# The role of calcium and activated oxygens as signals for controlling cross-tolerance

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**Plants are confronted on a regular basis with a range of environmental stresses. These include abiotic insults caused by, for example, extreme temperatures, altered water status or nutrients, and biotic stresses generated by a plethora of plant pathogens. Many studies have shown that the cellular responses to these environmental challenges are rather similar, which might be why plants resistant to one stress are sometimes cross-tolerant to others. To understand this phenomenon and to be able to take full advantage of it in agriculture, we must determine whether the individual biochemical pathways that make up the responses to each external stimulus are activated by unique, overlapping or redundant signalling systems. We discuss the potential role of signalling molecules, such as calcium and activated oxygen species, in underlying cross-tolerance.**

The existence of common defence systems to combat stress was first inferred from simple observations that plants resistant to one stress are often more resistant to others. In some cases the resistance phenotypes could even transcend the biotic–abiotic stress boundary. For example, ozone exposure can induce resistance to virulent phytopathogenic *Pseudomonas syringae* strains in *Arabidopsis*[1] and to tobacco mosaic virus in tobacco[2] (Fig. 1): the phenomenon is defined as cross-tolerance.

Cross-tolerance is extremely important for agriculture because plants can be selectively bred that are tolerant to more than one stress. Additionally, cross-tolerance allows us to compare and contrast individual responses and to examine the roles of common signal transducing molecules. Numerous studies have shown that calcium and activated oxygen species (AOS) exhibit important signalling functions in responses to both biotic and abiotic stresses, implying that they might be central components controlling cross-tolerance, at least at the cellular level. However, cross-tolerance is only possible if the whole plant is exposed to the primary signal or if systemic signals are also stimulated to ensure robust systemic resistance phenotypes.

## Calcium

Increases in intracellular calcium have been noted for a range of abiotic stresses, including chilling, heat shock, anaerobic stress, salinity and drought[3]. Therefore, cytosolic calcium might act at a convergence point for integrating different signals. In addition, abiotic stresses commonly mobilize abscisic acid (ABA), which is known to use calcium and calcium-dependent protein phosphatases for signal transduction. However, the increases in calcium and ABA levels generated by certain abiotic stresses are not in the same temporal range, which makes any association between the two rather tenuous. For example, ABA levels increase transiently only after 24 h of cold stress[4], whereas calcium changes occur immediately[5]. Furthermore, ABA-independent pathways controlling abiotic stress responses have been reported[6].

Ethylene is another common stress hormone whose mode of action requires calcium[7]. It can also promote ABA formation[8]. Therefore, a picture emerges in which abiotic stress responses are induced by a variety of primary and secondary (or reiterative) signals that can be temporally differentiated from each other.

Intracellular calcium homeostasis is also modulated during plant defence responses to pathogens. In this case, cytosolic calcium is increased in response to race-specific elicitors (e.g. AvrRpn1 in *Arabidopsis*[9] and Avr9 in tomato; J.D.G. Jones and M. Blatt, pers. commun.) as well as in response to some (but not all[10]) of the more general non-race-specific signals of pathogen attack or wounding, such as Pep13, chitin fragments, oligogalacturonides and salicylic acid[5,10–12].

How is specificity controlled through such a ubiquitous molecule? First, there are clear differences in the calcium signals arising from each response, for example, some are fast, others are slow, some are transient, others persist, oscillate or make waves, and some can be desensitized by repetitive stimulation whereas others cannot. These differences result in diagnostic calcium signatures for each signal[13].

One reason for these differences is the type of calcium channel through which calcium enters the cytoplasm. Often these channels are gated by other signalling molecules (such as calmodulin or specific nucleotides), by voltage or by stretch, and this can determine how long they are active and how long they remain recalcitrant to subsequent stimulations. The localization of the channel is also important because it determines where the calcium is derived from. Plasma membrane channels probably transport calcium derived from the cell wall, such as that dissociated from pectins. Internal channels can be localized on vacuolar, endoplasmic reticulum, nuclear, mitochondrial and plastid membranes, some of which might act as stores of internal calcium. Depletion and refilling of each of these stores will occur at different rates, which will also contribute to the desensitization and resensitization characteristics of each response.

Calcium homeostasis is maintained principally by the action of extrusion proteins, such as calcium ATPases and calcium antiporters[14,15]. Because calcium is toxic for most eukaryotic cells, resting concentrations remain in the nanomolar (50–100 nM) range. The cytosol is strongly buffered against high concentrations of calcium by a numerous range of calcium-binding proteins, such as calmodulins and calmodulin-binding proteins. A free molecule of calcium has been estimated to have a half-life of

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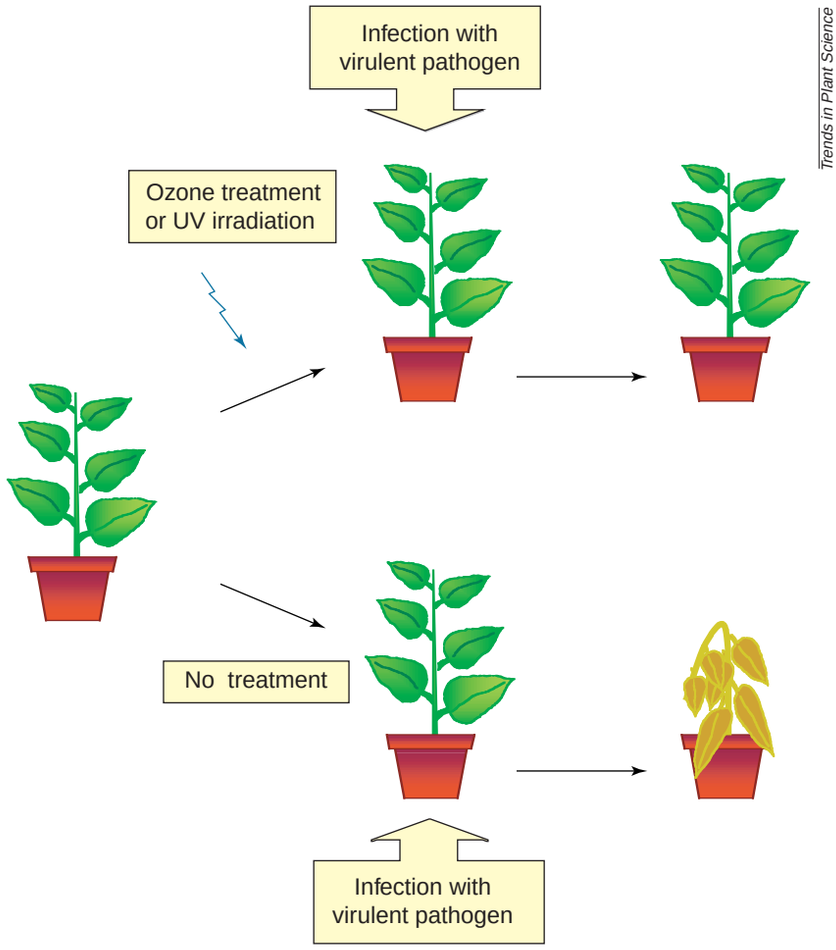


**Fig. 1.** The phenomenon of cross-tolerance. Pre-treatment of plants with a sub-lethal dose of ozone or ultraviolet (UV) irradiation can confer tolerance to a virulent pathogen. A plant not given this pre-treatment will die. Derived from experiments reported in Refs 1,2.

25 μs and to be able to diffuse no more than 100–500 nm within the cytoplasm of a eukaryotic cell[16]. Therefore, signalling specificity can be achieved by the spatial localization of molecules within the cytoplasm and by their controlled release of calcium. For this reason, signalling molecules have often been found in close proximity to each other, bound to scaffolding molecules or to the cytoskeleton that allow association and dissociation in response to specific signals such as calcium and protein phosphorylation.

### Activated oxygen species

Oxidative stress, resulting from the generation of activated oxygen species (AOS), such as superoxide ($O_2\cdot^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (OH·), is a common phenomenon in many stress responses, such as photoinhibition, hypo-osmotic stress, drought and cold shock[17]. Although the uncontrolled production of AOS caused by disequilibria in electron transfer reactions is a common phenomenon in aerobic organisms, the problem is exacerbated in oxygenic organisms such as higher plants. AOS generation leads to cellular damage and ultimately to cell death, primarily through damage to the photosystem II reaction centre and to membrane lipids. In addition, pathogen defence responses use deliberate AOS-generating systems based on plasma membrane-bound NADPH oxidases or peroxidases[18]. These systems might have been designed to kill pathogens and host plant cells, to reinforce the plant cell wall or to immobilize a pathogen within it, and lead to the phenomenon of hypersensitive cell death in incompatible plant pathogen reactions.

The role of $H_2O_2$ in inducing defence responses is supported by the finding that constitutive expression of an $H_2O_2$-generating glucose oxidase in transgenic plants confers broad-spectrum disease resistance, probably as a result of the induction of defence-related genes, cell wall lignification and salicylic acid[19].

Because many stress conditions cause cellular redox imbalances it has been proposed that oxidative stress defence responses might be a central component mediating cross-tolerance[20]. The first evidence for this came from the unicellular green alga *Chlorella*. It was found that prior growth on sublethal concentrations of the herbicide paraquat (which generates an oxidative stress) could decrease the damage caused by exposure to low temperatures[21] and to sulphite[22]. Such phenomena have now been reported for several plants, for example, paraquat-tolerant maize is also tolerant to drought and atmospheric pollutants such as $SO_2$ (Ref. 23), and ozone pre-treatment can induce pathogen resistance in *Arabidopsis* and tobacco[1,2].

But because of the toxic effects of AOS, it has been difficult to distinguish the AOS signalling roles from the secondary effects caused by cytotoxicity. For example, because much evidence has implicated AOS in hypersensitive cell death, it had been assumed that $H_2O_2$ is the cytotoxic agent. However, recent results using the cryptogein elicitor in tobacco implicate a 9-oxylipin pathway involving the lipoxygenase-dependent peroxidation of fatty acids as the major cause of cell death and suggest that $H_2O_2$ might act as a signalling molecule for the induction of the response[24].

Furthermore, in spite of the commonalities in plant defence responses to pathogens, not all elicitors of defence responses act in the same way. For example, oligogalacturonides use phospholipase C- but not phospholipase A-activated pathways, whereas an elicitor from *Verticillium dahliae* appears to operate via the opposite system[10]. Therefore, even though the net result of plasma membrane oxidase assembly and activation is the same, it would appear that distinct signal transduction pathways are used in each specific case.

Although the source of AOS in the hypersensitive response is normally assumed to be the NADPH oxidase, this might not always be the case. The interaction of *Xanthomonas* with cotton has provided some interesting insights[9,25]. In this case, although both peroxidase and NADPH oxidase are present, only the peroxidase system appears to generate AOS (Ref. 26). Therefore, precise differences might exist for specific plant–pathogen interactions and also between plant species.

Just as compartmentalization is important for regulating calcium-dependent responses, the effects of AOS are also dependent upon their site of generation. This can be extracellular,

cytoplasmic, organellar or nuclear[27]. AOS localization is particularly important because cytotoxic molecules such as $O_2^{\cdot -}$ and OH· cannot cross membranes whereas $H_2O_2$ can. For example, phytoalexin biosynthesis in parsley (*Petroselinum crispum*) can be induced only by apoplastic $O_2^{\cdot -}$ production[28], heat shock proteins can be induced by $H_2O_2$ but not by $O_2^{\cdot -}$ in tomato[29], and tomato extensins can be induced by both $H_2O_2$ and $O_2^{\cdot -}$ (Ref. 30). Chloroplast or mitochondrial $O_2^{\cdot -}$ production might also have different consequences, particularly concerning the promotion of cell death via apoptotic-like mechanisms. In animal cells, increased oxidative stress in the mitochondria can lead to apoptosis[31]. But although the regulatory mechanism in animal cells has been elucidated in detail it is largely unknown in plants, therefore it should not be assumed that programmed cell death occurs in the same way in both plants and animals.

AOS formation leads to alterations in intracellular redox homeostasis, a consequence of which is the activation of specific signalling pathways. To date, the membrane diffusible and comparatively stable molecule $H_2O_2$ is the most likely candidate for this intracellular signalling role. The addition of $H_2O_2$ or its experimental generation in catalase-antisense transgenic plants by exposure to high light irradiation can cause the induction of several defence-related genes[32] (Fig. 2). The use of plants with reduced $H_2O_2$-detoxifying capabilities exemplifies the potential role of this molecule as a signal. Indeed, enzymes that scavenge $H_2O_2$ are down regulated during pathogenesis[33]. Conversely, $H_2O_2$ is a potent activator of certain MAP kinase cascades [such as those involving wound-induced protein kinase (WIPK)][25], which are components of pathogen defence signalling, perhaps working upstream of the NADPH oxidase-derived oxidative burst[34].

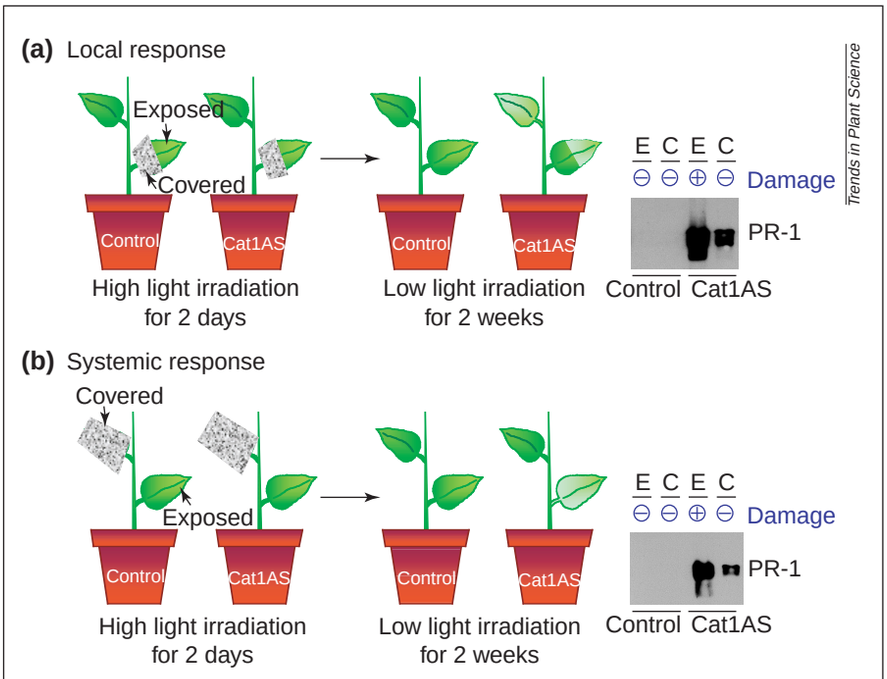


**Fig. 2.** Systemic expression of the pathogenesis-related protein PR-1 in local and distal leaves of catalase-antisense plants after light treatment. In transgenic tobacco plants expressing an antisense catalase construct (Cat1AS), $H_2O_2$ can be generated experimentally by shifting the plants to photoinhibitory light conditions[32]. This system has been used to investigate the role of $H_2O_2$ in (a) local and (b) systemic signalling. (a) Half a leaf was covered with foil during treatment with high light irradiation, whereas in (b) a top leaf was entirely covered to shield it from the high light irradiation. Following exposure to photoinhibitory light for two days the foil was removed and the plants were returned to low light intensity levels for two weeks before harvest. (a) Samples were collected from the exposed and unexposed areas of the same leaf, and in (b) from different exposed and unexposed leaves. The western blot analysis of PR-1 abundance (right) demonstrates that PR-1 levels increase only in antisense and not control plants and that both exposed (E) and covered (C) areas contain increased levels, even though the covered areas did not have any visible damage (indicated by a minus symbol, damage is indicated by a plus symbol, damaged areas at the time of harvest are indicated in light-green on the plants). (a) $H_2O_2$ can stimulate local responses, whereas in (b) a role for $H_2O_2$ is implicated in systemic signalling. *Reproduced, with permission, from Ref. 32.*

The role of $H_2O_2$ as an intracellular signal in animal cells is well known. For example, it activates the NF-κB transcription factor, which mediates inflammatory, immune and acute phase responses to diverse stress stimuli[35]. Several plant disease resistance genes share some homology with molecules involved in NF-κB-mediated responses[18], indicating that similarities exist between animal and plant stress signalling systems. A range of other transcription factors that are responsive to oxidative stress, such as SoxR/S, OxyR and AP1 (Ref. 36), has been described in other non-plant systems.

Pathogen defence in plants also involves the production of nitric oxide (NO), which, when combined with $O_2^{\cdot -}$, can generate highly toxic molecules such as peroxynitrite radicals. Although peroxynitrite is an important intermediate in the phagocytic oxidative burst in animals[37], this does not appear to be the case in plants. Instead, NO appears to act in conjunction with $H_2O_2$ rather than $O_2^{\cdot -}$ (Ref. 38). Nitric oxide has been proposed to activate responses via specific signalling pathways involving G proteins, cGMP and calcium[39] (G. Neuhaus, pers. commun.). The overlap among these pathways and the better-studied calcium–MAP kinase-NADPH oxidase pathways[9] remains to be elucidated. A further complication is that, as with other reactive oxygen species, NO can have opposite effects depending on the applied exogenous doses and the degree of insult that the plant is suffering[40]. Because no reliable data on endogenous NO concentrations are available, the elucidation of the physiological role of this gas is only just emerging. In addition, because NO is a free radical with high levels of reactivity towards other AOS, knowing the relative concentrations of each activated oxygen or nitrogen species will be of tremendous importance in understanding the basis of NO function and the plant's response to it.

A further possibility is that AOS signalling in abiotic and biotic stresses is mediated by thiol–disulphide exchange reactions involving glutathione[17]. Depletion of glutathione (a potent inducer of defence genes[41]) in *Arabidopsis* cell cultures renders them susceptible to oxidative damage[42]. Furthermore, in bacteria, glutathione acts as a sink for NO by generating nitrosoglutathione (GSNO), which can activate the OxyR transcription factor[43]. Although the generation of GSNO has not yet been established in plants, it is an interesting possibility for linking redox control with cellular signalling.

### Crosstalk between calcium and AOS

Changes in intracellular redox and calcium homeostasis are unifying consequences of biotic and abiotic stress. Furthermore, an oxidative stress *per se*, such as the administration of $H_2O_2$, can stimulate increases in cytosolic calcium[44], and when calcium signalling is blocked, elicitation of the oxidative burst by oligogalacturonides is prevented[10]. However, this is not the case for all elicitors, for example, the proteinaceous elicitor harpin does not appear to use calcium signals to stimulate the oxidative burst in tobacco cells[10]. Therefore, although overall responses might be conserved, there appear to be distinct differences between the regulatory systems in operation in each case; in some cases calcium is upstream of AOS production, in other cases it is downstream, and most commonly it is both upstream and downstream.

Communication between calcium and AOS production is mediated, at least in some cases, by calmodulin[45]. This has been proposed to occur through a calmodulin-dependent NAD kinase that supplies NADPH during assembly and activation of the oxidative burst oxidase. Consistent with the proposed role of AOS in the plant hypersensitive response, tobacco cells expressing a constitutively active calmodulin isoform show enhanced hypersensitive cell death in response to an incompatible but not a compatible pathogen[46]. Other possibilities for calcium–AOS crosstalk are the calcium-binding EF hands present in one of the gp91phox subunits of the NADPH oxidase[47].

### Systemic signals

The responses of single cells must be integrated with the response at the whole plant level. Systemic signalling has been intensively studied in response to localized pathogen attack and wounding, and a recent report proposes that systemic signals are also generated in response to abiotic stresses[48].

During pathogen infection, long-range signalling can lead to systemic acquired resistance (SAR), in which localized treatment with an avirulent pathogen gives robust whole-plant resistance to unrelated virulent pathogens, which can last up to several months. Conversely, systemic signalling in response to wounding can enhance defence against insects. In both cases, the signalling systems are highly complex and involve salicylic acid, jasmonic acid and ethylene[49]. Crosstalk has been observed in several instances, and although in some cases the pathways can be mutually antagonistic, in other situations they can be co-regulated and synergistic. The MAP kinase salicylic acid-induced protein kinase (SIPK)[50] and the NPR1 protein [also known as NIM1 (Refs 51,52)] might be convergence points that govern the final output in each case.

Calcium is unlikely to participate in systemic signalling because it is unlikely to diffuse from cell to cell. However, there is accumulating evidence that AOS or derived molecules are long-range signals. It has been observed that localized inoculation of *Arabidopsis* leaves with an avirulent bacterial pathogen induces secondary oxidative bursts in distant tissues that result in systemic microscopic lesions denoted 'micro-hypersensitive responses' (micro-HRs)[53]. Blocking AOS production at the infection site can inhibit the appearance of micro-HRs. Conversely, the localized generation of $H_2O_2$ results in the generation of micro-HRs at a distance. $H_2O_2$ has been proposed to be the systemic signal and the micro-HRs throughout the plant might serve a reiterative role for ensuring its efficient propagation[53].

Conversely, it has been reported that the controlled generation of $H_2O_2$ in plants that have decreased AOS scavenging capacity because of compromised catalase expression, results in both local and systemic induction of a pathogenesis-related protein (PR-1) (Fig. 2) and subsequently in inducible local resistance to bacterial pathogens[32]. This provides further experimental proof that the modulation of $H_2O_2$ levels can lead to enhanced disease resistance. However, as has been found in other cases (such as with NO), there appears to be a fine line between benefit and injury because the reduced capability of these plants to detoxify AOS *per se* results in hypersensitivity to pathogen infection[33].

Systemic signalling also occurs during abiotic stress, and AOS have again been implicated as prime players[48,54]. For example, it has recently been reported that high light irradiation can induce ascorbate peroxidase (APX) both locally and in adjacent leaves that have not been exposed to light[48]. APX induction at a distance is accompanied by increased $H_2O_2$ content and acclimation of the photosynthetic apparatus to high light intensity levels. The involvement of $H_2O_2$ is inferred from the observation that these responses can be inhibited by infiltration with catalase but not superoxide dismutase (an $O_2\cdot^-$ scavenger). These results give some clues as to the identity of the systemic signal(s) for disease resistance and for acclimation to abiotic stress. Are the same molecules involved in both cases? The induction of a systemic signal by an abiotic stress (high light irradiation) that modulates pathogen defence responses in catalase-compromised plants[32] might suggest that this is the case. Is $H_2O_2$ the systemic signal? Although $H_2O_2$ can be propagated over several cell lengths[27], there is no real evidence for longer-range propagation. An alternative candidate for a systemic signal is glutathione, which is readily modulated by AOS such as $H_2O_2$, which can cross biological membranes and which can diffuse or be transported long distances[17]. In this regard, it might be relevant that the PR-1 protein induced systemically in catalase-antisense plants is a glutathione *S*-transferase[55].

The relationship between glutathione and the more conventional systemic signals, jasmonic acid and salicylic acid, should therefore be investigated with some urgency, and future models must incorporate key protein components such as NIM1/NPR1 and its interacting partners[52].

### A unifying view of signalling in response to stress

It is clear that both calcium and AOS are important modulators of the cellular signal transduction events following biotic and abiotic stress insults, although the long-distance signalling capabilities of some AOS implicate that they, or derived molecules, are more important for the induction of cross-tolerance. The similarities among the plant stress responses are also striking. For example, cDNA-AFLP differential display has revealed that the majority of genes induced by the race-specific Cf9–Avr9 interaction in tobacco are also induced by wounding (J.D.G. Jones, pers. commun.), and many systemic responses to pathogens also occur at localized infection sites. On the one hand, such results might reflect the experimental difficulty of differentiating between primary and secondary responses (i.e. as a result of one stress and concurrent damage, further multiple stresses and ensuing damage will occur). On the other hand, they might infer a certain level of informational and/or functional redundancy (i.e. although responses are complex, they are flexible and unstable). Furthermore, the observed differences in the level and timing of the transcriptional readouts might suggest that plant acclimation to different stresses is controlled by sophisticated quantitative rather than qualitative effects. Plants are clearly highly tuned to the absolute levels of AOS, because small concentration changes can result in drastically different responses.

The similarities among intermediate signalling molecules used by diverse stresses imply the existence of intracellular networks rather than linear pathways[56]. If a limited number of signalling intermediates can interact in a combinatorial fashion, such networks could allow specific cellular responses to numerous,

potentially conflicting, signals. Some of the constituents of MAP kinase cascades are activated by cold, drought, salinity, $H_2O_2$, heat, shaking, wounding, pathogens, elicitors, ABA, salicylic acid and ethylene, suggesting that they might function as promiscuous networking molecules[57]. How biological specificity can be generated is a major problem that should be addressed. The availability of a range of experimental systems and technologies promise to resolve our current ignorance into coherent models of how this might function.

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# Colinearity and gene density in grass genomes

**Beat Keller and Catherine Feuillet**

**Grasses are the single most important plant family in agriculture. In the past years, comparative genetic mapping has revealed conserved gene order (colinearity) among many grass species. Recently, the first studies at gene level have demonstrated that microcolinearity of genes is less conserved: small scale rearrangements and deletions complicate the microcolinearity between closely related species, such as sorghum and maize, but also between rice and other crop plants. In spite of these problems, rice remains the model plant for grasses as there is limited useful colinearity between *Arabidopsis* and grasses. However, studies in rice have to be complemented by more intensive genetic work on grass species with large genomes (maize, Triticeae). Gene-rich chromosomal regions in species with large genomes, such as wheat, have a high gene density and are ideal targets for partial genome sequencing.**

The botanical family of the grasses (Poaceae) comprises >10 000 species. Their reproductive mechanism, plant anatomy and genetic variability results in a high level of adaptability enabling grass species to grow in most terrestrial habitats. In the past few thousand years, humans have taken advantage of these natural resources by domesticating and breeding a small subset of the grass species. These efforts have resulted in many important crop plants, such as wheat, rice, maize and sorghum. Many species, including wheat, are grown in different climate zones and environmental conditions, demonstrating the diversity in the gene pool of a single species. Wheat and rice each contribute ~20% of the calories ingested by the world's population (FAOSTAT home page; http://apps.fao.org/). In total, ~60% of the world's food production is obtained from grasses, which makes them economically by far the most important plant family.

In terms of genome organization, grasses represent a highly diverse family. Their chromosome number varies from 2n = 4 for the two species *Zingeria biebersteiniana* and *Colpodium versicola*[1], to 2n = 266 for the polyploid grass *Poa litorosa*[2]. Their genome sizes also vary greatly; for example, the genomes of the two crop species, rice ($4.3 \times 10^8$ bp) and bread wheat ($1.7 \times 10^{10}$ bp), differ by a factor of 40 (Ref. 3). Comparative genetics enables us to analyse the genome structure in these different species. If gene organization and order are conserved between species, a smaller reference genome can be used as a model for gene isolation from large genomes. In addition, comparative genetics provides the basis for understanding genome evolution.


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