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Iacr Report for 1998

Full Table of Content

Inherent Plant Productivity

Rothamsted Research

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Inherent Plant Productivity

P.R. SHEWRY

The yield, composition and end use properties of arable crops are determined by genetic and environmental factors and by the interactions between these. Work within the theme aims to dissect these factors in detail using a multi-disciplinary approach which combines physiological, biochemical, biophysical, cell biological and molecular genetic approaches. Studies are focused on the major arable crops grown in the UK (cereals, oilseeds, potatoes and sugar beet) with underpinning work on model plant species (notably *Arabidopsis* and tobacco) where appropriate. Studies of weed species, which are less welcome components of arable farming systems, are also included. Genetic transformation is being used in almost all the individual projects, both as a tool to explore the control of plant development and metabolism and to manipulate specific characteristics in order to improve crop performance and end use properties. The projects within the theme can be divided into five broad areas and selected highlights within these are reported below.

GENE TECHNOLOGY

Gene technology underpins the whole of molecular biology. With the advent of both physical and functional genomics, gene technology has undergone a revolution in both its scale and future possibilities. Through the efforts of the Cereal Transformation and Crop Genetics Groups, the Institute has established teams which are at the forefront of their respective areas. Both Groups are involved in a number of collaborative programmes with internal and external research teams. Significantly, a large number of the external collaborations involve international agri-seed companies. They are therefore well placed to take advantage of current and future developments within this exciting area.

Within the Crop Genetics Group, several international collaborative programmes are utilising both microsatellite markers and allele specific oligonuceleotides (ASOs) to develop rapid, cost-effective screening systems for the large-scale analysis of natural and man-made plant populations. For example, the Group has utilised the large number of available maize microsatellite markers to develop a prototype DNA chip . Although in the early stages of development, the chip is already proving to be much easier to use than the existing simple sequence repeat markers and should be more amenable to automation. It is intended to expand this technology to include wheat, brassica, sunflower and sugar beet for all of which the Group has developed numerous microsatellite markers.

Through the EU Framework IV Map Maize Programme coordinated by IACR-Long Ashton, the Crop Genetics Group has established a world-wide reputation for the development and utilisation of large insert YAC and BAC libraries to characterise the maize genome. In a collaboration which involves UK, EU and US laboratories, the Group has established for the first time that the *Rp1-D* disease resistance locus covers approximately 450 kb of chromosome 10 and includes seven copies of the NBS-LLR gene. It is this gene which is responsible for the resistance phenotype.

INSTITUTE OF ARABLE CROPS RESEARCH REPORT 1998

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In 1997, the Crop Genetics Group used the extensive facilities of the Long Ashton site to grow 5000 *Mutator* active maize plants. The seed for these plants had been specially bred at Long Ashton both to contain a large number of active *Mutator* elements and to cope with the vagaries of the UK summer. Seed collected from these plants is now a significant resource for a number of functional genomics-related programmes. For example, in collaboration with the Embryo Development Group, a high throughput screen, based upon hybridisation, is being developed to identify genes specifically involved in dormancy and genes whose sequence is conserved between rice, maize and Arabidopsis. In another collaboration with Professor Schuch at Zeneca Plant Sciences, the Group has identified over 60 interesting 'gene knock outs'. Both these and the other gene knock outs produced from this resource are now the subject of intense activity within several internal and external groups.

Fig. 2 *Partly harvested field trials of genetically modified wheat growing at Rothamsted in 1998*

The Cereal Transformation Group has developed technologies for transforming wheat, barley and tritordeum (a novel cereal species combining the genomes of durum wheat and wild barley) by particle bombardment and the regeneration of embryonic callus. These technologies are used to study the expression, stability and inheritance of genes conferring improved agronomic performance and end use quality and other valuable traits. In 1998 the first IACR field trials of genetically modified wheat were carried out in parallel at Long Ashton and Rothamsted (Fig. 2). The trials involved breadwheat which was modified with a gene that encodes the high molecular weight subunit of glutenin. It is these subunits that determine the strength (elasticity) of bread dough. Preliminary results of the trials show that field-grown plants express the glutenin transgenes in the same way as the plants grown in controlled environments.

One important challenge facing cereal transformation is the lack of well characterised tissue-specific and developmentally-regulated promoters. This lack is particularly acute in wheat and barley, which have lagged behind rice and maize as the focus of gene modification technology. The Cereal Transformation Group has used a promoter tagging approach together with direct DNA transfer to identify such promoter regions. Promoterless *uidA* constructs were used to transform tritordeum. Four of eight transgenic lines expressing *uidA* showed evidence that a regulatory sequence had been tagged. Analysis of these lines revealed a range of expression patterns including one line with expression restricted to the anther primordia within the developing spikelet (Fig. 3). The sequences upstream of this tag showed homology to previously identified MADS-box proteins, many of which have been implicated in flower organ identity. Cloning of this sequence represents the first report of a MADS-box gene promoter in cereals. A second line showed constitutive GUS expression, which may identify a novel wheat constitutive promoter.

Fig. 3 *A developing spikelet of tritordeum showing anther-specfic expression of GUS - one of the lines generated by a promoter-tagging experiment*

Although particle bombardment is the standard technique for introducing genes into cereals, the Group is interested in developing alternative methods to improve efficiency and robustness and to reduce cost. One such alternative method is *Agrobacterium*-mediated transformation. The use of *Agrobacterium* species to introduce genes into dicotyledonous plants has been routine for many years. However, until recently, wheat has remained recalcitrant to *Agrobacterium*-mediated gene transfer. The Group has initiated two projects aimed at understanding factors controlling *Agrobacterium*-mediated transformation in wheat, and applying the developed protocols to a wide range of germplasm. Early work has focused on optimising *Agrobacterium* strain/vector combinations, identifying explant

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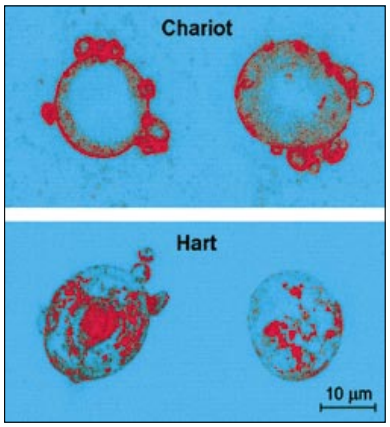

Fig. 4 *Immunochemical localisation of friabilin on the surface of starch granules prepared from soft (cv. Chariot) and hard (cv. Hart) cultivars of barley, revealed by confocal laser scanning microscopy with friabilin shown in red*

tissues such as inflorescence or scutellum which are amenable to transformation and investigating the effects of wounding and other physical treatments to explants prior to co-cultivation.

Keith Edwards - (IACR-Long Ashton) and Huw Jones - (IACR-Rothamsted)

SEED BIOLOGY

Oleosins and oil body biogenesis

Oleosins are a unique group of proteins present only on the surface of oil bodies, where they are thought to stabilise the structure and prevent coalescence. The proteins contain a central hydrophobic domain of amino acids which is thought to insert within the triacylglycerol matrix of the oil body. Fourier-transformed infra-red spectroscopy of intact oil bodies, carried out in collaboration with Dr Klaus Wellner of the Institute of Food Research, has shown that this domain adopts an α-helical conformation, disproving the generally accepted β-sheet model. This has been confirmed by spectroscopic analyses of the hydrophobic domain expressed in *E.coli* and a model structure comprising two coiled α-helix separated by a proline-rich turn region has been developed in collaboration with the Department of Biochemistry, University of Bristol .

Although the oil body is the final destination of the oleosin, the precise targeting and deposition pathway of this protein has until recently remained obscure. However, experiments using a cell-free *in vitro* targeting assay indicated that the oleosin protein was initially synthesised on the rough endoplasmic reticulum (ER) membrane, implying that the ER was likely to be the initial site of deposition. To corroborate these *in vitro* observations, the oleosin was expressed in both wild type and ER-targeting-defective sec mutants of yeast. This confirmed the role of the ER membrane in the targeting and accumulation of the oleosin, and more precisely, indicated a role for signal recognition particle-mediated co-translational insertion into the membrane. Therefore, the initial step in the targeting of the oleosin to the oil body involves insertion into the ER. Since the ER is also the site of storage lipid synthesis, it is likely that the deposition of oleosins and triacylglycerols is co-ordinated in such a way as to facilitate the biogenesis of the oil body.

Friabilins and grain texture

Grain texture (hardness) is a major determinant of the milling and processing properties of wheat and may also affect the malting quality of barley. It is thought to be determined by the degree of adhesion between the starch granules and the protein matrix with stronger binding in hard grain resulting in a greater energy input and more starch damage during milling. Published studies have shown that water-washed starch granules from soft wheats contain high levels of a low molecular mass group of surface associated proteins, called 'friabilins', and it has been proposed that these act as a 'non-stick' layer preventing adhesion to the matrix proteins. We have used a monoclonal antibody to friabilin and confocal laser scanning microscopy to confirm that friabilins are indeed present on the surface of starch granules prepared from soft textured varieties of wheat and barley (Fig. 4) using a dry sieving procedure, demonstrating that redistribution does not occur during water washing. Similar levels of friabilins were associated with granules prepared from hard textured cultivars of the two cereals, but in these the friabilins were associated with matrix proteins. This different distribution of friabilins in hard and soft textured grain is consistent with a role in determining grain texture, but the molecular basis for the binding properties is still not known.

Manipulation of embryo dormancy in wheat

The normal course of seed development in wheat involves embryogenesis, the accumulation of

Fig. 5 *Lucy Andrew (Sandwich Student from the University of Bath) and the first generation of transgenic wheat plants expressing the wild oat (*Avena fatua*) afVP1 gene*

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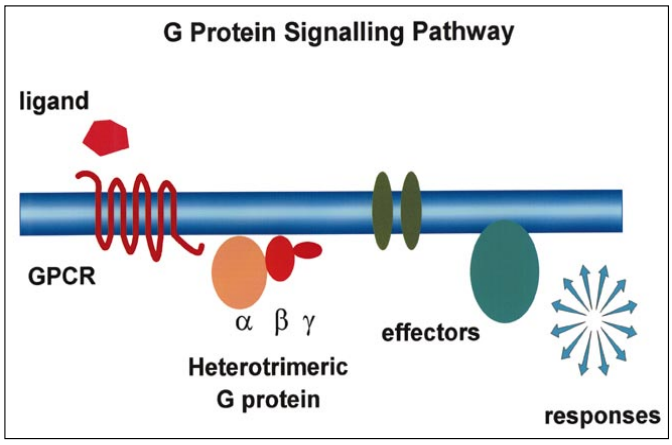

Fig. 6 *Diagram depicting a G protein signalling pathway. G protien coupled receptors (GPCRs) are integral membrane proteins with an extracellular N-terminus and intracellular C-terminus. They have seven transmembrane spanning domains connected by alternate intracellular and extracellular loops. Extracellular signals are perceived by GPCRs which become activated altering their conformation. Heterotrimetric G proteins comprise α, β and γ subunits. They interact with activated GPCRs causing the G_α subunit to exchange bound GDP for GTP. The G_α-GTP subunit dissociates from the β/γ complex. Each of these components is then capable of stimulating down stream signalling molecules such as ion channels or enzymes. Inherent GTPase activity of the G_α subunit restores the GDP-bound form which re-associates with the β/γ complex and is thus available for reactivation. Activated GPCRs can stimulate numerous cycles of GTP exchange by G proteins thus amplifying and transducing the signal across the cell membrane*

storage reserves in both the embryo and endosperm, seed desiccation and the induction of embryo dormancy during which seeds fail to germinate under otherwise favourable conditions. In certain circumstances, these processes can be bypassed allowing precocious expression of the germination pathway, as occurs in the viviparous mutants of maize that are blocked in either the production of, or responsiveness to, the plant hormone, abscisic acid (ABA). The Viviparous 1 (*VP*1) gene of maize encodes a transcription factor that upregulates other genes involved in the embryo-maturation program, whilst repressing germination-related genes, such as α-amylase.

The similarity of the phenotypes of the maize viviparous mutants and pre-harvest sprouting (PHS) in temperate cereals prompted a search for orthologues of the maize *VP*1 gene in wheat. The level of expression of a wild oat (*Avena fatua*) gene (*afVP*1) was directly related to the degree of embryo dormancy of different lines of this species at maturity and was upregulated during the induction of secondary dormancy, suggesting that this gene may have a similar function in wheat.

A cDNA library, generated from 35-day-old developing wheat embryos, was used to clone cDNAs representing the wheat *VP*1 genes (*taVP*1). In collaboration with colleagues at the John Innes Centre, Norwich, *taVP*1 was mapped to the long arm of chromosome 3 at a similar position to *VP*1 in maize, confirming synteny for this gene between the two species . Significantly, the map position of *taVP*1 was resolved from that of the *R* locus, which regulates pericarp colour and coat-imposed dormancy in wheat. Preliminary expression analysis of *taVP*1 transcripts showed similar abundance in developing embryos of two genotypes grown under standard controlled environment conditions. However, artificial wetting of ears during grain development induced PHS and an absence of *taVP*1 transcripts in sprouted embryos, showing that loss of wheat embryo dormancy is associated with a decrease in *taVP*1 gene expression.

A targeted approach to the generation of new wheat genotypes with altered dormancy characteristics has been undertaken, in collaboration with colleagues in the Cereal Transformation Group at Rothamsted. Transgenic wheat plants that should be more or less susceptible to PHS have been generated by either down-regulating the endogenous *taVP*1 genes or overexpressing *afVP*1 (Fig.5), thus providing a direct test of whether or not embryo dormancy can be successfully manipulated in transgenic wheat.

Peter Shewry and John Lenton - (IACR-Long Ashton)

SIGNALLING SYSTEMS AND THEIR INTEGRATION

One of the most highly conserved mechanisms that enables cells to sense and respond to changes in their environment is the G protein signalling pathway. Primary components of this are cell surface G protein-coupled receptors (GPCRs) that perceive extracellular ligands, and heterotrimeric G proteins (G proteins) that transduce information from activated GPCRs to down-stream effectors (Fig.6). It has been suspected that a signalling mechanism of this type operates in higher plants. Recent evidence we have obtained strengthens this concept and suggests that G proteins and a novel GPCR may be involved in transducing the plant hormones gibberellin (GA) and cytokinin.

Cereal aleurone cells respond to GA by expressing genes encoding a variety of hydrolases, including α-amylase. We examined the possible role of G proteins in GA signalling in aleurone using the wasp venom peptide analogue Mas7 that mimics GPCRs by stimulating GDP/GTP exchange by G proteins. We found that Mas7 stimulates aleurone protoplasts to produce and secrete α-amylase in a dose-dependent manner. Mas7 induces α-amylase mRNA and expression of an *α-Amy2/54:GUS* promoter:reporter construct. The inactive MasCP, differing from Mas7 by a single amino acid substitution, and Mas7-COOH, in which a free acid

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replaces the amine group at the C terminus, do not induce α-amylase. We obtained further evidence that GA signalling may involve a G protein by studying the effects of hydrolysis-resistant guanine nucleotides on GA-induction of *α-Amy2/54:GUS* expression. The hydrolysis-resistant GTP-γ-S and GDP-β-S hold G_α subunits in either the activated (GTP-γ-S-bound) or inactivated (GDP-β-S-bound) forms. GDP-β-S completely prevented GA induction of *α-Amy2/54:GUS* expression, whereas GTP-γ-S stimulated expression slightly. We also cloned a partial G_α subunit cDNA (*$AfG_\alpha1$*) and two related G_β cDNAs (*$AfG_\beta1$* and *$AfG_\beta2$*) from wild oat aleurone and confirmed that these are expressed in aleurone cells. Taken together these observations suggest that a G protein or proteins are involved in GA signal transduction in aleurone.

Because higher plants appear to have functional G proteins these might be regulated by GPCRs. We recently identified the first plant homologue of the GPCR superfamily. The *GCR1* cDNA encodes a 326 amino acid polypeptide with up to 23% amino acid identity (53% similarity) to known GPCRs. Hydropathy analysis indicates that GCR1 has seven potential transmembrane spanning domains and membrane topology prediction algorithms support a structure characteristic of GPCRs (Fig.7). Antisense suppression of *GCR1* expression in transgenic *Arabidopsis* gave rise to a specific reduction in sensitivity to the cytokinin benzyladenine in both root and shoot tissues, suggesting a role for GCR1 in the perception or transduction of this plant hormone. At present it is not known if GCR1 is a cytokinin receptor. It is equally likely to be a downstream component in cytokinin signalling, or a receptor for another ligand, the signalling pathway for which interacts with cytokinin signalling. In fact, another membrane protein, CKI1, that has sequence similarity to histidine-kinase two-component response-regulators, is also a candidate cytokinin receptor. Further research should help elucidate the function of, and relationship between, these molecules.

Higher plants have three essential components of a G protein signalling pathway, GCR1, a putative GPCR, a putative G_α subunit and putative G_β subunits. Further research will test the possibility that these components, or polypeptides related to them, may interact with one another as elements of a plant G protein signal transduction pathway. These new advances cast light on some of the earliest events that take place in plant hormone signalling systems and have identified new molecular targets for controlling specific aspects of plant growth and development.

Richard Hooley - (IACR-Long Ashton)

DEVELOPMENTAL AND CELL BIOLOGY

The complex nature of the tissues and cells which comprise the secondary vascular system (wood) can only be satisfactorily resolved by three-dimensional microscopy. This is also true of subcellular structures, such as the microtubular and actin cytoskeletons. The recent purchase of a confocal laser scanning microscope (CLSM) is proving useful and productive in furthering both the anatomical and cytological studies of woody cells and the meristem (cambium) which produces them. With respect to anatomical studies Figure 8 shows how fibre cells in wood are intermeshed within the water-conducting woody xylem tissue. The sinuous path of the fibres is indicative of their ability to slide between cells by means of tip growth. As a result, fibres fill up any spare intercellular space and hence confer additional mechanical strengthening upon the woody or root system. Note also the helical arrays of microtubules, revealed by immunofluorescence. These are responsible for the deposition of secondary wall in these fibre cells.

Previously it was difficult to see how microtubules and actin filaments related either to each other or to the topography of intracellular structures such as pit fields and wall thickenings, but now this is possible in a routine way as shown in Fig 9. Pit fields, simple pits and bordered pits are all areas of cell wall where the process of secondary thickening has been excluded. The resulting wall structures (pit domains) are used for water transport and cell communication.

Pit formation in vessels commences with a localised separation of microtubules within their characteristically dense spiral arrays. Each resulting 'eye' of microtubule-free cortex then becomes surrounded by a ring of microtubules. No wall deposition occurs within the 'eye' sites, which become the future pits. Deposition does, however, continue in the immediately surrounding area of the 'eye', where it is supported by the microtubule ring. This then builds the rim of the pit. 'Eye' formation is also reinforced by actin, which co-localises with the microtubules: together, these two cytoskeletal elements form a dynamic contractile ring which can regulate the size and orientation of the pit. The factors that determine where and when microtubular 'eyes' form are unknown, but it is likely that motor proteins are responsible in some way. Such proteins are also probably deployed in the further cytoplasmic structuring at the pit which

Fig. 7 *The predicted membrane topology of the G protein coupled receptor GCR1*

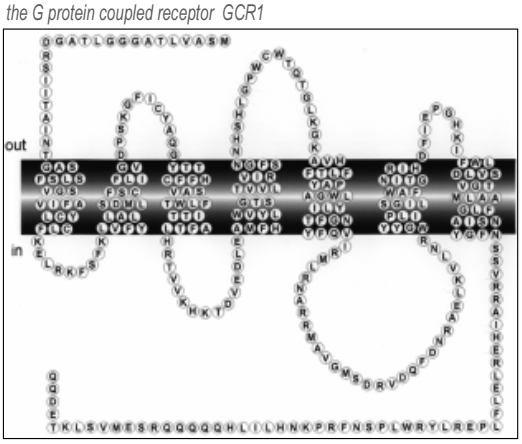

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Fig.8 *Confocal laser scanning microscope image of fibres from a stem of* Aesculus *(horse chestnut). Green flourescence highlights the microtubular cytoskeleton, red flourescence is due to the autoflourescence of cell walls*

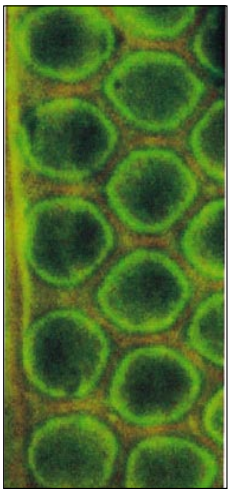

Fig. 9 *Confocal laser scanning microscope image of developing bordered pits in vessel of* Aesculus *(horse chestnut). The identities of the flourescent images are as in Fig. 8*

contributes to its function. Elsewhere in the cell, other aggregations of microtubules are instrumental in fabricating, first the thickened secondary wall (responsible for strengthening the vessel), and later, the specialized tertiary wall thickenings (responsible for some of the hydrodynamic properties of the water that flows through these vessels).

Root hairs play a crucial role in the uptake of nutrients and water into plants. *Arabidopsis* root hairs are also an increasingly important system for studies of higher plant cell biology, because they are accessible, transparent, and amenable to a variety of powerful research techniques. Root hairs form by tip growth, a precise mechanism where growth is localised to a small region of the cell. We are investigating how genes contribute to plant cell growth using mutants with altered root hair formation. One gene that has been identified by mutation, *TIP1*, is being isolated and its DNA sequenced. This is part of a project to understand the molecular basis of *TIP1* function. The requirement for *TIP1* is particularly strong in the tip-growing cells of root hairs and pollen tubes. The appearance of root hairs carrying loss of function mutations in the *TIP1* gene , suggests that the normal function of *TIP1* is to enable root hairs to grow long and narrow without branching. This is similar to the role of the *COW1* gene, which we reported last year [1]. Double mutants, carrying mutations in both the *TIP1* and *COW1* genes, have shorter and more branched root hairs than either single mutant, suggesting that these two genes act independently of each other during root hair growth. Mutations in the *TIP1* gene are not specific to tip-growing cells, and affect growth in every cell of the *Arabidopsis* plant. This shows that some mechanisms involved in tip growth are also involved in diffuse growth, which is the other main method of plant cell growth. As a first step towards isolating the *TIP1* gene, we have identified the location of *TIP1* on the top arm of chromosome 5 (Fig. 10).

[1] IACR Report for 1997, 13.

Peter Barlow and Claire Grierson - (IACR-Long Ashton)

RESOURCE CAPTURE AND ALLOCATION

Leaves power life. Photosynthesis is an integrated process in which resources are captured and assimilated. Chief among these are light, water, carbon, nitrogen, sulphur and phosphorus. All biomass production depends either directly or indirectly on light-driven photosynthetic electron transport processes. However, biomass production is not simply dependent on resource abundance. The partitioning of resources within each cell and within the plant is also crucial. We are seeking to understand the control of this partitioning with a view to improving the efficiency and sustainability of arable crops.

Co-ordinate use of different resources in leaves

Numerous studies have attempted to reconcile photosynthetic energy budgets solely in terms of assimilation of carbon. While carbon assimilation is incontestably the major sink for energy, variability in assimilatory quotients (net CO_2 fixed to net O_2 evolved) provides clear evidence for other processes in the illuminated photosynthetic cell (Fig. 11). Typical foliar carbon:nitrogen (C:N) ratios in plants growing under optimal N supply are in the region of seven. Although these ratios may seem to discount a marked influence of N assimilation on photosynthesis, closer examination reveals that this is not the case. Firstly, photosynthetic N assimilation requires C skeletons. Secondly, mole for mole, it requires more reductant than C assimilation. Our current studies consider the following questions: (i) the influence of N assimilation on gas exchange; (ii) the effect nitrate, nitrite and 2-oxoglutarate have as electron acceptors; (iii) likely rates of respiration necessitated by N assimilation and the potential contribution this may make to cellular ATP synthesis. A major question concerns the extent to which photosynthesis and respiration must interact to achieve maximal resource use efficiency over a wide range of environmental conditions. Such features ultimately determine the success or failure of manipulations attempting to improve resource use efficiency.

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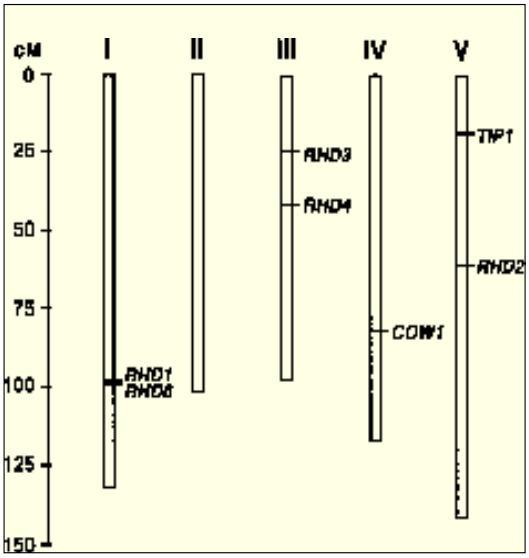

Fig. 10 *The Location of* TIP1 *and other genes involved in root hair formation on the* Arabidopsis *genetic map*

Improving the efficiency of nitrogen use

Crop breeding programmes have used plants growing under optimised conditions, when most resources are not limiting. Fertiliser applications ensure that nutrients are present in the soil at luxury concentrations. One consequence of this excess supply is that nutrient is leached from the soil. A principal challenge will be to engineer crops able to intercept and allocate resources more efficiently.

In wheat, we are attempting to increase the efficiency of N use and thereby decrease the requirement for fertilisation. This strategy involves manipulation of cytosolic glutamine synthetase activity. In collaboration with Professor R. Leigh, University of Cambridge, transformed wheat has been produced showing stable inheritance of the transgene (Fig. 12). At least one of the lines has increased cytosolic glutamine synthetase. CO_2 assimilation and photorespiration were also increased, indicating a change in nitrogen metabolism. We are now measuring nitrogen-use efficiency in this line, grown with varying amounts of N and CO_2 supply.

A second strategy aims to generate transgenic wheat with less of the primary CO_2-assimilating enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase, the most abundant protein in plants. Decreases of between 10% and 40% will confer greater N-use efficiency under current and future atmospheric CO_2 concentrations. These decreases are predicted not to affect whole plant photosynthesis.

Fig. 11 *The major flows of carbon in the photosynthetic cell. Inorganic substrates are CO_2, O_2 and nitrate. Starch and sucrose are shown in the compartments in which they are synthesised; amino acid synthesis, which can occur in several compartments, is shown as being cytosolic for reasons of diagrammatic simplicity*

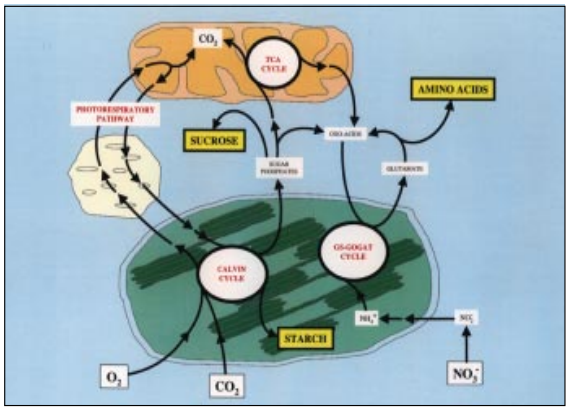

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Fig. 12 *Transformed wheat overexpressing cytosolic glutamine synthetase*

Sugar sensing in plants

As a major product of photosynthesis, sucrose acts as a regulatory signal that affects gene expression, growth and development. Photosynthetic capacity, resource allocation and yield are greatly influenced by this signal molecule. In order to realise the full potential of increased photosynthesis, the use of sucrose by sink organs (roots, seeds, fruits) must accompany any increase in photosynthetic capacity. If not, sugar accumulates in leaves and the consequent decrease in photosynthetic gene expression causes feedback regulation of photosynthesis. This effect may be even more important in the future, because of higher sugar contents due to increased environmental CO_2. A complete understanding of these signal mechanisms is necessary to allow effective targeting in the quest to achieve full resource use efficiency.

Arabidopsis mutants that, unlike the wild-type, will germinate on high concentrations of different sugars, are being used to identify the signal transduction pathways and their impact on the whole organism. Early indications are that networks of signalling mechanisms exist involving many other fundamental plant processes. Mutants selected in this way show similar rates of photosynthesis to the wild-type when grown in air, since sugars do not accumulate in the leaves. We have tried to induce sugar accumulation by growing plants at high CO_2 (700-3000ppm). Once conditions causing phenotypic alteration are identified (Fig. 12), we will be able to screen for expression of genes responsible for the sucrose insensitivity in the mutants.

Trehalose is a disaccharide like sucrose, but consisting of two glucose units rather than a glucose and a fructose unit. We are currently comparing the regulatory roles of the two sugars in the leaf's response to carbohydrate accumulation and allocation.

Christine Foyer - (IACR-Rothamsted)