysed by various electrophoretic and immunological techniques. As it has proved relatively easier to identify qualitative differences in mRNAs than in proteins, many cDNA clones have been described that correspond to mRNA species present only in specific organs, associated with morphogenesis, growth or development and the accompanying alterations in metabolism, or induced by changes in environmental conditions of an abiotic or a biotic nature. In some cases evidence has been obtained that different sets of genes are each co-ordinately expressed under the influence of developmental or environmental signals (Goldberg 1988).

By using specific cDNAs as probes, structurally homologous genes can be identified. In many cases it has been found that similar proteins are coded by families of related genes. Different members of such multigene families may be differentially expressed in tissue-specific or developmentally-controlled manners, even though the proteins may serve the same function. These observations point to a complex regulation of morphogenetic and metabolic activities in plants. This makes it difficult to understand the physiological significance of the particular contribution of each individual gene within a family.

On the basis of expression patterns the function of individual genes cannot be assessed, nor is it readily apparent what signals are required for the mRNAs to be transcribed.

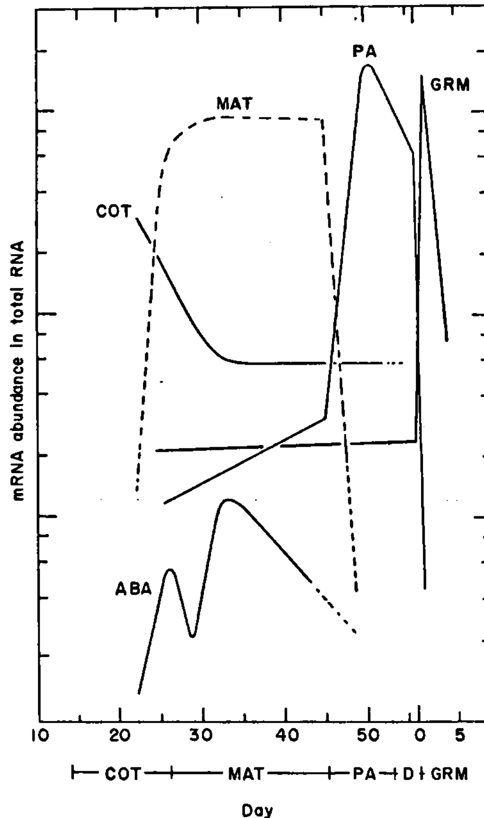

Fig. 2. Temporal components encompassing the expression of the various classes of mRNAs during cotton seed development and germination. The concentrations of the mRNAs are presented on an arbitrary logarithmic scale relative to standard developmental time. COT: cotyledon development, MAT: seed maturation, ABA: ABA content, PA: post-abscission, D: desiccation, GRM: germination. (From Hughes & Galau, 1989).

A singular analysis of the variations in the amounts of a large number of mRNAs in cotton embryos during development and germination has shown that accumulation kinetics distinguish 12 co-ordinately expressed mRNA classes, but all patterns are combinations of up to four out of five temporally discrete abundance components that seem to reflect specific developmental programs (Fig. 2) (Hughes & Galau 1989). One of these programs is associated with the transiently high ABA content during seed maturation but the chemical signals for the induction of the other stage-specific mRNA classes are unknown.

FUNCTIONAL ANALYSIS OF PLANT GENES

Several strategies are being employed to gain insight into the functional significance of specific gene transcripts. By determining the base sequence of the cDNA clone concerned, one can deduce the amino-acid sequence of the protein that the corresponding mRNA is coding for. Sometimes the function of the protein can then be inferred from homologies with sequences of proteins for which the function has been established otherwise. Thus, because of homology with DNA-binding proteins from yeast and human, it was deduced

that the flower-specific homeotic genes *agamous* from *Arabidopsis* and *deficiens* from snapdragon are similar and resemble transcription factors (Yanofsky *et al.* 1990; Sommer *et al.* 1990). Therefore, they may both regulate the expression of other genes that determine flower organ development. By in-situ hybridization of cDNA with the endogenous mRNA in tissue slices the cellular location of the transcripts can be microscopically established, yielding direct information on the expression of the gene at the cellular level. In agreement with the phenotypic absence of anthers and ovary in *agamous* mutants, the transcript was found to be present in developing anthers and stamen but virtually not in sepals and petals.

If sequencing and/or in-situ hybridization do not provide clues as to the function of the gene of interest, one can resort to manipulation of the expression of the gene transcript in the plant itself and analyse its physiological consequences. This can be done by either expressing the mRNA in a situation in which normally it is not expressed, or preventing its normal expression. Although sometimes effects are observed when cDNA or genomic sequences are introduced directly into plant cells and transiently expressed, a thorough functional analysis requires the stable transformation of plants through integration of the exogenous DNA into the plant genome.

The method that is most frequently employed makes use of the phytopathogenic bacterium *Agrobacterium tumefaciens* harbouring the Ti (tumour-inducing) plasmid (Klee *et al.* 1987; Schell 1987). After infection has been established part of the Ti-plasmid, the T region, is transferred into the nuclear DNA of the host plant. This region contains DNA coding for enzymes of auxin and cytokinin biosynthesis that are responsible for the induction of tumours. By substituting the genes responsible for tumorous growth by the gene(s) of interest together with a suitable marker gene, for instance for resistance to an antibiotic, it is possible to infect plant cells and transform them through transfer of these genes into the genomic DNA of the plant. After selection of transformed cells on antibiotic-containing medium and regeneration of the cells into plants, transgenic plants are obtained in which expression of the foreign gene can occur.

However, *A. tumefaciens* only effectively transforms dicotyledonous plants and for monocotyledons direct transformation protocols, in which the DNA is introduced directly into plant cells, are being employed (Steinbiss & Davidson 1989; Potrykus 1991). Currently the most advanced is 'biolistics', in which plant cells are bombarded directly with metal particles coated with DNA, using a particle gun. The major problem, however, is that from the genetically manipulated cells whole plants have to be regenerated for phenotypic effects to be properly observable. Many plant species, particularly the major grain crops, are refractory to current regeneration protocols. Although recently transformed maize plants have been obtained as a result of shooting foreign DNA directly into embryonic tissues (Gordon-Kamm *et al.* 1990), mainly representatives of the *Solanaceae* are being transformed to study the expression and effect of the integrated DNA.

To express a gene under conditions where it is absent or not normally expressed, the cDNA or that part of the gene that encodes the structural protein is fused to a constitutive promoter that enables transcription to take place independent of cell type or plant developmental stage. Often the cauliflower mosaic virus (CaMV) 35S promoter (Guilley *et al.* 1982) is used for this purpose. Under these circumstances the mRNA is, in principle, formed in all cells of the plant. A disadvantage of this method is that expression may occur in cells in which the mRNA would never be present naturally. This may lead to disturbances of growth or development but, nevertheless, may provide information about the function of the gene concerned. If the tissue in which the gene is expressed normally is

known, instead of a constitutive promoter a tissue-specific promoter may be used in order to obtain physiologically more relevant information.

Conversely, the stability and/or translation of an endogenous mRNA can be diminished by transforming plants with 'anti-sense RNA'. In that case plants are transformed with a DNA construct in which the promoter is attached to the non-coding strand. Upon transcription, a minus-strand RNA is formed with a sequence complementary to that of the plus-strand mRNA. When minus-strand RNA is present in excess, it hybridizes with the available plus-strand RNA and inhibits its further expression. The function of the corresponding protein may then be deduced from the absence of a phenotypic effect.

The same techniques can be employed to establish causal relationships between the expression of particular genes and specific processes, which previously could only be inferred from biochemical studies. The biosynthesis of flower pigments in *Petunia* may serve as an example. The key enzyme in the biosynthesis of flavonoid flower pigments is chalcone synthase (CHS). The resultant (iso)flavonoids are white or yellowish and may be converted by dihydroflavonol-4-reductase (DFR) into red or purple anthocyanidins. *Petunia* DFR does not recognize the substrate dihydrokaempferol which, when similarly converted, yields orange pelargonidin. By transforming *Petunia* with a DFR gene from maize that codes for an enzyme that does catalyse this conversion, *Petunia*'s with orange-red flowers have been obtained (Meyer *et al.* 1987). Conversely, formation of flower pigments was inhibited altogether when plants were transformed with an anti-sense CHS-gene (Van der Krol *et al.* 1988). However, in the latter case, also variegated flowers with coloured sectors or rings were encountered, indicating that the anti-sense RNA was not always fully effective. Similar effects have been found by others and still await explanation.

In an alternative approach, possibilities are being explored to transform plants with vertebrate genes coding for antibodies against plant proteins (Hiatt *et al.* 1989). When complexing with the antigen, such 'plantibodies' might inactivate the target protein and, thereby, inhibit its action.

It is clear that in this way specific phenotypic effects can be related directly to the activity of the introduced gene. Thereby, the molecular approach leads to an enormous step forward in our understanding of plant morphogenesis and metabolism. In view of the importance of auxin and cytokinin as plant growth regulators and the essential lack of mutants that are deficient in or insensitive to these hormones, the effects resulting from transformation of plants with T-DNA containing either the auxin or the cytokinin biosynthetic genes only, have been amply investigated. Tobacco callus expressing the auxin biosynthetic genes readily develops roots, but not shoots, whereas callus expressing the cytokinin gene gives rise to shoots, but not roots (Amasino & Miller 1982), thus fully corroborating the results obtained many years ago by Skoog & Miller (1957) upon exogenous application of these growth regulators. However, several additional processes can now be firmly attributed to the activity of these hormones (Klee & Estelle, 1991, and references cited therein). Plants overexpressing an auxin gene exhibit almost complete apical dominance and plants overexpressing the cytokinin gene show reduced apical dominance. Bud dormancy can be induced by raising the auxin level 10-fold but can be relieved by further increasing the cytokinin content. Lateral growth appears to be regulated by the ratio of the two hormones and absolute levels appear to be secondary. Auxin-overproducing plants contain many more xylem elements than control plants. The cells are, however, smaller. In contrast, in cytokinin-overproducing plants xylem formation is inhibited. All alterations in endogenous auxin and cytokinin content result in

slower growth of the root system. Increased auxin content does stimulate adventitious root formation but, overall, plants so treated have less root mass than controls. Both auxin and cytokinin manipulations can also have profound effects on leaf development: overproduction of auxin leads to epinastic growth, presumably owing to induction of ethylene, and leaves are generally smaller and narrower than those of controls (Klee & Estelle 1991). Tobacco plants with increased cytokinin levels were shorter, had larger side shoots and exhibited a decreased rate of leaf senescence (Smart *et al.* 1991).

Although these strategies provide important information on the functioning of plant genes, expression in heterologous hosts does not always faithfully reflect their activity in the native environment. Thus, tobacco plants that have been transformed with genes coding for storage proteins in soybean seeds, express these genes exclusively during seed development but at relatively low levels and in different relative proportions (Goldberg 1986). It is clear that the expression of the introduced gene is also dependent on plant factors. These factors may act at the level of transcription, mRNA stability, translation, protein processing, transport and stability. Moreover, the protein rather than the mRNA is the entity that influences the physiological process under study. Furthermore, questions remain as to how and by what signals the expression of genes is induced.

HORMONAL REGULATION OF GENE EXPRESSION

The considerations given above imply that functional analyses most readily improve our understanding of plant growth and development if applied to processes that previously have been extensively analysed physiologically and biochemically and where specific proteins are seemingly implicated to play key roles. Much effort is being directed to the study of the initial interactions between hormones and tissues and to the identification of hormone receptors (Klämbt 1987). So far, however, only auxin-binding proteins have been characterized that seem to fulfil criteria for true hormone receptors implicated in a defined hormone-dependent physiological process (Van der Linde *et al.* 1984; Löbler & Klämbt 1985). Even less is clear about the nature and complexity of the signal-transduction chains. In contrast, much progress is being made in the analysis of gene expression in systems that are characterized by a more or less specific physiological reaction to a hormone in a highly sensitive tissue or organ. Systems such as auxin-induced cell elongation in coleoptiles and mobilization of storage reserves in germinating cereal seeds under the influence of gibberellin (GA) have been thoroughly analysed physiologically and have provided good starting points for analyses of hormonal regulation of gene expression. How molecular-biological techniques are contributing to our further understanding of the regulation of physiological processes will be illustrated for three selected hormone-dependent systems.

Actions of GA and ABA in the cereal aleurone

More than 20 years ago it was established that in de-embryonated half seeds of barley, GA can substitute for the embryo in inducing in the aleurone layer the release of α-amylase that is necessary for the hydrolysis of starch in the endosperm during seed germination (Chrispeels & Varner 1967a). The question whether the hormone regulated the activity of the enzyme at the level of transcription, translation or post-translationally, or perhaps at more than a single level, could not be solved then, even though every available strategy was tried. The density labelling technique, in which newly synthesized enzyme can be distinguished from previously existing enzyme by an increase in density through the

```
Amy32b:       CCTTTT                          CTCG TAACAGAGT
19 kd zein:   CCTTTA       CAC          ATGTG TAACGATA
Amy6-4:       CCTTTTGAGCTCACCGTACCGGCCGATAACAAACTCCGGCC
```

Fig. 3. Similar base sequences within the promoters of a barley low pI (Amy32b) and high pI (Amy6-4) α-amylase gene and of a maize endosperm storage protein (19 kd zein). The arrangement CCTTTT—TAACA(G/A)A present in the Amy genes is thought to comprise a 'gibberellin response complex' within a larger sequence mediating GA- and ABA-responses. The latter part of this sequence has been underlined to ease identification. (From Rogers *et al.* 1992).

incorporation of ^{2}H, ^{15}N or ^{18}O, was developed specifically for this purpose and is still the method of choice when it has to be established whether all or only part of the amount of a protein present has been newly synthesized. When it was found that in response to GA all of the α-amylase was newly synthesized (Filner & Varner 1967), the evidence pointed to transcription as being the step regulated by the hormone. However, quantitative determinations of translatable α-amylase mRNA, together with results obtained with inhibitors of RNA and protein synthesis, indicated that there is also a requirement for synthesis of other proteins in order for α-amylase mRNA to be made or stabilized (Varner 1977).

In addition to α-amylase, several other hydrolytic enzymes are induced at the level of transcription by GA. Increases in additional hydrolytic enzymes occur independently of GA, but GA is necessary for the secretion of all the enzymes from the aleurone layer and their release into the endosperm (Laidman 1983, and references cited therein). One aspect of GA action related to enzyme secretion is the substantial rearrangement of cellular membranes elicited by the hormone. This is associated with increased membrane turnover, formation of Golgi vesicles and polysome formation. GA thus transforms the machinery of the highly specialized aleurone cells into that of a terminally differentiated, actively mobilizing and secreting tissue (Fincher 1991). This process is antagonized by ABA (Chrispeels & Varner 1967b). Using cDNA probes and specific antisera, the regulation of transcription and stability of the mRNAs, and processing and fate of the proteins under the influence of GA and ABA were analysed. Amylase mRNA is produced in the presence of combinations of GA and ABA, be it more slowly than with GA alone, but its translation is largely prevented, indicating that the effects of GA as well as ABA occur at the levels of both transcription and translation (Higgins *et al.* 1982).

Under the influence of GA, enzymes are synthesized and/or released that function in the hydrolysis of the endosperm reserves that need to be mobilized for growth of the developing seedling. Under these conditions, GA also promotes a general increase in RNA and protein synthesis (Varner 1977), but it is impossible to distinguish whether these increases are a direct result of GA action, or merely secondary consequences of an increased metabolism, once the preparatory stages for germination have been initiated. Thus, it would be of interest to see what happens if specific subsets of genes expressed under the influence of GA were either expressed at an inappropriate time or repressed at the appropriate time. However, transformation and regeneration of barley into fully fertile plants has not yet been accomplished and, thus, one has not yet succeeded in gaining insight into the significance of each of the enzymes induced.

The α-amylase in germinating barley seeds consists of a mixture of different isoenzymes that are derived from two different multigene families, coding for enzymes with high and low isoelectric points, respectively (Rogers 1985). Both families differ in biochemical properties and are expressed differentially in response to GA. Members of each family have been cloned. Promoter elements of various lengths have been fused to easily

quantifiable reporter genes, such as β-glucuronidase (GUS); the protein expressed enzymatically converts a chromogenic substrate into a blue product (Jefferson *et al.* 1987). In this way, the ability of the constructs to be expressed in response to the hormone has been examined in transient expression assays upon their introduction into barley aleurone protoplasts (Jacobsen & Close 1991). The protoplasts faithfully secrete α-amylase in response to GA and this response is abolished upon incubation in ABA. By varying the length of the promoter sequences hormone-responsive elements, conferring inducibility by GA and repression by ABA, have been delineated (Rogers *et al.* 1992). More than a single fragment is probably required and variations in the sequence appear to be tolerated. However, distinct homologies have been found among similarly regulated genes (Fig. 3), strengthening the notion that these elements function in the regulation of the expression of the genes under the influence of the hormone.

There is considerable evidence that regulation of gene transcription by these *cis*-acting promoter sequences is controlled by their interaction with *trans*-acting DNA-binding proteins (Goldberg 1986, 1988). The binding of such proteins must, therefore, be controlled by the hormone as a final step in its signal-transduction pathway. By directing research, on the one hand, to the identification of the receptors responsible for the initial interaction of the hormone with its target cell and, on the other hand, to the regulation of the expression of hormone-regulated genes, the intervening steps and the molecular mechanism of action of plant hormones may now be gradually elucidated.

Regulation of tomato fruit ripening by ethylene

Whereas the significance of each of the α-amylase isoenzymes for the hydrolysis of the starchy endosperm in cereals will become clear only after transformed plants can be analysed, the role of polygalacturonase in the ripening of tomato fruits has now been more clearly assessed in transgenic plants. Tomato fruit softening in the course of ripening is thought to result mainly from the dissolution of the middle lamellae that glue the cell walls in the fruit pericarp together. The middle lamella is composed of pectin that first has to be demethylated by a methylesterase, after which the polymer is hydrolysed by an endopolygalacturonase (PG). This latter enzyme is virtually absent from green fruit but the activity strongly increases at the start of ripening. The rise in activity and the ripening-associated fruit softening can also be induced prematurely by application of the ripening hormone, ethylene, suggesting that PG is responsible for fruit softening during ripening (Bruinsma *et al.* 1989).

PG mRNA is not present in green fruit but accumulates to extremely high levels, accounting for over 1% of total mRNA, during normal ripening after the start of ethylene synthesis, or about 24 h after treatment of mature green fruits with ethylene (Grierson *et al.* 1989). The *Never ripe (Nr)* mutant produces less ethylene, turns only to orange, softens less and produces much less PG mRNA and active enzyme, while the *ripening inhibitor (rin)* mutant fails to show the ripening-related increase in ethylene production, softens extremely slowly, and produces neither PG nor its mRNA (Brady 1987). The latter mutant also fails to synthesize PG mRNA when given ethylene, suggesting that it is unresponsive to the hormone. The observations indicate that the increase in PG activity depends on transcription of PG mRNA and show a good correlation between PG activity and softening, supporting the view that PG activity is necessary for softening to occur (Grierson *et al.* 1989).

The situation is considerably more complex, however. *In vitro*, PG activity is the result of at least two isoenzymes with molecular weights of c. 100 kDa (PG I) and 45 kDa (PG II), respectively (Knegt *et al.* 1988). As only a single PG gene has been identified in

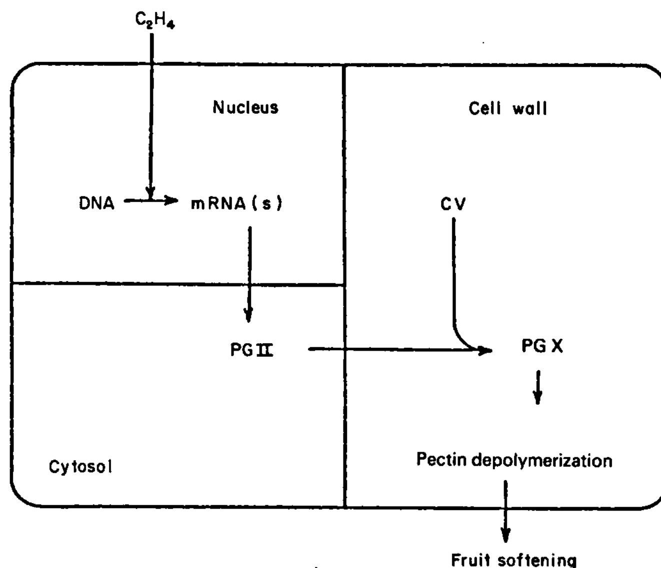

Fig. 4. Hypothetical scheme of polygalacturonase production and action in ripening tomato fruit. (After Bruinsma *et al.* 1989, modified).

tomato, consistent in length with the occurrence of the latter, but not the former (Bird *et al.* 1988), the situation appeared enigmatic and was only clarified by physiological and biochemical analysis. As it turned out, the rise in PG activity during ripening or after ethylene treatment is due primarily to PG II (Knegt *et al.* 1988). The large amount of PG II is present within the cells and not in contact with the middle lamellae of the cell walls. However, some of the PG II migrates through the plasmalemma into the cell-wall space, where it associates with another, constitutively present, wall-bound glycoprotein, the converter (CV) (Bruinsma *et al.* 1989). The combination is fixed, stabilized and activated and can degrade demethylated pectin (Fig. 4). This combination is most probably not PG I. The CV appears to have two binding sites. *In vivo*, one site binds to the cell-wall carbohydrates, the other to PG II, rendering the wall-bound PG X of 71 kDa. PG I is considered to be an artefact of extraction, arising from free CV and an overdose of PG II occupying both binding sites (Knegt *et al.* 1991).

Whether the relatively small amount of PG X would be the controlling factor in the fruit softening process was still unproven, however. In an attempt to specifically block the rise in PG activity, tomato plants were transformed with anti-sense constructs using the CaMV 35S promoter to transcribe cloned PG cDNA sequences in a reverse orientation relative to the endogenous gene. In the ripening fruits of these transgenic plants the anti-sense RNA reduced the PG protein by 90%. However, colour change of the fruits was not affected and neither was fruit softening (Smith *et al.* 1988). This result did not contradict the theory that softening is a PG-dependent process. PG II is produced in large excess and combination with CV to active PG X could still be sufficient to allow for the softening. However, in a further analysis plants homozygous for the anti-sense gene were found to have PG enzyme expression reduced by more than 99% (Smith *et al.* 1990). In the fruits with a residual 1% enzyme activity pectin depolymerization was inhibited, indicating that PG is involved in pectin degradation *in vivo*, but the compressibility of the fruit, taken as a parameter for softening, remained unaffected (Smith *et al.* 1990).

```
       1                 10                20                            30
Hv-1   A T F N I K N N C G S T I W P A G I P V - - - - - G G G F E L X S
PR-5   A T F D I V N K C T Y T V W A A A S P - - - - - - G G G R R L N S
Osm    A T I E V R N N C P Y T V W A A S T P I - - - - - G G G R R L D R
NP24   A T I E V R N N C P Y T V W A A S T P I - - - - - G G G R R L N R
MAI    A V F T V V N Q C P F T V W A A S V P V - - - - - G G G R Q L N R
Tha    A T F E I V N R C S Y T V W A A A S K G D A A L D A G G R Q L N S
```

Fig. 5. N-terminal amino acid sequences of 'thaumatin-like proteins': the pathogenesis-related proteins Hv-1 from barley and PR-5 from tobacco, the osmotic-stress proteins osmotin (Osm) from tobacco and NP 24 from tomato, a maize seed trypsin/α-amylase inhibitor (MAI), and the sweet-tasting protein thaumatin from the fruit of *Thaumatococcus daniellii*. Amino acids are indicated in the one-letter code; — denotes gaps introduced to optimize alignment. (From Bryngelsson & Green 1989).

In an alternative approach, the *rin* mutant, that does not ripen in response to ethylene, was transformed with a PG gene under the direction of a fruit-specific promoter and expressed in response to the ethylene analog propylene (DellaPenna *et al.* 1990). Although PG mRNA was produced later, the level of extractable PG enzyme activity was approximately 60% of that found in wild-type fruit. Cell-wall pectin was degraded and the period of maximum polyuronide solubilization and depolymerization occurred coincident with the appearance of bound PG (DellaPenna *et al.* 1990). These results demonstrate a primary role of PG in pectin degradation and suggest that, indeed, the larger isoenzyme is sufficient to carry out both solubilization and depolymerization of pectin. However, under these conditions no significant effect on fruit softening or other ripening-related parameters was detected (DellaPenna *et al.* 1990). Thus, contrary to common belief, PG-mediated pectin degradation seems neither necessary, nor sufficient for tomato fruit ripening (Fischer & Bennett 1991). Softening apparently requires also (an)other, as yet unknown, factor(s) that is (are) regulated by ethylene. Indeed, when ethylene production during fruit ripening is blocked through transformation of plants with an anti-sense gene of 1-aminocyclopropane-1-carboxylic acid synthase, the regulatory enzyme in ethylene biosynthesis (Adams & Yang 1979), the fruits remain green and firm, indicating that all characteristics of ripening are abolished (Oeller *et al.* 1991).

Besides PG, ethylene induces several other ripening-related mRNAs in tomato, some of which appear substantially earlier than PG mRNA (Lincoln & Fischer 1988). Although several ethylene-induced clones have been sequenced, the functions of almost all of the proteins are unknown. Progress is being made in defining promoter elements in the genes that confer inducibility by ethylene, but the regulation of the ripening process by the hormone is still far from being resolved.

It is quite obvious from these examples that molecular-biological and genetic approaches are as indispensible for further progress in plant physiology as biochemical methods have been hitherto. However, for the introduction of novel traits or suppression of undesirable characters by plant transformation, physiological analysis of transgenic plants will continue to depend on classical physiological and biochemical practice for at least two reasons. One is that the signals and mechanisms by which the plant regulates its genetic expression are inherently complex and still far from understood, the second that transgenic plants may show unexpected phenotypic deviations that require clarification through physiological and biochemical analysis.

Induction and significance of stress proteins

One of the mRNAs that is rapidly induced in tomato fruits by ethylene shows 53% homology to the sequence of a protease inhibitor from potato (Lincoln & Fischer 1988)

and, on this basis, is tentatively assumed to protect the ripening fruit from certain proteases produced by bacteria or fungi attempting infection. In this way, this ripening-associated gene resembles genes coding for pathogenesis-related proteins (PRs), a class of stress proteins in infected plants (Van Loon 1988, and references cited therein). These proteins were first discovered in tobacco plants reacting hypersensitively to tobacco mosaic virus (TMV). Whereas in the mosaic type of disease the virus rapidly spreads throughout the entire plant, in resistant plants it is halted by the hypersensitive reaction of the host cells: the primarily infected cells die, forming necrotic spots from where the virus is unable to escape (Van Loon 1983).

Characteristic of the hypersensitive reaction is the rapid *de novo* formation of a set of low-molecular-weight proteins, comprising up to 10% of the total soluble leaf protein, which accumulate in the intercellular space of the leaf. PRs have now been identified in many plant species and consist of several families, comprising chitinases and β-1,3-glucanases, whereas others show sequence homology to protease inhibitors or to proteins induced as a result of osmotic stress (Fig. 5) (Bryngelsson & Green 1989). Similar proteins are constitutively present in tubers and seeds (Dure 1985); some accumulate in vegetative tissues in a developmentally-controlled manner (Neale *et al.* 1990), but a common feature is their strong induction upon pathogenic attack. Their activity may be linked to their peculiar solubility at low pH values and resistance to common proteases (Van Loon 1985). Although the activities of other PRs have not been identified, all might act as defenses against invading fungi and other pathogens.

In tobacco, part of the set of PRs can also be chemically induced by ethylene, whereas a complementary, partly overlapping part occurs upon treatment with salicylic acid (SA) (Van Loon 1988). An important feature of the hypersensitive reaction is that, although spread of the initial infection is blocked, some factor acts as a message from the infected leaf to the uninfected parts of the plant, inducing them, too, to produce PRs. Both ethylene and SA are implicated in the generation, transport and activity of this factor (Van Loon & Antoniw 1982; Yalpani *et al.* 1991). As a result, if such healthy parts become infected, their defenses are triggered earlier and to a greater extent, and this systemic acquired resistance offers additional protection of the plant to a variety of pathogens. The practical implications appear obvious. PRs are induced at the transcriptional level and cDNAs have been isolated by differential hybridization. Most of these cDNA clones, as well as the corresponding genes, have been characterized by sequence analysis (Bol *et al.* 1990). Transformation of plants with these stress protein genes fused to an efficient promoter might bestow them with field resistance against a variety of infections and perhaps other stresses. This would have considerable impact on the protection of crops and reduce the use of chemical means as pesticides.

Indeed, tobacco plants transformed with a protease-inhibitor gene from potato have been found to be resistant to the tobacco hornworm (Johnson *et al.* 1989). Resistance to particular viruses has been engineered by transforming plants with viral coat protein genes (Beachy *et al.* 1990). These exogenous defense genes are active against insect predators and an individual virus, respectively, but not against different types of pathogens. So far, expression of single PR genes from tobacco in transgenic plants has not led to enhanced resistance against different pathogens (Linthorst *et al.* 1989), although some PRs appear to reduce the severity of disease caused by specific individual fungi or bacteria (J. Ryals, personal communication). This raises the question as to how far the PRs are involved in the general plant's defense. A chitinase and a glucanase, for example, can hardly be expected to be effective against virus infection. On the one hand, infection, ethylene, and

SA also induce substances other than PRs that might play a much more important role in the defense system of the plant. On the other hand, the massive accumulation of several different PRs in concert may point to the activation of an integrated set of responses that may need to function as a network for substantial enhancement of resistance to be achieved. Interestingly, isolation and characterization of antifungal and antiviral proteins recently revealed that these are intracellular isoforms of two families of PRs (Edelbaum *et al.* 1991; Woloshuk *et al.* 1991), indicating that such additional proteins may be at least as important as the PRs themselves.

Peroxidase is also stimulated by infection and contributes to the synthesis of lignin, involved in mechanically blocking infection spread (Van Loon 1983); it can likewise be induced by ethylene (Van Loon & Antoniw, 1982). Transformation of tobacco with one of its peroxidase genes under the direction of the CaMV 35S promoter increased peroxidase activity 2- to 10-fold (Lagrimini *et al.* 1990), an effect similar to that brought about by virus infection. Yet, no correlation between increased peroxidase activity and resistance has been found (Van Loon 1976). Moreover, in the transgenic plants chronic wilting appeared from the onset of flower formation, an effect not encountered in virus-infected plants (Lagrimini *et al.* 1990). The severe disturbance of the water balance was caused neither by limited uptake or translocation of water by the plant, nor by enhanced stomatal water loss. This inexplicable correlation between enzymatic activity, morphogenetic development and water relationships provides an unexpected anomaly due to genetic engineering and will require a physiological analysis at all levels of organization.

Like infections, abiotic stresses such as drought, heat, anaerobiosis, etc. give rise to the induction of sets of novel proteins, at least some of which are induced as a result of accumulation of the stress hormone, ABA (Sachs & Ho 1986; Skriver & Mundy 1990). From homologies at the cDNA level it has become clear that these sets of stress proteins are partly overlapping, also with ethylene-induced PRs occurring upon biotic stress. Thus, both osmotic stress proteins and members of the PR-5 family show sequence homology to thaumatin, for which reason they have been grouped within a single family of 'thaumatin-like proteins' (Bryngelsson & Green 1989; Fig. 5). Such partly overlapping sets of proteins are assumed to enable plants to acclimate and survive in the constantly changing external environment. These observations clearly suggest that such acclimation depends on integrated cellular responses. Consequently, exploitation of acclimation mechanisms present in plants for practical purposes will be exceedingly difficult if pursued by genetic engineering of single genes. Presumably only a limited set of signals suffices to induce a full response. Therefore, the identification and manipulation of these signals and/or the regulatory DNA sequences involved holds at least as much promise for enhancing resistance to stress and disease.

SIGNALS FOR GENE REGULATION

Thus, plant physiology, molecular biology and genetics provide complementary approaches and tools for the further analysis of the external and internal stimuli that regulate the expression of the genetic code in the higher plant. Such morphogenetic signals can be of a physical nature, e.g. light, or chemical, such as inorganic ions and hormones that modulate and integrate plant growth and development.

The light signals are perceived by at least three different morphogenetic pigments: the red-far red sensitive phytochrome as the main pigment, the blue-dependent cryptochrome that acts in, e.g., phototropism, and the UV-B receptor. The latter is operative in,

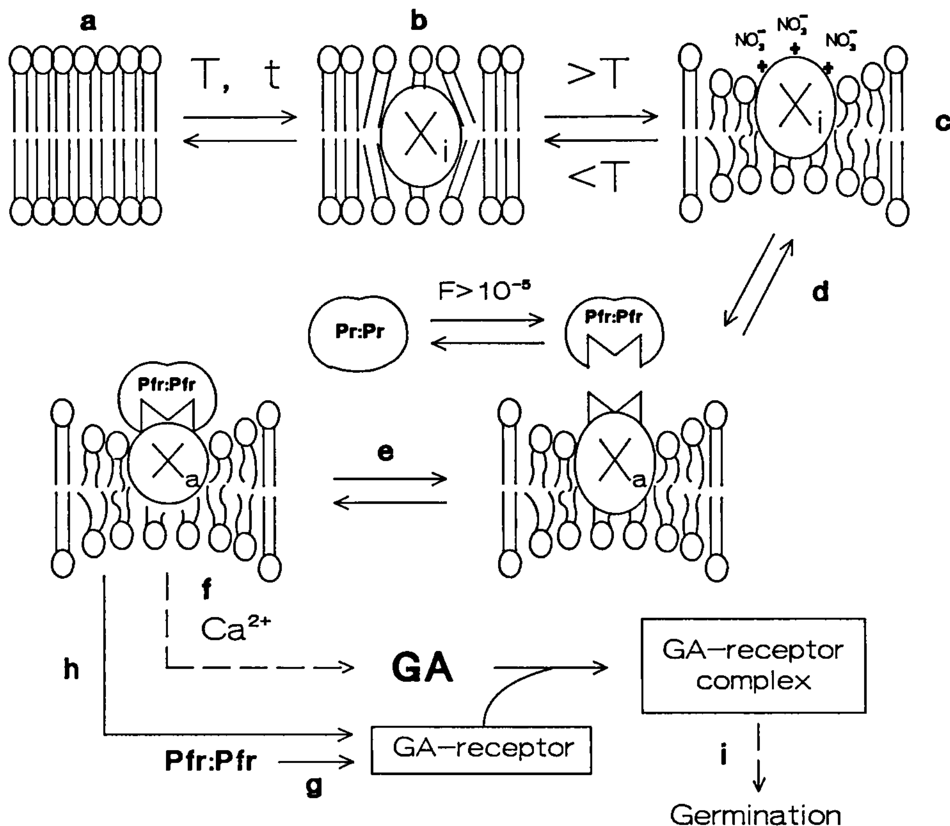

Fig. 6. Model for dormancy and germination of seeds of *Sisymbrium officinale*. The phytochrome receptor, X, is assumed to be a membrane protein. F: fluence (mol m^{-2}), Pr:Pr and Pfr:Pfr: phytochrome dimer in the red- and far-red-absorbing form, respectively, T: temperature, t: time, X_i: inactive receptor, X_a: activated receptor. (From Hilhorst 1990).

e.g., light-induced pigment formation in plant-cell cultures: mRNAs for enzymes of anthocyanin synthesis start to rise within 3 h after a 10-min UV irradiation (Schäfer *et al.* 1990). However, when the UV treatment is followed by 10 min red, the messenger accumulation is halved. Still more peculiar is that 6 h pre-illumination with blue light completely removes the lag time after the UV induction and doubles the amount of mRNA (Schäfer *et al.* 1990). This effect, too, is reduced by red light following the UV. This example shows the complexity of the light induction of gene expression. It is very common that effects, specifically induced through a single pigment, are nonetheless modified by the other photomorphogenetic systems. Somewhere in the transduction of the signal these systems interact.

There is not only intertwining of the transduction pathways of light signals. It is well known that light can sometimes be replaced by a hormone, e.g., GA. Light may induce changes in both the level of and the sensitivity to this hormone. These integrated systems are still largely black boxes, and a variety of hypotheses must lead to a great deal of further physiological research. A complicated model, in which a number of physical and chemical factors are hypothesized to co-operate, has recently been suggested for the germination of the seed of *Sisymbrium officinale* (Hilhorst 1990). This germination depends on light, that

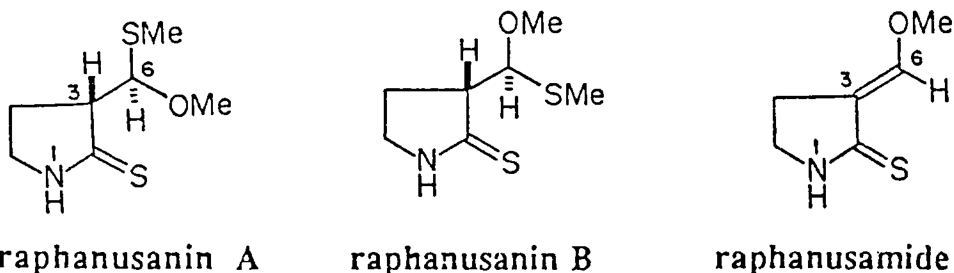

Fig. 7. The natural anti-auxins causing phototropic curvature in radish seedlings. (From Harada *et al.* 1991a,b).

can be replaced by GA, as well as on nitrate and temperature. Extensive dose-response analyses led to the speculation that the phytochrome receptor is a membrane-bound protein that at a sufficiently high temperature becomes exposed and can then be activated by nitrate ions (Fig. 6). In the activated form the receptor can bind light-induced dimers of P_{fr}. The receptor-pigment complex produces two signals, one of which leads to GA biosynthesis and may involve calcium ions; the other increases the number of GA receptor molecules. The resulting enhanced formation of GA-receptor complexes then gives a signal leading to the expression of genes coding for cell wall-solubilizing enzymes that weaken the seed coat, so that the radicle can break through and germination is established. Needless to underline the necessity of much research provoked by such a hypothesis.

A final problem with hormone-induced gene expression is that for a quarter of a century only five groups of hormones have been distinguished: indoleacetic acid (IAA) as the auxin, several gibberellins and cytokinins, at least two abscisins (ABA and xanthoxin), and ethylene. Clearly, the list cannot be complete: the hormonal requirements of such developmental processes as flower induction and tuberization cannot be met by combinations of these compounds. We know that both these processes are induced by leaf-produced hormonal substances, but their identification is hampered, on the one hand, by the difficulty of finding an easy-to-handle, reliable and sensitive bioassay and, on the other hand, by their probable lability *in vitro* during isolation, purification and determination. Preliminary observations suggest that tuberization in potato is induced by a hydroxylated derivative of jasmonic acid (JA) (Yoshihara *et al.* 1989; J. Helder, D. Vreugdenhil and J. Bruinsma, unpublished observations). We have indications that JA leads to sucrose synthase activity in stolon tips, which may lead to starch synthesis as a prerequisite for tuber development. Other investigations have implicated oligosaccharins (Ryan & Farmer 1991) (carbohydrate fragments derived from cell walls; Albersheim *et al.* 1983; Aldington *et al.* 1991), polyamines (Galston and Kaur-Sawhney 1988), JA (Parthier *et al.*, 1992) and salicylic acid (Raskin *et al.* 1987) as hormone-active substances.

Natural anti-hormones may help in elucidating hormone-receptor interactions. For example, the three light-induced growth-inhibiting substances that regulate phototropism in radish hypocotyls by antagonizing the cell-elongating activity of IAA, apparently do so because of their structural resemblance to the auxin (Fig. 7) (Harada *et al.* 1991a,b). It is to be expected, therefore, that the list of the five types of classical plant hormones will have to be extended before long and that it will be found that the classical and newly recognized growth regulators interact in novel ways. All such physiological and biochemical research opens up new prospects for molecular-biological and genetic developments as well.

CONCLUSIONS

After the benefits to plant physiology from biochemistry, now the contributions from molecular biology and genetics have to be incorporated into plant-physiological research to explore further possibilities and prospects. Such studies reveal that the regulation of plant metabolism, growth and development is inherently complex. Plant morphogenesis is modulated by both internal and external signals, which may interact in different ways to achieve alterations in gene expression associated with cell division and extension, differentiation, growth, maturation and senescence of plant organs, and its acclimation to changes in the external environment. It has become clear that biotic and abiotic stresses induce substantial changes in the expression of many genes. Apparently, the interaction of several factors is required for optimization of the physiological response.

To understand morphogenesis, the chemical nature of the signals should be further explored and their action in sensitive tissues probed, on the one hand, by identifying and characterizing receptor molecules and, on the other hand, by analysing the resulting changes in gene expression, ultimately aiming at a full elucidation of the intervening signal-transduction pathways. For these molecular analyses genetic, morphological and physiological studies remain essential to widen their scope and applicability. Genetically characterized mutants that are deficient in or insensitive to a hormone, or exhibit specific morphogenetic defects, are of enormous value in dissecting the molecular mechanism of action of hormones, the interrelationships of various hormone-controlled processes, and the pathways of tissue and organ differentiation. Further questions to be addressed by both physiological and molecular-biological approaches are the control of hormone metabolism and the control of sensitivity to hormones. Microscopic analysis at the (sub)cellular level will be required to provide information on structural aspects of hormone action and gene expression.

Physiological and biochemical characterization of the signals involved and their interactions remains a prerequisite to our understanding of the induction of morphogenetic processes. Likewise, biochemical pathways have to be more fully explored in order to understand the regulation of primary metabolism and the complex arrays of secondary metabolites and their ecological significance. Moreover, the tools of plant physiology and biochemistry remain indispensable for physiological analyses of transgenic plants. The new plant physiology emerging from this mutual co-operation requires the interaction of plant physiologists, cell biologists, biochemists, geneticists and molecular biologists. From such co-operations mutual profits, both theoretical and practical, are already arising, leading to better understanding of plant growth and development and its applications.

REFERENCES

Adams, D.O. & Yang, S.F. (1979): Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc. Natl. Acad. Sci. USA* **76:** 170–174.

Albersheim, P., Darvill, A.G., McNeil, M., Valent, B.S., Sharp, J.K., Nothnagel, E.A., Davis, K.R., Yamazaki, N., Gollin, D.J., York, W.S., Dudman, W.F., Darvill, J.E. & Dell, A. (1983): Oligosaccharins: naturally occurring carbohydrates with biological regulatory functions. In: Ciferri, O. and Dure III, L. (eds): *Structure and Function of Plant Genomes*. pp. 293–312. Plenum, New York.

Aldington, S., McDougall, G.J. & Fry, S.C. (1991): Structure-activity relationships of biologically active oligosaccharides. *Plant Cell Environ.* **14:** 625–636.

Amasino, R.M. & Miller, C.O. (1982): Hormonal control of tobacco crown gall tumor morphology. *Plant Physiol.* **69:** 389–392.

Beachy, R.N., Loesch-Fries, S. & Tumer, N.E. (1990): Coat protein-mediated resistance against virus infection. *Annu Rev. Phytopathol.* **28:** 451–474.

Bird, C.R., Smith, C.J.S., Ray, J.A., Moureau, P., Bevan, M.W., Bird, A.S., Hughes, S., Morris, P.C., Grierson, D. & Schuch, W. (1988): The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Mol. Biol.* **11**, 651–662.

Bleecker, A.B., Estelle, M.A., Somerville, C. & Kende, H. (1988): Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* **241**: 1086–1089.

Bol, J.F., Linthorst, H.J.M. & Cornelissen, B.J.C. (1990): Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* **28**: 113–138.

Bowman, J.L., Smyth, D.R. & Meyerowitz, E.M. (1989): Genes directing flower development in *Arabidopsis*. *Plant Cell* **1**: 37–52.

Brady, C.J. (1987): Fruit ripening. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **38**: 155–178.

Bruinsma, J., Knegt, E. & Vermeer, E. (1989): Polygalacturonase in the ripening tomato fruit. In: Osborne, D.J. and Jackson, M.B. (eds): *Cell Separation in Plants*. pp. 127–138. Springer-Verlag, Berlin.

Bryngelsson, T. & Green, B. (1989): Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. *Physiol. Mol. Plant Pathol.* **35**: 45–52.

Chandlee, J.M. (1991): Analysis of developmentally interesting genes cloned from higher plants by insertional mutagenesis. *Dev. Genet.* **12**: 261–271.

Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S. and Meyerowitz, E.M. (1988): Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **85**: 6856–6860.

Chrispeels, M.J. & Varner, J.E. (1967a): Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiol.* **42**: 398–408.

Chrispeels, M.J. & Varner, J.E. (1967b): Hormonal control of enzyme synthesis: on the mode of action of gibberellic acid and abscisin in aleurone layers of barley. *Plant Physiol.* **42**: 1008–1016.

Coen, E.S. (1991): The role of homeotic genes in flower development and evolution. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**: 241–279.

DellaPenna, D., Lashbrook, C.C., Toenjes, K., Giovannoni, J.J., Fischer, R.L. & Bennett, A.B. (1990): Polygalacturonase isozymes and pectin depolymerization in transgenic *rin* tomato fruit. *Plant Physiol.* **94**: 1882–1886.

Dooner, H.K., Keller, J., Harper, E. & Ralston, E. (1991): Variable patterns of transposition of the maize element *Activator* in tobacco. *Plant Cell* **3**: 473–482.

Dure III, L. (1985): Embryogenesis and gene expression during seed formation. *Oxf. Surv. Plant Mol. Cell Biol.* **2**: 179–197.

Edelbaum, O., Sher, N., Rubinstein, M., Novick, D., Tal, N., Moyer, M., Ward, E., Ryals, J. & Sela, I. (1991): Two antiviral proteins, gp35 and gp22, correspond to β-1,3-glucanase and an isoform of PR-5. *Plant Mol. Biol.* **17**: 171–173.

Filner, P. & Varner, J.E. (1967): A test for *de novo* synthesis of enzymes: density labelling with $H_2{}^{18}O$ of barley α-amylase induced by gibberellic acid. *Proc. Natl. Acad. Sci. USA* **58**: 1520–1526.

Fincher, G.B. (1991): Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**: 305–346.

Finkelstein, R.R. & Somerville, C.R. (1990): Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. *Plant Physiol.* **94**: 1172–1179.

Fischer, R.L. & Bennett, A.B. (1991): Role of cell wall hydrolases in fruit ripening. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**: 675–703.

Galston, A.W. & Kaur-Sawhney, R. (1988): Polyamines as endogenous growth regulators. In Davies, P.J. (ed.): *Plant Hormones and Their Role in Plant Growth and Development*. pp. 280–295. Kluwer, Dordrecht, The Netherlands.

Goldberg, R.B. (1986): Regulation of plant gene expression. *Phil. Trans. R. Soc. Lond.* **B314**: 343–353.

Goldberg, R.B. (1988): Plants: novel developmental processes. *Science* **240**: 1460–1467.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, Jr., W.R., Willetts, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P. & Lemaux, P.G. (1990): Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* **2**: 603–618.

Grierson, D., Smith, C.J.S., Morris, P.C., Watson, C., Bird, C.R., Ray, J., Schuch, W., Knapp, J.E., Davies, K. & Picton, S.J. (1989): Signals for gene expression in ripening tomato fruit. In Osborne, D.J. & Jackson, M.B. (eds): *Cell Separation in Plants*. pp. 1–9. Springer-Verlag, Berlin.

Guilley, H., Dudley, R., Jonard, G., Balazs E. & Richards, K. (1982): Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization of transcripts. *Cell* **30**: 763–773.

Harada, N., Hagiwara, H., Ono, H., Uda, H., Ohba, S., Kubota, M., Nishiyama, S., Yamamura, S., Hasegawa, K. & Sakoda, M. (1991a): Revision of the structure of raphanusanins, phototropism-regulating substances of radish hypocotyls. *Tetrahedron Lett.* **32**: 6757–6760.

Harada, N., Ono, H., Hagiwara, H., Uda, H., Hasegawa, K. & Sakoda, M. (1991b): Revision of the structure of raphanusamide, a phototropism-regulating substance of radish hypocotyls. *Tetrahedron Lett.* **32:** 6761–6762.

Harpham, N.V.J., Berry, A.W., Knee, E.M., Roveda-Hoyos, G., Raskin, I., Sanders, I.O., Smith, A.R., Wood, C.K. & Hall, M.A. (1991): The effect of ethylene on the growth and development of wild-type and mutant *Arabidopsis thaliana* (L.) Heynh. *Ann. Bot.* **68:** 55–61.

Hiatt, A., Cafferkey, R. & Bowdish, K. (1989): Production of antibodies in transgenic plants. *Nature* **342:** 76–78.

Higgins, T.J.V., Jacobsen, J.V. & Zwar, J.A. (1982): Gibberellic acid and abscisic acid modulate protein synthesis and mRNA levels in barley aleurone layers. *Plant Mol. Biol.* **1:** 191–215.

Hilhorst, H.W.M. (1990): *Seed Dormancy and Germination: Light and Nitrate.* Ph.D. Thesis, Agricultural University Wageningen.

Hughes, D.W. & Galau, G.A. (1989): Temporally modular gene expression during cotyledon development. *Genes Dev.* **3:** 358–369.

Jacobsen, J.V. & Close, T.J. (1991): Control of transient expression of chimaeric genes by gibberellic acid and abscisic acid in protoplasts prepared from mature barley aleurone layers. *Plant Mol. Biol.* **16:** 713–724.

Jefferson, R.A., Kavanagh, T.A. & Bevan, M.W. (1987): GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6:** 3901–3907.

Johnson, R., Narvaez, J., An, G. & Ryan, C. (1989): Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. USA* **86:** 9871–9875.

Kamalay, J.C. & Goldberg, R.B. (1980): Regulation of structural gene expression in tobacco. *Cell* **19:** 935–946.

Karssen, C.M., Brinkhorst-Van der Swan, D.L.C., Breekland, A.E. & Koornneef, M. (1983): Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid-deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. *Planta* **157:** 158–165.

Karssen, C.M., Hilhorst, H.W.M. & Koornneef, M. (1990): The benefit of biosynthesis and response mutants in the study of the role of abscisic acid in plants. In: Pharis, R.P. and Rood, S.B. (eds): *Plant Growth Substances 1988.* pp. 23–31. Springer-Verlag, New York.

Klämbt, D. (ed.) (1987): *Plant Hormone Receptors.* Springer-Verlag, Berlin.

Klee, H. & Estelle, M. (1991): Molecular genetic approaches to plant hormone biology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 529–551.

Klee, H., Horsch, R. & Rogers, S. (1987): *Agrobacterium*-mediated plant transformation and its further application to plant biology. *Annu. Rev. Plant Physiol.* **38:** 467–486.

Klerk, H. & Van Loon, L.C. (1991): Two-dimensional gel electrophoretic analysis of the changes in soluble proteins during development of primary oat (*Avena sativa* L.) leaves. *J. Exp. Bot.* **42:** 1295–1304.

Knegt, E., Vermeer E. & Bruinsma, J. (1988): Conversion of the polygalacturonase isoenzymes from ripening tomato fruits. *Physiol. Plant.* **72:** 108–114.

Knegt, E., Vermeer, E., Pak, C. & Bruinsma, J. (1991): Function of the polygalacturonase converter in ripening tomato fruit. *Physiol. Plant.* **82:** 237–242.

Koornneef, M., Van Eden, J., Hanhart, C.J., Stam, P., Braaksma, F.J. & Feenstra, W.J. (1983): Linkage map of *Arabidopsis thaliana. J. Hered.* **74:** 265–272.

Koornneef, M., Hanhart, C.J., Hilhorst, H.W.M. & Karssen, C.M. (1989): *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana. Plant Physiol.* **90:** 463–469.

Koornneef, M., Van Tuinen, A., Kerckhoffs, L.H.J., Peters, J.L. & Kendrick, R.E. (1992): Photomorphogenetic mutants in higher plants. In: Karssen, C.M., Van Loon, L.C. and Vreugdenhil, D. (eds): *Progress in Plant Growth Regulation.* Kluwer, Dordrecht, The Netherlands. (in press).

Kuhlemaier, C., Green, P.J. & Chua, N.-H. (1987): Regulation of gene expression in higher plants. *Annu. Rev. Plant Physiol.* **38:** 221–257.

Lagrimini, L.M., Bradford, S. & Rothstein, S. (1990): Peroxidase-induced wilting in transgenic tobacco plants. *Plant Cell* **2:** 7–18.

Laidman, D.L. (1983): The role of gibberellins in controlling cellular processes in germinating cereals. *Biochem. Soc. Trans.* **11:** 534–537.

Lincoln, J.E. & Fischer, R.L. (1988): Regulation of gene expression by ethylene. In: Verma, D.P.S. & Goldberg, R.B. (eds): *Plant Gene Research; Temporal and Spatial Regulation of Plant Genes.* pp. 155–167. Springer-Verlag, Wien.

Linthorst, H.J.M., Meuwissen, R.L.J., Kauffmann, S. & Bol, J.F. (1989): Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. *Plant Cell* **1:** 285–291.

Löbler, M. & Klämbt, D. (1985): Auxin-binding protein from coleoptile membranes of corn (*Zea mays* L.) I. Purification by immunochemical methods and characterization. *J. Biol. Chem.* **260:** 9848–9853.

Marcus, A. (ed). (1989): *The Biochemistry of Plants. Vol. 15. Molecular Biology.* Academic Press, New York.

Meurs, C.M., Basra, A.S., Karssen, C.M. & Van Loon, L.C. (1992): Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana*. *Plant Physiol.* (in press).

Meyer, P., Heidmann, I., Forkmann, G. & Saedler, H. (1987): A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature* **330:** 677–678.

Meyerowitz, E.M. (1989): Arabidopsis, a useful weed. *Cell* **56:** 263–269.

Nam, H.-G., Giraudat, J., Den Boer, B., Moonan, F., Loos, W.D.B., Haye, B.M. & Goodman, H.M. (1989): Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana*. *Plant Cell* **1:** 699–705.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnet, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J. & Dennis, E.S. (1990): Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. *Plant Cell* **2:** 673–684.

Oeller, P.W., Wong, L.M., Taylor, L.P. Pike, D.A. & Theologis, A. (1991): Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* **254:** 437–439.

Parthier, B., Brückner, C., Dathe, W., Hause, B., Herrmann,. G., Knöfel, H.-D., Kramell, H.-M., Kramell, R., Lehmann, J., Miersch, P., Reinbothe, S., Sembdner, G., Wasternack, C. & Zur Nieden, U. (1992): Jasmonates: metabolism, biological activities, and modes of action in senescence and stress responses. In: Karssen, C.M., Van Loon, L.C. and Vreugdenhil, D. (eds): *Progress in Plant Growth Regulation*. Kluwer, Dordrecht, The Netherlands. (in press).

Potrykus, I. (1991): Gene transfer to plants: assessment of published approaches and results. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 205–225.

Raskin, I., Ehman, A., Melander, W.R. & Meeuse, B.J.D. (1987): Salicylic acid: a natural inducer of heat production in *Arum* lilies. *Science* **237:** 1601–1602.

Reid, J.B. (1990): Gibberellin synthesis and sensitivity mutants in *Pisum*. In: Pharis, R.P. and Rood, S.B. (eds): *Plant Growth Substances 1988*. pp. 74–83. Springer-Verlag, Berlin.

Rogers, J.C. (1985): Two barley alpha amylase gene families are regulated differently in aleurone cells. *J. Biol. Chem* **260:** 3731–3738.

Rogers, J.C., Lanahan, M., Rogers, S.W. & Mundy, J. (1992): The gibberellin response element: a DNA sequence in cereal α-amylase gene promoters that mediates GA and ABA effects. In: Karssen, C.M., Van Loon, L.C. and Vreugdenhil, D. (eds): *Progress in Plant Growth Regulation*. Kluwer, Dordrecht, The Netherlands (in press).

Ryan, C.A. & Farmer, E.E. (1991): Oligosaccharide signals in plants: a current assessment. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 651–674.

Sachs, M.M. & Ho, T.-H.D. (1986): Alteration of gene expression during environmental stress in plants. *Annu Rev. Plant Physiol.* **37:** 363–376.

Schäfer, E., Bruns, B., Frohnmeyer, H., Hahlbrock, K., Harter, K., Merkle, T. & Ohl, S. (1990): Phytochrome in plant cell cultures. In: Nijkamp, H.J.J., Van der Plas, L.H.W. and Van Aartrijk, J. (eds): *Progress in Plant Cellular and Molecular Biology*. pp. 355–365. Kluwer, Dordrecht, The Netherlands.

Schell, J.S. (1987): Transgenic plants as tools to study the molecular organization of plant genes. *Science* **237:** 1176–1183.

Scott, I.M. (1990): Plant hormone response mutants. *Physiol. Plant.* **78:** 147–152.

Skoog, F. & Miller, C.O. (1957): Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. *Symp. Soc. Exp. Biol.* **11:** 118–131.

Skriver K. & Mundy, J. (1990): Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* **2:** 503–512.

Smart, C.M., Scofield, S.R., Bevan, M.W. & Dyer, T.A. (1991): Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. *Plant Cell* **3:** 647–656.

Smith, C.J.S., Watson, C.F., Ray, J., Bird, C.R., Morris, P.C., Schuch, W. & Grierson, D. (1988): Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* **334:** 724–726.

Smith, C.J.S., Watson, C.F., Morris, P.C., Bird, C.R., Seymour, G.B., Gray, J.E., Arnold, C., Tucker, G.A., Schuch, W., Harding, S. & Grierson, D. (1990): Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Plant Mol. Biol* **14:** 369–379.

Sommer, H., Beltrán, J.-P., Huijser, P., Pape, H., Lönning, W.-E., Saedler, H. & Schwarz-Sommer, Z. (1990): *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. *EMBO J.* **9:** 605–613.

Steinbiss, H.H. & Davidson, A. (1989): Genetic manipulation of plants: from tools to agronomical applications. *Sci. Prog., Oxf.* **73:** 147–168.

Trewavas, A. (1991): How do plant growth substances work? II. *Plant Cell Environ.* **14:** 1–12.

Ulrich, T.H. & Key, J.L. (1988): Comparative analysis of polyadenylated RNA complexity in soybean hypocotyl tissue and cultured suspension cells. *Plant Physiol.* **86:** 482–490.

Van der Krol, A.R., Lenting, P.E., Veenstra, J., Van der Meer, I.M., Koes, R.E., Gerats, A.G.M., Mol,

J.N.M. & Stuitje, A.R. (1988): An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature* **333**: 866–869.

Van der Linde, P.C.G., Bouman, H., Mennes, A.M. & Libbenga, K.R. (1984): A soluble auxin-binding protein from cultured tobacco tissues stimulates RNA synthesis in vitro. *Planta* **160**: 102–108.

Van Loon, L.C. (1976): Systemic acquired resistance, peroxidase activity, and lesion size in tobacco reacting hypersensitively to tobacco mosaic virus. *Physiol. Plant Pathol.* **8**: 231–242.

Van Loon, L.C. (1983): Mechanisms of resistance in virus-infected plants. In: Bailey, J.A. and Deverall, B.J. (eds): *The Dynamics of Host Defence* pp. 123–190. Academic Press Australia, Sydney.

Van Loon, L.C. (1985): Pathogenesis-related proteins. *Plant Mol. Biol.* **4**: 111–116.

Van Loon, L.C. (1988): Stress proteins in infected plants. In: Kosuge, T. and Nester, E.W. (eds): *Plant-Mirobe Interactions; Molecular and Genetic Perspectives* **3**: 198–237. McGraw-Hill, New York.

Van Loon, L.C. & Antoniw, J.F. (1982): Comparison of the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance in tobacco. *Neth. J. Plant Pathol.* **88**: 237–256.

Varner, J.E. (1977): Hormonal control of protein synthesis. In: Bogorad. L. and Weil, J.H. (eds): *Nucleic Acids and Protein Synthesis in Plants.* pp.293–307, Plenum Press, New York.

Verma, D.P.S. & Goldberg, R.B. (eds) (1988): *Plant Gene Research; Temporal and Spatial Regulation of Plant Genes.* Springer-Verlag, Wien.

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., Van den Elzen, P.J.M. & Cornelissen, B.J.C. (1991): Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans. Plant Cell* **3**: 619–628.

Woolhouse, H.W. (1978): Senescence processes in the life cycle of flowering plants. *Bioscience* **28**: 25–31.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A. & Raskin, I. (1991): Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell* **3**: 809–818.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. & Meyerowitz, E.M. (1990): The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature* **346**: 35–39.

Yoshihara, T., Omer, E.-S.A., Koshino, H., Sakamura, S., Kikuta, Y. & Koda, Y. (1989): Structure of a tuber-inducing stimulus from potato leaves (*Solanum tuberosum* L.). *Agric. Biol. Chem.* **53**: 2835–2837.