Ethanolic fermentation: new functions for an old pathway

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Ethanolic fermentation is an ancient metabolic pathway. In plants, it is a major route of ATP production under anaerobic conditions. In addition, recent developments suggest that the pathway has important functions in the presence of oxygen. Both of the enzymes required for the production of acetaldehyde and ethanol, pyruvate decarboxylase and alcohol dehydrogenase, are highly abundant in pollen, resulting in fermentation in fully oxygenated cells. Acetaldehyde toxicity is an inevitable side effect of aerobic fermentation. Could acetaldehyde be the elusive pollen factor that contributes to male sterility in *cmsT* maize? The versatility of this ancient pathway is also illustrated by the induction of aerobic fermentation by environmental stress and activation of a defense response by overexpression of pyruvate decarboxylase.

In 1897 the Büchner brothers demonstrated that a cell-free yeast extract could convert glucose to CO_2 and alcohol. That simple experiment demonstrated for the first time that the reactions of life can proceed outside the living cell, and marked the beginning of modern biochemistry. By the 1930s it had become clear that fermentation is a complex metabolic process that results from the orderly succession of chemical reactions, each catalysed by a specific enzyme. The pathway was essential in the early anaerobic atmosphere because it produces ATP without the consumption of O_2. In the atmosphere today, ethanolic fermentation is used only by specialized organisms or under special conditions. In plants, it has been studied because of its relevance to ATP production during flooding. Recently there have been some interesting developments concerning this ancient pathway in the context of:

- Flooding tolerance.
- During anther development.
- Its possible relevance to disease resistance and stress.

Survival under limited oxygen supply

The marsh plant *Acorus calamus* can survive two months under anoxia[1], but wheat and barley seedlings survive only hours[2]. This difference in flooding tolerance is based on complex anatomical and biochemical adaptations. But even morphologically comparable land plants can show a wide range of tolerance in flooded soils, mainly because of differences in metabolic adaptations. Numerous studies have addressed the issue of metabolic change in oxygen-limited environments[1,3–5]. Different fermentation pathways and products of anaerobic metabolism play essential roles in surviving prolonged periods under anoxia. Here we focus on two of the most common pathways, lactic acid and ethanolic fermentation, that regenerate NAD^+ for the continuation of glycolysis (Fig. 1).

Lactic acid fermentation is a one step conversion from pyruvate, which is catalysed by lactate dehydrogenase (LDH) with a concomitant oxidation of NADH. Ethanolic fermentation is a two step process in which pyruvate is first decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC), and acetaldehyde is subsequently converted to ethanol by alcohol dehydrogenase (ADH), regenerating NAD^+ (Fig. 1). Both lactate and ethanol are produced to a varying degree by most plants under oxygen stress. However, lactate is an acid and its accumulation in the cytoplasm could alter cellular pH and cause damage, whereas ethanol diffuses to the extracellular medium and poses no major problem except at high concentrations. This raises a number of questions: does ethanolic fermentation have advantages over lactate fermentation under anoxia? How do plants regulate the concentrations of lactate and ethanol under such conditions? The answers to these questions are not straightforward and remain controversial. Let us examine some of the most relevant experiments and the latest strategies undertaken to address this issue.

On the basis of *in vitro* LDH and PDC enzymatic activity, a self-controlling system for lactate and ethanol production called the 'pH-stat' hypothesis was proposed[6]. LDH has an alkaline pH optimum whereas that of PDC is acidic. At the onset of anoxia when oxidative phosphorylation is blocked, LDH is active at the alkaline pH of the cytoplasm and shunts pyruvate to lactate. The accumulation of lactate reduces cytoplasmic pH, which, in turn, inhibits LDH and activates PDC leading to ethanol production[6]. This was later supported by *in vivo* nuclear magnetic resonance (NMR) studies in maize root tips where accumulation of lactate and a dramatic decrease in cytoplasmic pH (from pH 7.4 to 6.8) were reported in the first few minutes of anoxia[7,8]. Ethanol production was detected only after a lag phase of about 10 min (Ref. 7). Thus, a lactate-modulated cytoplasmic pH shift was postulated to be the signal for the induction of ethanolic fermentation[7]. Furthermore, studies on *Adh1*-null mutants indicated that maize root tips that are unable to regulate lactate production are unable to stabilize cytoplasmic pH and succumb more rapidly to anoxia[7,8]. However, several observations do not fit well with the pH-stat hypothesis. For example, in oxygen-stressed wheat seedlings, cell sap acidification stops after 2 h whereas lactate accumulation lasts more than 10 h (Ref. 2). In rice shoots, cytoplasmic pH decreases immediately in spite of the low lactate production[9]. Furthermore, in maize root tips it has been reported that hypoxia stimulates a drop in cytoplasmic pH long before the lactate concentration reaches a steady-state level[10]; on subsequent re-oxygenation the pH returns to normal values long before lactate concentrations decrease. Thus, the data regarding the role of lactate as the cause of cytosolic pH change are not in agreement with one another – a settlement of this issue awaits further investigation.

However, the decrease in cytoplasmic pH at the onset of anoxia, whatever the mechanism might be, appears real[2,7,10,11] and if unabated might well be the cause of cell death. A decrease in cytoplasmic pH at the onset of stress is not restricted to hypoxia.

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For instance, challenging plant cells with pathogens or elicitors causes a similar drop in cytoplasmic pH (Refs 12–14). In most of these examples, it has been assumed that the drop in cytoplasmic pH serves as a 'second messenger' and mediates subsequent changes in plant metabolism and gene expression. But whether there is any relation between cytoplasmic pH, intracellular calcium concentrations, and other signal transduction cascades is not clear. Most of the controversies over the pH-stat hypothesis arise from the use of different plant species, tissues and experimental conditions. Recently, attempts have been made to standardize experimental systems and genetic background. Two of these approaches, hypoxic acclimation and transgene expression are considered here.

When maize root tips are pre-exposed to hypoxic conditions, survival under subsequent anoxic conditions is significantly improved[15,16]. Pretreatment leads to increased lactate secretion into the medium, reduced intracellular lactate concentrations, better pH regulation and improved survival under anoxia[16]. However, these data remain correlative and direct evidence of cause and effect has not been obtained. Some experiments correlate hypoxic pretreatment with factors other than pH regulation. For example, it has been demonstrated that in hypoxically pretreated roots, increased hexokinase activity is the critical factor that modifies glycolytic flux and energy production for improved survival under anoxia[17]. More recently, promoting glycolytic flux through NADH oxidation in low oxygen environments is attributed to non-symbiotic plant haemoglobins, which are up-regulated by hypoxia[18]. Hypoxic acclimation probably leads to several changes making it more difficult to correlate anoxia tolerance with one or two biochemical traits.

Both lactate and ethanolic fermentation are simple pathways, making them ideal targets for genetic manipulation. Overexpressed barley LDH cDNA in tomato roots has been used to evaluate the role of lactate in the control of ethanolic fermentation[19]. According to the pH-stat model, increased lactate flux and lower cytoplasmic pH at the onset of anoxia and thus a much earlier start in the kinetics of ethanolic fermentation would be expected in the transgenics as compared with the wild type. These authors reported a 50-fold increase in *in vitro* LDH enzymatic activity but found no difference in lactate and ethanol production between the transgenics and wild type[19]. PDC has been expressed from the obligate anaerobe *Zymomonas mobilis* in tobacco under the strong CaMV 35S promoter[20]. In leaves, acetaldehyde and ethanol are not detectable under aerobic conditions in either the transgenics or the wild type, indicating that the accumulation of PDC alone is insufficient for ethanol production under aerobic conditions in tobacco leaves. But when respiration is blocked by anoxia or respiratory inhibitors, the transgenics produce up to 35- and 20-fold higher acetaldehyde and ethanol, respectively, compared with the wild type[20]. This experiment demonstrated that PDC activity is limiting ethanolic flux *in vivo* under anoxic conditions in tobacco leaves. More recently, the question of energy maintenance and survival under anoxia has been addressed in the roots of these transgenic tobacco[21]. Both transgenic and wild-type roots produce acetaldehyde and ethanol at low levels even under aerobic conditions. Production increases dramatically under anoxia, being higher in the transgenics than in the wild type. However, the concentration of lactate is at the limit of detection at any time point during the first 4 h of anoxia[21]. This suggests that ethanol production does not require activation by lactate. Because the transgenics did not show increased flooding tolerance, this work indicates that sugar availability is a critical factor contributing to ethanolic flux and survival[21]. This is consistent with the observation in rice that the ability to use starch under anoxia is one of the major factors that contribute to anoxia tolerance[22].

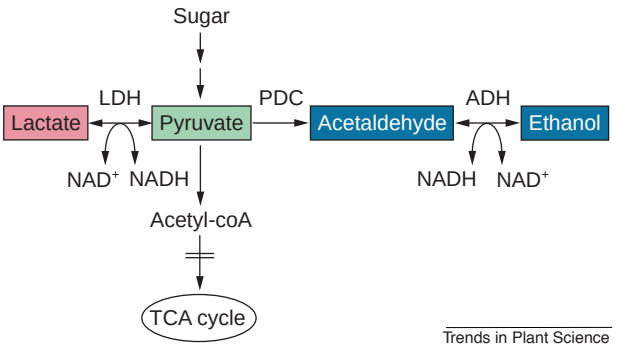

Fig. 1. Metabolic routes of lactate and ethanol production during oxygen stress. When respiration is impaired by lack of oxygen, plants resort to fermentation for regenerating NAD^+. In lactic acid fermentation, pyruvate is converted to lactate by lactate dehydrogenase (LDH). In ethanolic fermentation, pyruvate is converted to acetaldehyde by pyruvate decarboxylase (PDC) and acetaldehyde is subsequently converted to ethanol by alcohol dehydrogenase (ADH). In many plant species, both lactate and ethanol have been shown to accumulate during hypoxia and anoxia.

It has been proposed that the different K_m of PDH and PDC for pyruvate are the controlling factors that regulate the entry of pyruvate into the TCA cycle or the ethanolic fermentation pathway[21]. The K_m of plant PDHs for pyruvate is in the μM range whereas that of PDCs is in the mM range. The internal pyruvate concentration in plants is between 0.1 and 0.4 mM (Refs 6,23), which is too low for PDCs to compete with PDHs. Pea PDH, for example, has a K_m of 57.0 μM for pyruvate[24] whereas the K_m of pea PDC is 1.6 mM and 4.1 mM at pH 6.5 and 6.9, respectively[25]. Thus, at the aerobic pH even if PDC remains active, pyruvate preferentially enters the TCA cycle. But when respiration is blocked by inhibitors or lack of oxygen, pyruvate concentration increases considerably[26,27] and pyruvate becomes available for the PDC reaction. In rice, where the lowest K_m for PDC is reported (i.e. 0.25 mM at pH 6.5), the lag phase in enzyme activity is avoided by the presence of 3 mM pyruvate[23] suggesting that pyruvate concentration is more important than pH. For example, at pH 7.0 and a concentration of 33 mM pyruvate, pea PDC exhibits 85% of its activity at pH 6.0 (Ref. 25). The lag phase of ethanol production at the onset of anoxia, therefore, might not be the result of a need for a drop in cytosolic pH because the pH falls rapidly within the first 2 min of anoxia. Rather, the lag phase might be required for a build up of pyruvate. The presence of low concentrations of ethanol in maize root tips[5,11] and tobacco roots[21] also suggests that PDC activity *in vivo* might not be under strict pH control. In light of the abundance of acetaldehyde and ethanol in tobacco pollen under aerobic conditions, we consider it unlikely that a lactate-modulated pH-stat is the regulator of ethanolic fermentation under anoxia. Therefore, we favour a PDH/PDC stat to explain the versatile nature of ethanolic fermentation with its limited existence under aerobic conditions[21].

Aerobic fermentation in pollen

The known function of ethanolic fermentation is to regenerate NAD^+ for limited glycolytic ATP production in the absence of oxygen. Only two enzymes (PDC and ADH) are required to produce ethanol from pyruvate, and these are usually present at low levels in aerobic tissues and strongly induced by low oxygen. There is at least one tissue that is an exception: ADH is an abundant protein in pollen of maize[28] and tobacco[29,30]. Because of the abundance of

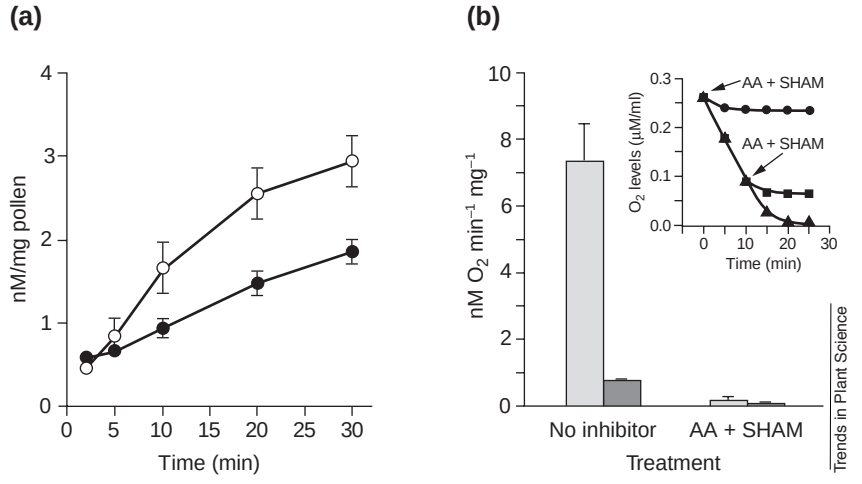

Fig. 2. Fermentation and respiration functioning concurrently in tobacco pollen. (a) Mature pollen incubated in the presence of glucose[30] under 40% oxygen produces acetaldehyde and ethanol without any measurable lag. Filled circles represent acetaldehyde, unfilled circles represent ethanol. (b) Oxygen uptake by mature pollen (light grey) in the same medium used in (a) indicates that pollen respires much faster than leaf tissue (dark grey), and this is strongly inhibited by cytochrome and alternative oxidase inhibitors. The insert shows the kinetics of oxygen consumption, which is linear for the first 15 min (triangles). Respiration can be inhibited at the beginning (circles) or 10 min after the start of the measurement (squares) by Antimycin A (AA) + salicylhydroxamic acid (SHAM). AA is an inhibitor of the cytochrome pathway and SHAM is an inhibitor of the alternative oxidase pathway. *Reproduced, with permission, from Ref. 30.*

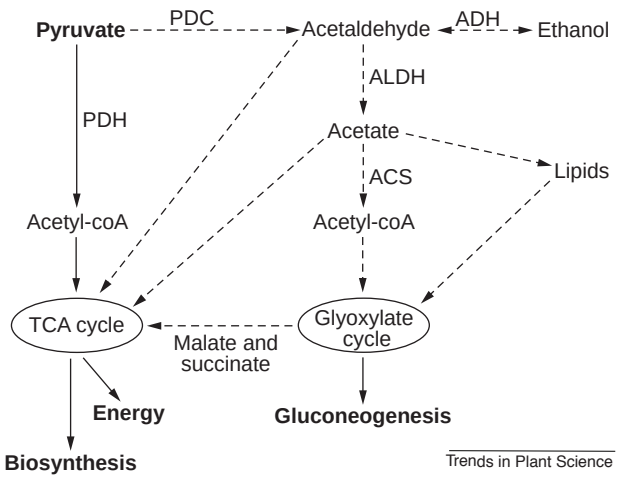

Fig. 3. The pyruvate dehydrogenase complex (PDH) bypass for pyruvate utilization in tobacco pollen. As an alternative to the direct route of pyruvate to acetyl-coA via PDH, pyruvate could enter the fermentation pathway via pyruvate decarboxylase (PDC) and form acetaldehyde. Acetaldehyde could then be converted to acetate by mitochondrial or cytosolic aldehyde dehydrogenase (ALDH). Cytosolic acetate could be used for lipid biosynthesis or converted into acetyl-coA by acetyl-coA synthetase (ACS), which is further processed in the glyoxylate cycle to regenerate malate and succinate. B oxidation of fatty acids in the glyoxylate cycle could also yield malate and succinate, especially during pollen germination. These intermediates could join the tricarboxylic acid cycle (TCA) for energy production or to replenish biosynthetic intermediates. Unbroken arrows indicate the usual route of pyruvate utilization in all tissues, broken arrows indicate the PDH bypass in pollen.

Adh mRNA in anoxic roots it was one of the first plant cDNAs cloned, and has been the subject of numerous elegant genetic studies[4,28] – and yet, the function of ADH in pollen remains a mystery. The existence of ADH as a major soluble protein in maize pollen is somewhat paradoxical because *Adh1*-null mutants have no obvious phenotype[28]. Neither pollination nor heritability is affected by *Adh1*-null pollen in an *Adh1*+/*Adh1*− null heterozygote[28].

Why then, does pollen synthesize ADH when it is not needed and the energy demand is extremely high? Tobacco pollen has the capacity to synthesize ethanol from pyruvate, because a pollen-specific isoform of PDC is also highly expressed[29,30]. More importantly, significant flux through the ethanolic pathway occurs throughout tobacco pollen development. Ethanol production takes place concomitantly with respiration [i.e. this is true aerobic fermentation (Fig. 2)]. The levels of acetaldehyde and ethanol are much higher than that produced by anoxic leaves and are not influenced by increased oxygen supply[30]. In addition, pollen respires much faster than vegetative tissues and the ethanol flux is regulated by sugar concentration rather than oxygen availability[30].

What could be the function of aerobic fermentation in pollen? There are three possibilities. First, at a high rate of sugar metabolism in developing and germinating pollen, the transport of pyruvate into the mitochondria and the activity of PDH might limit ATP production. In such a situation, ethanolic fermentation could be used to generate additional ATP. Although fermentation produces ATP inefficiently, it is fast and could substantially contribute to energy production. However, we consider this to be unlikely because *Adh1*-null mutants of maize appear to be unaffected in survival as well as heritability of the *Adh1*-null allele under normal atmospheric conditions. Second, there could be another pathway that bypasses PDH and funnels acetaldehyde into general metabolism. This pathway, which we refer to as the PDH bypass, could yield acetate, acetyl-coA, malate or succinate from acetaldehyde (Fig. 3). One or more of these intermediates could join the TCA cycle for additional ATP production. A third possibility, which is also based on the PDH-bypass model, is that the critical requirement for this pathway is biosynthetic demand. Acetate derived from acetaldehyde in the bypass could be used directly for fatty acid and lipid biosynthesis[30]. These lipids might be of specific classes that are required during pollen development or might be of a general class that is required in large amounts compared with vegetative tissues. Glyoxylate cycle intermediates, such as malate or succinate, could also be used for gluconeogenesis or to feed the TCA cycle and replenish biosynthetic intermediates (Fig. 3). Thus, the PDH bypass might be required to accommodate the increased demand for energy and/or biosynthetic building blocks during pollen development and germination. Although some points remain hypothetical, the core of this model is supported by concrete evidence. For instance, aldehyde dehydrogenase (ALDH) cloned from a tobacco pollen cDNA library has been shown to be highly expressed in pollen[31]. The recombinant protein uses acetaldehyde as a substrate. Moreover, the activity of ALDH was shown to be indispensable for pollen tube growth.

Inhibiting ALDH activity by disulfiram, arrests pollen tube growth and leads to cell death whereas the same inhibitor has little or no effect on seedling development[31]. Preliminary experiments indicate that pollen can indeed convert ^{14}C-ethanol into both lipids and amino acids (S. Mellema, M. Tadege and C. Kuhlemeier, unpublished). According to this bypass model, the function of ADH would be that of a safety valve to protect pollen against the accumulation of toxic acetaldehyde and redirect ethanol into metabolism when required. Thus, ADH could be optional in tissues where ALDH is abundant (Box 1), explaining the absence of an obvious phenotype in plants of *Adh1*-null genotype. If the second and/or third alternatives are true, PDC knockouts should show a phenotype during pollen development and germination.

Box 1. Alcohol and cytoplasmic male sterility

In cmsT maize, cytoplasmic male sterility is caused by a novel mitochondrially encoded protein called URF13 (Ref. 37). URF13 resides in the inner mitochondrial membrane of the cmsT line where it confers sensitivity to fungal pathotoxins called T-toxins and to the insecticide methomyl. Although URF13 is expressed in all tissues, only pollen is affected. One long-standing theory postulates the existence of a pollen-specific substance called 'factor X', which interacts with URF13 to cause pollen abortion[38]. The nature of the pollen factor has remained a mystery. Fertility is restored by two dominant nuclear-encoded genes called *Rf1* and *Rf2*. *Rf1* decreases the expression of URF13 by about 80%, whereas, *Rf2* has no effect on the accumulation of URF13. *Rf2* was recently cloned by transposon tagging and found to encode a putative aldehyde dehydrogenase (ALDH)[39]. How might an ALDH restore fertility? One possibility is that the *Rf2* gene product interacts directly or indirectly with URF13 and inactivates it[39]. The other possibility is that the substrate of the *Rf2* gene product is an aldehyde that interacts with URF13. There might be many aldehydes in pollen but it is possible that acetaldehyde is factor X. This is suggested by the observation that under aerobic conditions high concentrations of acetaldehyde can only be measured in pollen, as demonstrated in tobacco and maize[30].

Chemically, acetaldehyde is a reactive aldehyde and a strong cell toxin[40–42]. It binds to nucleic acids and proteins forming stable acetaldehyde–protein adducts. Thus, acetaldehyde could interact with URF13 in a similar manner to the fungal pathotoxins and the insecticide methomyl to cause mitochondrial dysfunction. Even if acetaldehyde does not interact with URF13, the presence of URF13 could make the tapetum more susceptible to acetaldehyde. Yeast cells expressing URF13 that are grown on ethanol as the sole carbon source are more susceptible to methomyl compared with other growth substrates[43] suggesting that the acetaldehyde derived from ethanol is interacting with URF13. In tobacco, ALDH is highly expressed in pollen and expression is restricted to the tapetum at earlier stages of pollen development (R. Op den Camp and C. Kuhlemeier, unpublished) suggesting that the activity of ALDH is important for pollen development and maturation.

Finally, as ALDH uses acetaldehyde as a substrate[31] we envisage that the function of ALDH is independent of fertility restoration to direct acetaldehyde into general metabolism, but becomes a restorer in the URF13 background because it mollifies the toxicity of acetaldehyde. It is therefore plausible that acetaldehyde is the mysterious factor X. Overexpression of PDC in leaves of cmsT maize should prove useful for testing whether leaf mitochondria with URF13 are sensitive to the accumulation of acetaldehyde.

Ethanolic fermentation and stress response

Another new and exciting feature of ethanolic fermentation is its connection with stress-signal transduction and the disease-resistance response. The observation that *Arabidopsis Pdc* and *Adh* genes are induced by abiotic stresses, such as cold and dehydration[4,32], raises the question as to whether fermentation plays a role under environmental stress. What is even more intriguing is that some stresses lead to the functional activation of the ethanolic pathway. For example, several plant species exposed to environmental insults, such as water deficit, SO_2 fumigation, ozone exposure and low temperature, produce considerable amounts of acetaldehyde and ethanol at ambient or even at elevated oxygen concentration[33]. Thus, it appears that ethanolic fermentation has a general function in aerobic metabolism under stress conditions. It would make sense that under adverse conditions, which damage the intricate mitochondrial ATP-generating machinery, the cell resorts to inefficient but robust fermentation. This is also suggested by experiments with transgenic potatoes. Constitutive high level expression of *Zymomonas* PDC in potato results in the accumulation of high levels of acetaldehyde and a lesion mimic phenotype[34]. Lesions appear in fully expanded leaves (Fig. 4) and the severity of the phenotype is correlated with the amount of PDC expressed. Transgenic plants express several markers normally associated with plant defense to pathogen attack: callose deposition at the lesions sites, induction of pathogenesis-related proteins and

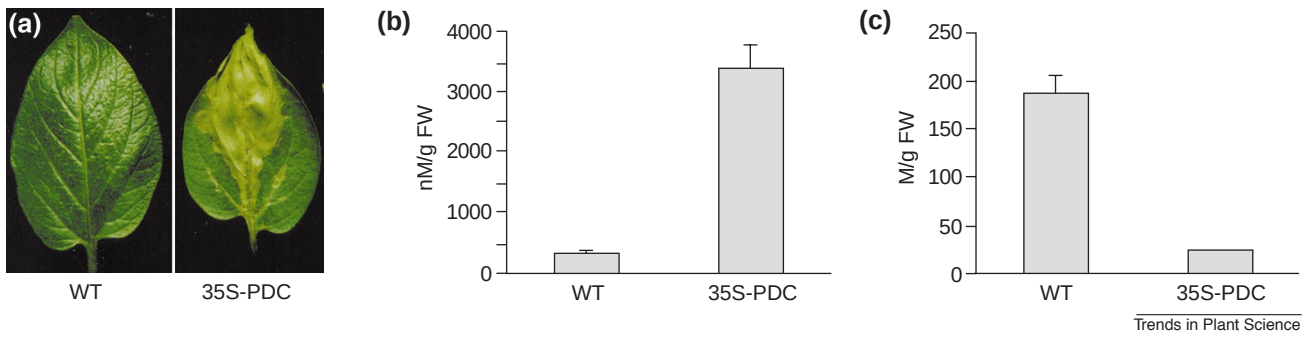

Fig. 4. Lesion formation and alteration of sugar metabolism in pyruvate decarboxylase (PDC) transgenic potatoes. (a) Transgenic potatoes expressing high concentrations of PDC develop extensive lesions on their source leaves. (b) Healthy transgenic leaves at the onset of lesion formation export up to ten times more sucrose than wild-type (WT) leaves. (c) A dramatic decrease in leaf starch content is found in the transgenic leaves compared with the wild type (data from Ref. 34).

enhanced disease resistance to fungal[34] and viral pathogens (I. Dupuis and C. Kuhlemeier, unpublished). The transgenic potatoes show reduced starch accumulation and enhanced sucrose export, indicating that the main pathways of sugar metabolism are affected (Fig. 4). A change in sugar metabolism during pathogen infection has been documented, and several genes involved in stress and defense response appear to be sugar-responsive[35,36]. As sessile organisms, it is perhaps not surprising that plants respond to environmental cues by altering their sugar metabolism. But, the connection between PDC expression, sugar metabolism and defense response is a formidable challenge. These lines of evidence fuel speculation that fermentation might be an important switch in regulating carbohydrate metabolism under stress conditions, but the functional significance is not clear.

Perspectives

For decades ethanolic fermentation has been studied because of its importance in flooding tolerance, and it is clear that this will remain an exciting field of study. For the future it will be of great interest to extend molecular methods to a wide variety of plants with different survival strategies under oxygen limitation, which will hopefully give insight into the ecological significance of the pathway. Importantly, ethanolic fermentation can occur in the presence of ambient oxygen. In pollen the pathway is highly active concomitantly with respiration. To understand the function of this aerobic fermentation and the negative side effects it might have, a genetic analysis will be required, and gene-inactivation approaches now available in several plant species will be invaluable. Finally, overexpression of PDC in potato leads to a lesion mimic phenotype and altered sugar metabolism. It makes sense for plants to remodel their basic metabolism in response to the environment. The currently available data suggest that such cross-talk occurs, but working out the molecular architecture of the system will require further detailed study.

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Floral mimicry: a fascinating yet poorly understood phenomenon

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Flowers of different species that resemble each other are not necessarily mimics. For mimicry to be occurring, the similarity must be adaptive. Unfortunately, no case of floral mimicry has ever been fully verified and it is important that we move beyond these perceived similarities to testing whether they are truly adaptive. Here we explain the differences between Batesian and Müllerian floral mimicry, illustrate what should be done to test mimicry hypotheses, and discuss how interspecific pollen transfer influences the evolution of mimicry.

The concepts of Batesian and Müllerian mimicry have been developed by zoologists and are commonly associated with protective mimicry in animal systems[1,2]. However, these concepts also apply to plant systems because the same evolutionary processes that form them (negative and positive frequency-dependent selection) occur whether an animal is being warned away (protective mimicry) or invited in (floral mimicry) (Fig. 1). In animal Batesian mimicry, selection favors resemblance of a palatable mimic to an unpalatable model. Similarly, in Batesian floral mimicry, selection favors resemblance of a non-rewarding mimic to a rewarding model[3,4]. In animal Müllerian mimicry, selection favors convergence on a single, 'aposematic', warning pattern as a defense against predators, such as the yellow and black striped pattern of bees, wasps and hornets. Similarly, in floral Müllerian mimicry, selection favors similar floral appearance among rewarding plants for the sake of attracting pollinators. The view that some floral mimicry systems fall within the concept of Batesian mimicry is now well established[3–5] although experimental tests remain few. Floral Müllerian mimicry is both less commonly accepted and less studied.

For floral mimicry to be established as occurring between two or more similar species, they must:

- Have strongly overlapping distributions, and must have done so long enough for coevolution to have occurred.
- Require pollinators for seed set.
- Overlap substantially in flowering phenology.
- Share the same pollinator species and the same individual pollinators must move freely between the species.
- The similarity must be important for fitness[6–8].

The majority of floral mimicry studies establish the first four points, but either neglect or incompletely address the last point – the critical question of whether the similarity is actually adaptive. Before we suggest the tests necessary to assess the fitness consequences of similarity, we would first like to further describe the basic kinds of floral mimicry, because the type of mimicry influences the kinds of tests performed.

There are two basic types of floral mimicry, Batesian and Müllerian, which are governed by different selection regimes (Fig. 1). In Batesian floral mimicry, the mimic produces no nectar reward, whereas the model does (Fig. 2). Hence the mimic's chances of visitation should be increased through its similarity to a nectar-producing model. Further, because the Batesian mimics do not have nectar, the more frequent they are in the population, the lower their pollination success becomes because pollinators can learn to avoid flowers that look a certain way, and indeed, both mimic and model might be avoided[9]. Thus, new Batesian mimic phenotypes that mimic a different model will enjoy a pollination advantage and this type of negative-frequency-dependent selection should select for increased diversity of model–mimic pairs (Fig. 1).

In Müllerian floral mimicry, two or more rewarding flower species gain a collective advantage as a result of convergence on a 'common advertising display'[4,7,10–15]. The similarity of Müllerian mimics increases the 'perceived' density of rewarding flowers and, thus, might increase the probability of pollination (Fig. 3). When pollinator visitation is positively density-dependent, greater similarity among flower species implies higher pollination success. Thus, Müllerian mimics are undergoing positive frequency-dependent selection (Fig. 1), and are all converging on a similar phenotype. In spite of selective pressure towards similarity, variation in Müllerian mimics probably exists because pollinators

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