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The evolution of plant biochemistry and the implications for physiology

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Introduction

Plant physiology and plant biochemistry are seen by some as brother and sister but by others as distant cousins. All would agree that there is a relationship but few would agree how close it is. The fact that books have been written on plant biochemistry with very little discussion of the physiology of plants shows that families can lose touch with each other. The aim of this chapter is to show that a functional understanding of plants can be gained by integrating physiological and biochemical knowledge. Our attempts to bring about integration start with an evolutionary perspective.

When introductory biochemistry is taught, there is a tendency initially to concentrate on the central metabolic pathways of 'the cell'. The default cell is usually mammalian. Plant cells are usually treated as unusual, but only in so far as they have walls and chloroplasts. This emphasis on the commonality of the basic metabolism of organisms is helpful to those learning the subject but it does tend to limit conceptual approaches to biochemistry. The reality is that most cells are biochemical specialists. Evolution has selected for biochemical traits of cells that are appropriate for their particular cellular environment in a manner analogous to the selection of organisms that are more suited to their environment. This specialization is found in both unicellular and multicellular organisms. However, in multicellular organisms, higher order coordination and control – physiological processes – can have a marked effect on the environment of specialist cells. To provide this coordination, specialist cells have evolved to play a central role in controlling many plant physiological processes (e.g. gas exchange is regulated by the guard cells, abscission depends on the abscission layer, seed reserve mobilization in grasses is dependent on the aleurone cells, etc.). Consequently there will be very clear links between physiological and biochemical processes. In this chapter we will argue that the selection pressures operating on the evolution of metabolism will have given rise to certain metabolic traits and, because of the intimate links between biochemistry and physiology, those traits have helped shape physiological processes. The intimate connection between the physiology and biochemistry of plants is well established in the case of some aspects of physiology, especially where there are dramatic morphological and anatomical adaptations which make the links clear – photosynthesis, germination, stomatal functioning, for example – and the links between the physiology and biochemistry of these processes will not be considered here. Instead, some more general principles concerning the evolution of metabolism will be discussed. These simple principles will then be used to consider the links between metabolism and physiology in cases where the anatomical and morphological signposts are less clear.

Molecular evolution, biochemical evolution and metabolic evolution – hierarchical terms

The term *molecular evolution* is currently used to refer to the way in which the evolution of protein structure relates to protein function, frequently with emphasis on tracing the lineage of base sequences in specific genes. Those working on molecular evolution generally focus on the role of one protein (or family of proteins). *Biochemical evolution* overlaps with molecular evolution but can extend to consider more than one enzyme in a pathway and the control of that pathway. *Metabolic evolution* could be considered to operate at the next higher level, determining the way in which the whole of metabolism evolves, with extensions or deletions of metabolic steps being the result of descent via modification in lineages subjected

to natural selection. It is *metabolic evolution* that determines the scope of metabolism in an organism, with biochemical evolution and molecular evolution determining the degree to which operation of the enzymes and their controlling elements effect organismal fitness. It is *metabolic evolution* on which we focus, because it is at this level that links between physiology and biochemistry are most apparent.

Metabolic evolution – what determines whether a new enzyme is retained?

New enzymes usually arise as a result of gene duplication and subsequent mutation of one gene copy such that the mutated enzyme has an altered substrate tolerance and can act on a new substrate to produce a new product (Petsko *et al.*, 1993). Whether that new metabolite enhances the fitness of the producer depends entirely on whether the benefits of possession of the new product exceed the costs of producing it. In turn, the benefits depend upon the properties that the new metabolite brings to the organism. We identify three major classes of property that natural selection appears to have favoured during metabolic evolution.

Biomolecular activity – the evolution of ‘secondary metabolism’

It has been well established from screening trials of collections of synthetic or naturally occurring molecules that the probability of any individual chemical possessing potent biological activity is very low (Jones and Firn, 1991; Firn and Jones, 1996). We proposed that this fact must have been a great constraint on the evolution of pathways leading to molecules that benefit their maker by possessing biological activity. However, the relevance of this fact to the understanding of the evolution of secondary metabolism has been challenged (Berenbaum and Zangerl, 1996). Berenbaum and Zangerl argued that the analogy between humans screening for a useful biological activity and organisms evolving chemical diversity in order to gain fitness by making biologically active chemicals was inappropriate – the nub of their argument was that vagueness in the definition of the term ‘biological activity’ led to a false analogy. However, by defining the term *biomolecular activity* quite precisely (as the ability of a molecule to interact with a biologically functional molecule such that its biological function is significantly changed), Firn and Jones (2000) countered this objection and provided evidence that the low probability of any chemical possessing potent *biomolecular activity* is a predictable and well understood consequence of ligand–protein interactions. Consequently, they reiterated a refined argument stating that the low probability of specific ligand–protein binding has been a significant evolutionary constraint on the production of biologically active molecules by organisms. Humans and other organisms may adopt different means of trying to reduce the impact of this fundamental constraint but they have both had to evolve ways of doing so. The methodology that humans have used when seeking to exploit the biological activity of chemicals (for example as pesticides or pharmaceuticals) has been the development and use of screening trials. These trials provide useful data about the frequency of any particular biological activity occurring in random collections of chemicals. Because such trials have been used to screen synthetic and naturally-occurring chemicals, and because they have been used against a wide range of biological targets, the information available from such trials is very extensive.

Do screening trials reveal any other relevant information? Indeed they do. They show that the type of biomolecular activity possessed by any individual chemical is unpredictable. Humans synthesizing new chemicals to test for biological activity are often surprised that a chemical made with the intention of producing one type of biological activity actually turns out to possess a very different, equally valuable activity. The discovery of Viagra is a recent well known example of human serendipity, but previous examples abound (Roberts, 1989). The herbicides paraquat and diquat emerged from an observation that a surfactant used in an experimental formulation was surprisingly phytotoxic, which led to some diquaternary dyestuff intermediates being examined in a herbicide screen with the result that the bipyridylum herbicides were discovered. Another example would be that the discovery of the pyrimidine fungicides started with attempts to make insecticides. In other words, the structure of any individual molecule is only partly predictive as to whether a molecule will possess biomolecular activity and is poorly predictive as to the type of biomolecular activity. The successful organism, like the successful company, exploits fortuitous events. Consequently, it is reasonable to predict that as a result of evolutionary adaptation, a pathway in an organism initially leading to one form of biomolecular activity can eventually lead to a quite different form of biomolecular activity. Hence we have two constraints that must be taken into account when considering the evolution of chemical diversity in plants – any new molecule has a very low probability of possessing biomolecular activity and the type of biomolecular activity cannot be reliably predicted from the biomolecular activity (if any) of the chemical(s) from which it derived.

Molecules retained because of their physicochemical properties

The terms 'primary metabolism' and 'secondary metabolism' are very commonly used, however, there are large groups of chemicals in organisms that sit between these two traditional groupings. Lipids, many pigments such as the carotenoids, many polysaccharides and some anthocyanins fall into this category. For example, consider lipids. All cellular organisms need lipids but they do not need them all – as a group lipids are essential but individually they are not. The paradox can be resolved by recognizing that the properties that have been selected for are physicochemical traits – lipophilicity, light absorption, structural properties, etc. These properties depend on the molecular properties shared by large numbers of structurally similar chemicals. Minor changes to part of the molecular structure might predictably make little difference to these properties – this is a marked contrast to the impossibility of predicting how such changes might alter the biomolecular activity of the same molecule. (Once again the analogy with chemicals made by humans is appropriate. A chemist who has synthesized a novel chemical can predict with some confidence its lipophilicity or its spectral properties but cannot reliably make any precise predictions about the biomolecular activity of the molecule.) Thus an organism that gains fitness by making a chemical that protects it against harmful irradiation, might as a result of extending the pathway leading to that pigment, produce another chemical with predictably similar useful properties. Slightly different extensions of the pathway leading from that point might have similar chances of creating new molecules with similar properties to the common precursor hence it is predictable that different species will produce a rather different mix of such chemicals – their overall requirement for a certain mix of chemicals with an average physicochemical property can be met in many different ways. The diversity of molecules selected for their physicochemical properties is thus predictable but the contribution that any one metabolite will make to the overall requirements is unpredictable. Under these

circumstances, it would seem that the advantage given to the producer of a new chemical depends greatly on the existing overall mix of existing similar chemicals.

The diversity of molecules selected for their contribution to the overall physicochemical requirements carries with it a chemical diversity that can be exploited as a route to molecules possessing useful biomolecular activity. Thus it is predictable that compounds with useful biomolecular activity will sometimes arise from pathways usually considered to operate principally for other purposes. Thus the phenylpropanoid pathway can generate chemicals that have a role in absorbing visible or non-visible light but this pathway may also lead to compounds that can enhance the fitness of the producer because they possess useful biomolecular activity (Winkel-Shirley, 2001). Likewise, the isoprenoid pathway can give rise to photoprotecting pigments such as the xanthophylls (Taiz and Zeiger, 1991) or carotenoid pigments in flowers or fruits (Harborne, 1988), yet also gives rise to the plant hormones such as the gibberellins or abscisic acid (Davies, 1995).

Primary metabolism – canalized metabolism, each step depending on other pre-existing metabolic capabilities

For the purpose of this discussion we define ‘primary metabolism’ as metabolic pathways where there is a great interdependence of the individual steps and where the individual molecules made by an enzyme serve only to feed into another enzyme – the complete pathway is greater than the sum of the parts. A significant difference between ‘primary metabolism’ and the previous two categories of metabolism, is that in primary metabolism selection is less environmentally contingent and the evolved homeostatic mechanisms of the organism and of the cell make the selection pressure more constant. At some stage early in the evolutionary history of primary metabolism the incorporation of a new metabolite into the developing primary metabolism could only have occurred if the new molecule fitted usefully into the specific scheme of existing pathways. The actual advantage to the producer of this new metabolite would arise solely from the ability of the new molecule to be acted upon by an existing enzyme(s) to produce another molecule(s) that had properties that enhanced fitness. The most extreme outcome would be the production of a metabolic cycle where there is no single ‘end point’ on which selection can act but the overall net efficiency of the cycle is subject to intense selection. However, in such cycles (e.g. Calvin cycle, the photorespiratory carbon oxidation cycle, the C_4 cycle, etc.), selection that has fitted existing capabilities to local circumstance makes it much harder to introduce a new, compatible transformative capacity. Consequently, the pathway becomes severely canalized – the optimization of the coordinated processes increasingly reduces the opportunities to evolve radically different methods of achieving the same goal. (A dramatic example of canalization would be the genetic code – it is only one possible code of many that could have been used but once organisms evolved with a workable system on which selection could improve, the die was cast.) The powerful selection against new chemical diversity is in contrast to the previous property classes where there is a tolerance of chemical diversity (physicochemical properties) or selection for chemical diversity (biomolecular activity).

Selection for different molecular properties has consequences for metabolic evolution

Although it has been biochemical dogma that enzymes are highly substrate specific, this dogma has largely arisen from studies of enzymes involved in primary metabolism where

canalization is severe. The biochemical properties evident in a highly evolved and specialized metabolism tell us about the outcome of the selection processes operating on that sphere of metabolism and provide little reliable guidance as to the properties of individual new enzymes arising as a result of mutation. We would argue that a mutant enzyme would have a high probability of possessing a broad substrate tolerance initially. The reasoning is that a mutant enzyme that cannot access an existing substrate because it has narrow substrate specificity cannot produce a new product. Hence the enzyme will not be selected for in any circumstance in which new products would confer evolutionary advantage. In contrast, a mutant with a broad substrate tolerance has a greater initial probability of producing new substances with useful properties, irrespective of the type of property considered. However, it is clear that the selection pressures that would operate after the new product(s) have been generated would differ greatly depending on the type of property brought to the cell. For example, gaining fitness by producing biomolecular activity requires chemical diversity to be generated and maintained, hence there might be little selection pressure narrowing substrate tolerance (Jones and Firn, 1991). Indeed, enzymes capable of acting on more than one substrate might bring benefits. So if a new enzyme produces a new lipid that adds to the physicochemical properties of the cell, as long as the enzyme does not generate a chemical with adverse cellular properties, there may be little selection to narrow the initial broad substrate tolerance. Where there will be strong selection for narrow substrate tolerance is in the final property class – participation in primary metabolism. Here an enzyme accessing a common, important intermediate would be expected to have a negative effect on the overall metabolic and homeostatic mechanisms due to substrate competition and the possible generation of compounds that act allosterically to hinder rather than aid metabolic control. These simple concepts predict that metabolic evolution will have produced rather different metabolic traits in different pathways and even at different stages within a pathway.

Biochemical evolution and physiology

Why should the ideas outlined above be of interest to physiologists or biochemists? The fact that these issues have rarely been addressed previously suggests that both physiologists and biochemists have been comfortable working without such general principles for most of the 150 years that these disciplines have existed. In order to help promote interest in building the evolutionary frameworks underpinning the links between plant biochemistry and physiology, we will use two topics as examples of how simple ideas about metabolic evolution could guide experiments:

1. the physiology of chemical interactions between plants and other organisms
2. the physiology of intraplant signalling (plant hormones).

The former area is chosen because it is one where an alternative evolutionary model – based on ecological rather than metabolic considerations – was well developed and widely accepted. That model is now challenged by our metabolic evolutionary model. The second area – the evolution of plant hormonal control – is chosen because plant hormones have been shown to play a role in many very important, basic physiological processes.

The interaction of plants with other organisms

The human experience

Human experience has been both a useful guide and a distraction in understanding the role of plant chemicals in the coevolution of plants with other organisms. Humans are themselves massively influenced by plant chemistry, although most people go about their lives unaware of this fact because plant chemicals are so embedded in most cultures. Few readers will be reading this sentence without some plant chemicals being active in their bodies – it will be the rare reader who starts this chapter (and an even rarer reader who finishes this chapter) without having taken one of the following during the previous few hours – coffee, tea, tobacco, chocolate, a recreational or prescription drug, a tasty wine, a flavoursome beer, a fruit drink or a piece of confectionery. If a reader cannot concentrate on this chapter because their mind would prefer to think of the meal that awaits them, it will not be the expectation of a plate of starch or a piece of protein that excites their mind but the thought of the pasta sauce, the exciting curry, the sharp onions, garlic or interesting green salad. Plant secondary chemicals feed the mind while plant storage products feed the body. The human craving for particular plant chemicals has been so powerful that it has driven colonial expansion in the past and today many national economies are dependent on such chemicals. The power that a few plant species exert over humans by making just a few strange chemicals is quite remarkable and yet traditionally we have considered such chemicals ‘secondary products’! If we delve a little deeper into the human experience with such chemicals we find that humans value such chemicals for different reasons:

1. such chemicals attract or repel humans by acting on sense organs – smell and taste mechanisms have been honed by evolution in many animals just to identify potential food sources and avoid intoxication based on their chemistries. Had there been no chemical diversity in the plant or microbial derived material would we have such fine senses?
2. the ability of these chemicals to influence mental processes (behaviour, well-being, etc) means that we can change our perception of the world
3. less commonly in humans, these chemicals can have a physiological effect, acting on some metabolic pathways in a positive or negative manner.

Each of these apparently different modes of action shares a common feature in that the effect of the chemical at a cellular level is caused by the chemicals binding to a specific receptor. Here the rules of ligand/receptor interactions apply. However, there is one crucial difference in the taste/smell receptors that differentiates them from the neurological and physiological receptors. In the case of the taste/smell receptors, the receptors have evolved to detect ligands and that means that there is potential for coevolution of the ligand/receptor interaction. A pollinator that is attracted to a food source by a plant-derived odour gains fitness if it is a mutant that has an odour binding protein that better matches the structure of the odorous chemical – the evolution of the detection system becomes ‘locked on’ to the chemistry of the producer. In contrast, neurological and physiological receptors are usually ‘fortuitous receptors’ – the receptor proteins have roles unrelated to their fortuitous ability to bind the plant-derived chemical and selection for their primary role would be paramount. If the plant-derived chemical has a serious adverse effect on the organism by interrupting its normal function there will be massive selection pressure to select individuals that make a mutant protein which functions in its original role but which cannot bind

the plant-derived chemical at the concentration that it occurs – the evolution of detection is ‘locked off’ (this is why insecticide-resistant pests and antibiotic-resistant microbes are an inevitable consequence of human attempts to use chemicals to reduce the fitness of competing organisms).

The human experience of plant and microbe-derived chemical diversity has thus been very important in human affairs and this experience can inform us about ligand/protein interactions but it can tell us little about the role of plant chemicals in the organisms that make them. However, this human experience does tell us a final, very important lesson. The great majority of chemicals made by plants or microbes have no direct impact on humans in any observable way. Even in plants grown and consumed by humans, the majority of plant chemicals made by those plants are unsensed by humans, these chemicals have no unambiguous physiological or neurological effect and there must be many such chemicals that are as yet uncharacterized by humans (who have tended to concentrate on the compounds that occur in large amounts or which are physiologically or neurologically active).

Plant/microbe and plant/insect interactions

Being (with a few exceptions) primary producers, plants are subject to attack by other organisms that seek access to the resources captured by the plant, resources that are now in a form usable by attackers. The evolution of plant physical and life cycle strategies which can reduce the rate, frequency or effectiveness of attack has obviously been an important feature of plant evolution (Rauscher, 1992) but such strategies will not be considered further, rather we shall concentrate on chemical defences. However, it is worth noting that there is an intimate link between physical defences and chemical ones. As in the case of physical defences used by humans, the chemistry of the material used to construct the physical form is crucial to its effectiveness. Likewise in plants, the cuticle, some trichomes and cell walls have chemistries that make the physical structure more suited to its purpose. The evolution of the chemistries of such structures is a topic which the following discussion might inform but it will not be considered further. We will concentrate on low molecular weight chemicals which are made by plants to gain fitness by acting directly on the interacting organism.

Because many organisms interacting with plants use volatile phytochemicals in order to locate the plant, plants will have evolved in response to the selection pressure that is associated with these volatile-mediated interactions (the clearest example of such selection comes from human selective breeding where human preferences for certain scents and flavours has resulted in plants (for example roses, carnations, apples, etc.) with extreme characteristics being bred and widely propagated throughout the globe by humans). The attraction of insects to a few plant volatiles can result in increased fitness for the plant in the case of pollination or in attraction of the insect natural enemies of plant-feeding insects (Harborne, 1988; Vet, 1999). However, a role can only be assigned (as an attractant or repellent) to a very few plant volatiles in the complex mixture made by any single plant species. There has certainly been a tendency to focus attention on the very small fraction of plant chemistry that possesses clear biological activity when building an evolutionary framework explaining all plant ‘secondary’ chemical diversity. Being impressed by the potent biological activity of some secondary chemicals, it was argued that these chemicals are used by the plant to increase fitness by negatively or positively affecting the metabolism or behaviour of other organisms (Fraenkel, 1959; Ehrlich and Raven, 1964; Harborne, 1988). To account for the tens of thousands of secondary chemicals for which there was no convincing evidence that the production enhanced the fitness of the producer, various

explanations were offered. For example, given the diversity of other plants, animals and microbes that come into contact with a single plant species over evolutionary time and throughout the contemporary geographical range, it was argued that in order to confer specificity to the chemical interactions between a plant and these other organisms, a very large number of biologically active compounds would be expected to be found in any single plant species. This idea, supplemented with an idea of evolutionary relics, led to the view that 'every natural product has, or had, a purpose in the evolutionary strategy of the taxon concerned' (Swain, 1975). This model ignores the fact that the probability of any compound possessing any biomolecular activity is very low (see earlier). To generate a new chemical with useful biomolecular activity, repeatedly at each stage of a linear pathway, is extremely unlikely. Furthermore, there are countering selection pressures which would further reduce the chances of such linear pathways evolving. Consider a scenario, where by such extreme chance, a plant species has evolved a four-step synthesis of a compound that reduces the fitness of a herbivorous insect. Once the end product of the pathway has lost its effectiveness (which it will do rapidly judging by >100 years experience with evolution of resistance to many synthetic pesticides and antibiotics), the chances of the plant gaining fitness by evolving another novel new biologically active chemical from the now-redundant chemical is much lower than the chance of gaining fitness by reducing the costs by eliminating the now redundant four-step pathway. The longer the pathway the greater the problem becomes because the number of genes in which a mutation can give cost savings is greatly in excess of the number of genes that can give rise to a new useful product. Furthermore, in each gene, there will be a greater chance of destroying enzyme activity via mutation than changing it usefully. The alternative model to explain secondary product chemical diversity (The Screening Hypothesis; Jones and Firn, 1991), was based on well-defined general principles of ligand/protein interactions instead of being based on limited and maybe selective ecological evidence. The Screening Hypothesis proposes that organisms that gain fitness by making compounds with potent biomolecular activity are selected for because of the overall metabolic traits they possess that enhance the generation and retention of chemical diversity. Some of the chemicals made by this metabolic capacity will possess fitness enhancing properties but many (the great majority?) chemicals made will possess no properties that contribute to the current fitness of the maker. Many concepts that have driven previous studies should now be questioned:

1. *Presence indicates purpose?* Not necessarily. The presence of a chemical in a plant, even if the chemical is shown to possess interesting biomolecular properties in some assay, is insufficient evidence that selection has operated to promote the synthesis of this specific property. The safer deduction is that selection has operated to retain the overall metabolic capacity which gave rise to this and other molecules, one or more of which must possess properties that enhance fitness, provided that the overall fitness benefits outweigh the overall metabolic costs.
2. *Quantity indicates purpose?* Not necessarily. It is known from many structure-activity studies that the relative biomolecular activity of several structurally related molecules can vary by many orders of magnitude (Firn and Jones, 1996) hence the amount of chemical made by a plant provides little evidence of role.
3. *Pathways indicate purpose?* Not necessarily. Because the biomolecular properties of a chemical are unpredictable, a pathway at one stage in evolution contributing to plant defence against insect herbivores could subsequently contribute to another property (such as reducing the fitness of a pathogen).

4. *Compound X has been shown to defend plant A hence surely a similar chemical in plant B plays a similar role?* Not necessarily. The different evolutionary experience of different species and the flexibility of the metabolic traits used to make molecules possessing biomolecular activity means that similar compounds could serve different roles in different species and the different compounds could serve the same role in different species.
5. *'A well-known physiological function of the anthocyanin pigments and flavonol copigments is the recruitment of pollinators and seed dispersers....'* (Winkel-Shirley, 2001). *'Glucosinolates are biologically active secondary metabolites....'* (Kliebenstein *et al.*, 2001). Both these statements come from papers published recently and they are inaccurate in that they generalize for a pathway and imply that all the compounds made from a particular pathway play a particular role. It has not been shown that all flavonoids play a role in attracting insects. It has not been shown that all glucosinolates are biologically active (and it would mean little unless that activity was shown to be of benefit to the producer). It might be true to say that some members of these chemical groups play a particular role but there needs to be the recognition that most chemicals made by these pathways have not been shown to play any role.

The Screening Hypothesis clearly places great demands on those studying the role of chemicals in plants (and microbes). The 'rules' which have shaped metabolism in plants are operating at the molecular level and the outcome of these rules is usually studied at higher levels of organization. The rules do not predict outcomes. As in a game of chess, the few simple rules cannot predict the outcome. The operation of any rules simply opens up multiple opportunities and it is the players that ultimately shape the game.

The evolution of regulatory systems for secondary metabolism

As in the case of the immune system (another strategy evolved to counter the low probability of any antibody possessing the correct properties to enable it to bind at low concentrations to a specific hapten), an ability to induce the chemical defence only when needed provides a very great cost saving. To achieve cost saving by having inducible defences three extra elements are needed:

1. a sensing system(s) that can detect the conditions that are an accurate indicator of a need for defence
2. a regulatory step in the chemical production capacity (by either regulating transcription, translation or by allosteric means)
3. a linkage mechanism between the detection system and the regulatory system.

Evidence is accumulating that plants possess all three of the above abilities and evolution may have provided multiple ways of linking the three elements. Damage or invasion detection mechanisms involving the detection of low molecular weight compounds or proteins, either arising from the attacker or as a result of the attacker creating low molecular weight compounds as they break into the plant, have now been found in many plants (Karban and Baldwin, 1997; McDowell and Dangel, 2000). One linkage between the detection system and the regulation of gene expression involves transcription factors (Tamagnone *et al.*, 1998), however, we shall not consider the detailed mechanisms involved in such responses.

Rather, we will consider the evolutionary strategy that has given rise to inducibility. How could an organism best gain the cost-saving benefits of inducibility yet retain the flexibility to generate and retain chemical diversity? Might not a well-defined regulatory system operating on a pathway begin to canalize an area of metabolism in a manner which ultimately constrains the production and retention of new chemical diversity?

A speculative scenario for the evolution of the control of pathways leading to compounds retained because they possess biomolecular activity

Given the advantages that inducibility confers (reducing costs increases chances that benefits outweigh costs), it is expected that inducibility should have evolved at an early stage in the evolutionary history of any secondary product pathway. Once the inducibility had evolved at that position in a pathway, as long as the mechanisms provided adequate control of the amount of active compound made at a later stage along the pathway, the selection pressure to regulate the pathway at a later stage might be small. Thus regulation of flux through a pathway could be achieved by regulation at an entry point or early stage of a metabolic sequence. An immediate implication of this logic is that inducibility becomes quite a poor predictor of the role of any chemical made as a consequence of a pathway being induced by a particular stimulus. Just as the inducibility of the immune system tells one little about the role of each type of antibody, maybe the inducibility of secondary chemicals after a biotic challenge indicates a mechanism of response and not a role for each chemical made. However, a further complexity is introduced because of the predicted multifunctionality of secondary metabolic pathways. Because a pathway may serve different roles at different stages of evolution, or in different organisms, how can natural selection result in a sensing/induction system that adapts to the new role that a pathway may best serve? Consider two extreme scenarios:

1. At one extreme, a particular pathway could evolve with associated regulatory processes finely tuned to deal with one specific type of challenge only – an insect herbivore defence system using products of an alkaloid pathway, for example. This would result in excellent cost savings when it first evolved, but the chances of evolving novel anti-insect compounds (i.e. compounds that are sufficiently different in their mode of action from a now redundant one for which the insect now has evolved to resist) would be greatly reduced if there was a reliance on this one pathway. If the insect damage sensing system is uniquely linked to this alkaloid pathway then to evolve an extension of a non-alkaloid pathway brings a requirement for a whole new regulatory system for that pathway in order to gain cost savings.
2. At the other extreme, regulatory systems for several pathways could be evolved which could respond to one or more of several different challenges (insects, fungal, physical damage, etc.). Such a strategy would increase the chances of evolving new compounds with biomolecular activity able to serve any purpose. Thus a pathway evolved because it enhanced the fitness of a plant by making compounds that reduced the fitness of insects could at any time produce a compound that enhanced plant fitness by reducing the fitness of an invading pathogen (see the earlier analogous human experience of using whatever biological activity one finds despite the original purpose). The multifunctionality of the pathways maybe dictates a multifunctional sensing system.

These extremes are not mutually exclusive and a plant may have different pathways which fall anywhere along the spectrum of the extremes. However, in recent years there has been

a growing awareness of 'cross talk' between signalling and response mechanisms in plants (Felton *et al.*, 1999a,b; Feys and Parker, 2000). This is precisely what would be predicted by one of the extreme scenarios just discussed where the multifunctionality of a pathway leads to a flexible response system which has 'cross talk' built into it. The possibility that cross talk is an inevitable consequence of the metabolic traits of such pathways has many consequences for those studying the role of products made by such pathways. Inducibility becomes a very poor predictor of the role of a chemical because inducibility has possibly been evolved as a general means of cost-saving to be applied in a non-specific manner because the underlying metabolism needs to retain the ability to generate chemical diversity.

Signalling molecules within plants

The concept of specific chemicals acting as the controllers of developmental and functional processes in plants has dominated the thinking of plant physiologists for many decades. At the centre of this thinking are the well established roles for the five major groups of plant growth substances (auxin, ethylene, abscissic acid, cytokinins and the gibberellins (Davies, 1995)). However, there are numerous reports in the literature of a very wide range of other chemicals (nearly always secondary plant metabolites) purportedly playing a regulatory role (Gross, 1975). It is often suggested that these secondary metabolites either replace or supplement the five major types of plant growth substances in particular circumstances (Gross, 1975). During the 35 years (1928–1963) that the major hormone groups were being discovered, a large number of plant extracts were tested for biological activity in plant-based bioassays, and many reports of new endogenous regulators appeared during that period. Possibly because the discovery of each of the major groups of plant hormone was unusual in some respect, with the active compounds first being isolated from unlikely sources or in a study that did not establish an unambiguous role (Table 4. 1), close scrutiny was not always given to other claims that new endogenous regulators had been discovered in certain plants. The result is that many substances or groups of substances have been ascribed roles as endogenous regulators in plants. The most commonly discussed examples are the polyamines (Evans and Malmberg, 1989; Bagni and Torrigiani, 1992), oligosaccharines (Albersheim, 1985), acetyl choline (Tretyn *et al.*, 1990) and the jasmonates (Pathier *et al.*, 1992). However, claims were also made for a much larger number of compounds of more much limited taxonomic distribution. Gross (1975) reviewed over 100 such substances that were considered to play a role as endogenous regulators, these included representatives of aliphatic and aromatic carboxylic acids, phenols, alkaloids, terpenes and S- and N-heterocyclic compounds. However, a satisfactory evolutionary explanation to explain why different species use different chemicals to control the same basic physiological process does not yet exist. Why should plant X use compound A to control flowering when plant Y uses compound B? Are the links between physiology and biochemistry in plants really anarchic? An answer to this question might be formed by considering the links between secondary metabolism and plant 'hormones'.

The link between secondary metabolism and hormonal control

Are plant hormones 'secondary metabolites'?

It is known that chemical communication is important in some simple organisms as a way of coordinating sexual reproduction. For example, in the water mold *Achlya*, the terpenoid compounds oogonol or antheridiol are used to coordinate the sexual reproduction of

Table 4.1 The major groups of plant growth substance and their discovery

Hormone group	Biosynthetic pathway or precursor	Discovery
Indolyl-3-acetic acid (IAA) auxin	Tryptophan (?)	First isolated in the 1930s during a search for auxin activity from human urine, <i>Rhizopus</i> and <i>Saccharomyces</i> cultures. At that time plants were thought to contain a cyclopentane auxin (<i>auxin a</i> - now known not to exist). IAA identified in corn seed extracts in 1946 and widely reported in other plant extracts subsequently.
Gibberellins	Isoprenoid	First isolated from fungal cultures by Japanese phytopathologists investigating a disease of rice (1926). Several related compounds were subsequently found in fungal cultures. Gibberellins were not isolated from plants until the 1950s, some decades after some of their effects on plant growth had been described.
Abscissic acid	Isoprenoid	Isolated and characterized from abscising cotton bolls and dormant tree buds in 1963. No longer thought to play an important role in abscission or bud dormancy but good evidence for a role in controlling stomatal aperture and seed dormancy.
Cytokinin	Isoprenoid and purine	Searching for a substance capable of promoting cell division in cell and tissue cultures, coconut milk, malt extract, yeast extract and autoclaved herring sperm DNA were found to be active. A purine was isolated from the latter source by the mid-1950s. A decade elapsed before a related compound was isolated from maize endosperm.
Ethylene	Methionine	As a constituent of town gas, ethylene was known to have a potent effect on plants since 1906 and this compound was isolated from ripening apples in 1935. Some physiologists did not accept ethylene as a true plant growth substance until well into the 1960s, despite its widespread occurrence and high biological activity.

All the major hormones were either first isolated from unusual sources or were discovered as a result of the study of a physiological process in which the hormone now plays a disputed role.

colonies and in some simple fungi, trisporic acid (C15 isoprenoid) is used as a sporulation coordinator (Gooday and Adams, 1993). Thus the roots of plant hormonal control may lie in simple organisms communicating between cells of the same species in *different* individuals. With the evolution of true multicellular organisms, the need for coordinated development and functioning would have extended the scope for chemical signalling and using chemicals to coordinate the functioning or fate of cells within the *same* individual would be a small step. The extent and way in which such chemical signalling would have evolved would have depended on the nature of the specialized functions that appeared in various types of organism over evolutionary time. It can be postulated that in all early multicellular organisms, secondary metabolites, already selected for the capacity to generate compounds with potent biomolecular activity were put to a new role. Evidence for such a scenario can be found in the multiple roles played by members of the isoprenoid pathway. Individual isoprenoids function as plant growth substances, plant defence compounds, fungal sexual coordinators, animal hormones and animal olfactory attractants and repellents. Of the plant growth substances, three are derived fully or partly from the isoprenoid pathway – the gibberellins, abscissic acid and cytokinins (see Table 4.1). The other two plant hormones, auxin and ethylene, are derived from amino-acid precursors and amino acids also serve as the precursors for some chemical regulators in animals and play a part in plant defences, possibly because amino acids have commonly been used as precursors in ‘inventive biosynthesis’ (Wong, 1981). It is interesting that the exact route leading to the biosynthesis of IAA has been debated for nearly half a century, with the expectation that the biosynthesis of such an important molecule would be finely controlled. Traditional biochemical investigations could not provide definitive evidence for such a pathway, but unexpected phenotypes resulting from the manipulation of cytochrome P450 genes suggests that, in some species, there may be an intimate link between the synthesis of indole glucosinolates and the synthesis of IAA (Feldmann, 2001). IAA could be considered to be a ‘secondary product’ with a primary role. If the evolutionary recruitment of a ‘secondary metabolite’ to serve a role as an endogenous coordinator or regulator in plants has occurred, it is likely that the event will have brought to the emerging ‘hormonal control’ a series of metabolic traits that have evolved to serve a quite different purpose – the generation and retention of chemical diversity. If there is not a duplication of all the enzymes involved in the pathway leading to the plant hormone, the plant hormonal control will inevitably be somewhat compromised by the metabolic features of a pathway which is even more multifunctional in that it now includes a role as an internal signalling molecule. Could such multifunctionality explain some previously puzzling aspects of plant hormone biosynthesis? Maybe this multifunctionality explains why so many stimuli (from insects and fungi to many forms of physical stimuli such as various wavelengths of light, low and high temperatures, too little or too much water, etc.) can change the hormone content of plants (Davies, 1995).

Gibberellin synthesis – generating diversity?

Over 100 different gibberellins have been isolated and characterized – some are found in many species but others have a much more limited taxonomic distribution. A single plant species usually contains several gibberellins. The great majority of gibberellins do not possess high biological activity (or possess activity only because they are converted to other more active gibberellins) (Davies, 1995). The fact that the great majority of gibberellins possess low activity is itself consistent with the Screening Hypothesis, but is harder for other models of secondary metabolite evolution to explain. But why do plants (and some

fungi) make so many gibberellins? We would argue that this group of plant hormones are showing their evolutionary origin as secondary products. Even when there is a need for a gibberellin to act as an essential regulator in the plant, the metabolic traits that generate chemical diversity are retained (or at least are not selected against). The particular trait of relevance to gibberellin biosynthesis is the proposal that some enzymes involved in secondary metabolite biosynthesis may possess a relatively broad substrate specificity, leading to matrix grid transformations. In such matrix grid biosynthetic routes, a few enzymes could add or transform substituents to a carbon skeleton and the order in which they are added or transformed is not fixed. Hence a number of intermediates can be generated. As long as the possession of at least one of the compounds made results in a net gain in plant fitness, the chemical diversity represented in all the intermediates can be retained. Evidence in support of this prediction can be found in the study of the gibberellin 20-oxidase which can convert two precursors into at least 11 products because it is multifunctional (Lange *et al.*, 1994). The fact that some parts of the biosynthetic pathway leading to the active gibberellin, GA₁, can operate as a matrix (Taiz and Zeiger, 1991) would be evidence that this type of mechanism does operate in plants even in a pathway used to make compounds which are central to controlling plant growth and development and that some enzymes involved in the pathway are following rules that would allow the extension and retention of chemical diversity. A similar complex metabolic grid may also exist in the biosynthetic pathways leading to brassinosteroids (Wang and Chory, 2000).

Plant hormone degradation – another role?

For many decades it has been argued by some that enzymes involved in metabolizing plant hormones (to give either breakdown products or conjugates) may play an important role in plant hormone homeostasis (Bandurski *et al.*, 1992). The control of hormone concentration by controlling the breakdown, rather than the synthesis, seemed counter intuitive to those schooled in hormonal control in mammals. However, if the biosynthetic machinery leading to plant hormone production carries with it some of the flexibility (and maybe the inducibility) of a metabolism evolved for multifunctionality, and where precise control of the amount of product may not be something that has been highly selected for, the evolution of other means of controlling the amount of any hormone by degradation perhaps deserves attention. However, an alternative explanation could be that, in some cells (maybe cells that are insensitive themselves to hormones), an ability to generate and retain chemical diversity has resulted in a production of hormones and it is in these cells that selection has resulted in a fairly crude method (degradation) of hormone concentration regulation so as to avoid disturbing more carefully regulated hormone levels in other cells. However, if these cells retain a capacity to make hormones and genes can be induced in them, possibly the extra burst of hormone synthesis could give rise to the hormone level changes that are sometimes associated with insect or fungal attack. This may be yet another opportunity for cross talk?

Summary

The aim of this chapter has not been to inform readers how plants work or indeed how plants have evolved. Rather it has been to try to engage readers in at least considering whether there might be an appropriate evolutionary framework for metabolism that could help us investigate and eventually understand many physiological processes. We have indulged in

speculation in order to provoke readers and some may find the lack of certainty unsatisfactory. Readers provoked into rejecting the evolutionary model that has been advanced should feel free to make such a rejection ... as long as they have a better model with which to replace it. After any discovery, the question 'why do plants do that?' needs some evolutionary explanation. Maybe any plant physiology or plant biochemistry textbook without a section on evolution should be regarded as seriously incomplete.

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