Beyond 2R

Although recent studies have focused on 2R, Ohno's seminal work [1] also presented a broader vision of whole-genome duplication as a force that could enable the leap in morphological and developmental complexity seen in modern vertebrates. Since 1970, genomics has revealed several other mechanisms that promote diversity of protein function on a genomic scale, including alternative splicing [19] and domain shuffling [6]. Widespread segmental duplication and domain accretion (recently reported by Eichler *et al*. [20]) could be evidence of ongoing gene formation through domain shuffling in the modern genome. The challenge for the future is to forge a comprehensive view of the interplay of all of the forces that drive vertebrate evolution.

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The genes of the Green Revolution

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The spectacular increases in wheat and rice yields during the 'Green Revolution', were enabled by the introduction of dwarfing traits into the plants. Now, identification of the genes responsible for these traits shows that they interfere with the action or production of the gibberellin (GA) plant hormones. We knew that the wheat *Rht* genes encode growth repressors that are normally suppressed by GA, and recent work shows that the rice *sd1* gene encodes a defective enzyme in the GA-biosynthetic pathway.

In the past 40 years the population of the world has doubled to more than 6.1 billion people. Fortunately, this increase has been more than matched by increases in global cereal production so, despite gloomy predictions of impending famine [1], world-wide food production per capita is higher than it was in 1960 [2]. A major factor that enabled these impressive yield increases was the introduction of high-yielding varieties of wheat and rice, in combination with the application of large amounts of fertilizer and pesticides. The impact of these advances was termed the 'Green Revolution'.

The introduction of dwarfing genes into cereal crops was crucial to this revolution. The stems of tall wheat and rice plants were not strong enough to support the heavy

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grain of the high-yielding varieties so that plants fell over, a process known as lodging, with consequent large yield losses. SEMI-DWARF (see Glossary) plants possessed short, strong stalks and did not lodge. Furthermore, a greater proportion of ASSIMILATE PARTITIONED into the grain, resulting in further yield increases. Now, the genes responsible for the semi-dwarf growth habit of the wheat and rice varieties that enabled the Green Revolution have been identified. About three years ago, the *Reduced height* (*Rht*) genes of wheat were isolated and shown to interfere with the signal transduction pathway of the gibberellin (GA) growth hormone [3]. Now, the *semidwarf1* (*sd1*) gene has been isolated from rice simultaneously by three research groups and shown to impair the biosynthesis of the same hormone [4–6]. Thus it seems that this hormone is central to the control of plant stature.

The origins of the dwarfing genes

The wheat dwarfing genes of the Green Revolution originated in Japan (reviewed in [7]). Early in the 20th century, the Japanese crossed a semi-dwarf wheat variety called Daruma with American high-yielding varieties to produce Norin 10. Although it was of little importance in Japan, Norin 10 was used in breeding programmes in the USA after 1945 to produce a number of high-yielding semi-dwarf CULTIVARS. One cross, Norin 10-Brevor 14, was sent to Norman Borlaug (later to win the Nobel Peace prize for this work) at the Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT) in Mexico and was bred with varieties adapted to grow in tropical and sub-tropical climates. The progeny were then distributed to Latin America and South and Southeast Asia, where they were rapidly adopted, allowing spectacular increases in wheat yields in these countries. Although the American cultivars were winter wheat that required vernalization (exposure to low temperatures) to flower, the new CIMMYT lines were spring wheat that could be grown in warm climates in any season, allowing two crops a year. Norin10-Brevor 14 was also the origin of dwarfing genes in many wheat cultivars suitable for temperate countries. The Norin 10 dwarfing genes are now present in >70% of current commercial wheat cultivars world-wide [8].

Bread wheat is hexaploid, its three sets of chromosomes, A, B and D, having been contributed by its wild diploid progenitors. Norin 10 contains two dwarfing genes, which are semi-dominant (change of function) alleles of HOMOEOLOGOUS GENES on chromosomes B and D. These dwarfing alleles are named *Rht-B1b* (formerly *Rht1*) and *Rht-D1b* (*Rht2*) to reflect their chromosomal location [9]. The effect of each gene on plant height is similar and their combined effect is additive.

Glossary

Assimilate partitioning: Distribution between plant tissues of the products of assimilation, the process whereby simple inorganic molecules are incorporated into complex organic compounds, principally by photosynthesis.
Cultivar: A plant variety that is found only in cultivation.
Homoeologous genes: Equivalent genes that are present on the different sets of chromosomes in polyploid species.
***Indica* and *japonica* rice:** Subspecies of rice (*Oryza sativa*). *Indica* is grown mostly in tropical and subtropical regions. It tends to be tall and, therefore, prone to lodging. *Japonica* rice is grown typically in temperate regions and is shorter than *indica* rice.
Semi-dwarf: A mutant variety that is moderately reduced in height relative to the normal plant. It has been defined as having a height that lies between 50–100% that of the normal plant, whereas a dwarf is <50% the height of the normal plant [26].

Fig. 1. Semi-dwarf rice cultivars and their tall isogenic lines. From left to right: Dee-geo-woo-gen (dwarf *indica* cultivar), woo-gen (tall equivalent), Calrose 76 (dwarf *japonica* cultivar), Calrose (tall equivalent).

The history of semi-dwarfing genes in rice closely parallels that in wheat, and also begins in the Far East. The Green Revolution in rice was dependent on the introduction of semi-dwarf, high yielding *INDICA* cultivars for growing in tropical areas. The dwarfing gene originated from a Chinese cultivar, Dee-geo-woo-gen (Fig. 1), which was used in a breeding program in Taiwan during the 1950s to produce the highly successful Taichung Native 1 (TN-1), and later at the International Rice Research Institute (IRRI) in the Philippines to produce IR-8, the so-called 'miracle rice'. TN-1 and IR-8 have subsequently been used as parents in breeding programs to produce many of the commercial semi-dwarf *indica* cultivars grown in tropical and semi-tropical areas, and also in developing *JAPONICA* cultivars for growing in the more temperate Republic of Korea and California [10]. Semi-dwarf, high yielding cultivars have also been produced independently in the People's Republic of China, Japan and the USA. The semi-dwarf trait turns out to be due to different alleles of a single recessive gene, *sd1*, even where the parent strains have been selected or produced independently [10].

Wheat *Rht* and gibberellin signal transduction

The *Rht* dwarfing alleles cause a reduced response to the GA class of plant hormones [7]. These growth regulators are diterpenoid carboxylic acids, which participate in many developmental processes in higher plants, including stem elongation [11]. The *Rht* gene was identified by Peng *et al.* [3] and shown to be an orthologue of the *Arabidopsis GAI* and maize *dwarf8* genes, for which mutations that result in GA-insensitive dwarfs were also known. Rht-1a/d8/GAI (these symbols refer to the wild-type proteins) form a sub-group of the GRAS family of proteins, which are thought to function as transcriptional regulators [12]. Members of this sub-group uniquely contain two conserved regions at the N-terminus, including a 27-amino acid motif known as the DELLA domain. Peng *et al.* [3] identified base substitutions in the *Rht-B1b* and *Rht-D1b* dwarfing alleles that introduce

stop codons within the DELLA region. They suggested that translational reinitiation at one of several methionines that follow the stop codons might result in the formation of truncated Rht proteins lacking the DELLA domain, and that these proteins function as constitutive (GA-insensitive) repressors of growth. The *D8* [3] and *gai* [13] mutations also result in partial or complete deletion of one or both of the conserved domains. Thus, the Rht-1a/d8/GAI (wild-type) proteins function as negative regulators of GA signalling and GA acts by repressing their function, provided the N-terminal domains are present [14,15]. In support of this concept, ectopic expression of *gai* in rice induced dwarfism [3] and loss-of-function mutations in *Rht*-like genes produce an overgrowth phenotype in some cases [16,17].

In addition to *d8*, orthologues of *Rht-1a* have been identified from rice (known as *OsGAI* [18] or *SLR1* [16]) and barley (*SLN1* [17]). Although cereals possess a single example of the Rht-1a/d8/GAI-type proteins, *Arabidopsis* contains a gene family encoding the related proteins RGA and three RGA-like proteins (RGL1, -2, -3) in addition to GAI. The *Arabidopsis* homologues appear to have overlapping functions in terms of their involvement in different GA-regulated developmental processes (reviewed in [19]). It is unclear how a single protein in the cereals is functionally equivalent to five proteins in *Arabidopsis*; this difference might indicate considerable functional redundancy in *Arabidopsis* or fundamental differences in GA signalling pathways between *Arabidopsis* and members of the Gramineae.

Some progress has been made recently in understanding the function of the Rht-like proteins and their repression by GA (Figs 2,3b). RGA [14], SLR1 [20] and SLN1 [21] are located in the nucleus and are rapidly degraded in the presence of GA, the DELLA domain being required for this process. The signal transduction pathway upstream of Rht is still unclear, but the GA-induced degradation is thought to involve ubiquitin-mediated proteolysis [19]. As well as the GA signal domains, which consists of the DELLA motif and a second conserved region (TVHYNP motif) at the N-terminus, other functional elements have been identified in the rice protein SLR1 by deletion analysis [20] (Fig. 2). These include a leu-heptad domain involved in dimerization, a nuclear localization domain and the repression domain at the C-terminus. Paradoxically, SLR1 can function as an activator of gene expression, suggesting that it acts indirectly by stimulating production of an as-yet-unidentified repressor [18]. The targets for repression could be transcriptional activators, such as GAMYB, which has been shown in barley aleurone to be induced by GA and to be repressed by SLN1 [21]. GAMYB is known to activate transcription of GA-induced genes, such as α-amylase, by interacting with their promoters [19].

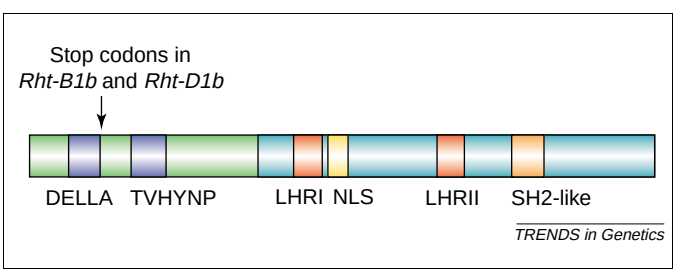

Fig. 2. Structure of Rht/GAI and related proteins. The C-terminus (light blue) is highly conserved in all GRAS proteins [12] and contains the repressor activity. Functional domains identified in this region include two leucine heptad repeats (LHR), the first of which mediates dimerization [20], a nuclear localization signal (NLS) and a SH2-like domain, which could indicate the involvement of phosphotyrosine signalling [3]. The N-terminus (green) contains the GA-signalling domain. It is more variable, but includes two highly conserved motifs (dark blue) that are required for GA-induced degradation [20]. The arrow indicates position of stop codons in *Rht-B1b* and *Rht-D1b*.

Rice *sd1* and gibberellin biosynthesis

In contrast to *Rht*, the *sd1* mutation of rice is recessive, and normal height can be restored in mutants by application of GA [22], indicating that they are defective in GA production. Three research groups have isolated the *sd1* gene independently and shown that it encodes a GA 20-oxidase (GA20ox), an enzyme involved in GA biosynthesis [4–6]. Two of these groups used positional cloning to detect a GA20ox open reading frame that was closely linked to the *sd1* locus on the long arm of chromosome 1 [4,6]. Furthermore, they could identify mutations in the corresponding genes from semi-dwarf cultivars. The third group, having deduced the identity of the gene by the effect of mutations on GA content, used PCR to amplify DNA fragments corresponding to two *GA20ox* genes, one of which mapped to the *sd1* locus [5,22]. Semi-dwarf rice varieties possessing the *sd1* allele from Dee-geo-woo-gen contain a 383-bp deletion in the GA20ox gene (known as *OsGA20ox2*) that introduces a stop codon, which probably results in a highly truncated, inactive enzyme. The Japanese varieties Jikkoku and Reimie, and the USA variety Calrose76, which are the γ irradiation-derived progenitors of semi-dwarf *japonica* lines grown in those countries, contain point mutations in *OsGA20ox2* resulting in single amino acid substitutions [4,6,22]. The dwarfing alleles used in the *japonica* lines are generally weaker than that in the *indica* varieties, indicating that the mutant enzymes might possess some activity.

Gibberellin 20-oxidases are 2-oxoglutarate-dependent dioxygenases that catalyze the loss of carbon-20 in the penultimate step of GA biosynthesis [23] (Fig. 3a). They are encoded by small gene families, members of which have partial functional redundancy because of overlapping (but distinct) expression patterns or because of movement of the intermediates produced by the enzymes between tissues. Thus, loss-of-function *GA20ox* mutants are usually only moderately GA-deficient and are semi-dwarfs, in contrast to severely GA-deficient plants, which are extremely dwarfed and often sterile. Two *GA20ox* genes have been described in rice: *OsGA20ox1* [24] and the newly identified *OsGA20ox2*. It is remarkable that selection for semi-dwarfism in rice has consistently yielded mutations in *OsGA20ox2* rather than in *OsGA20ox1* or another GA-biosynthesis gene (GA 3-oxidase, for example, is also encoded by a multi-gene family). Mutations in other genes could have severe developmental consequences or have detrimental effects on yield and so have never been selected in breeding progammes. *OsGA20ox1* is more highly expressed in flowers than is *OsGA20ox2* [4], and could be required for grain formation.

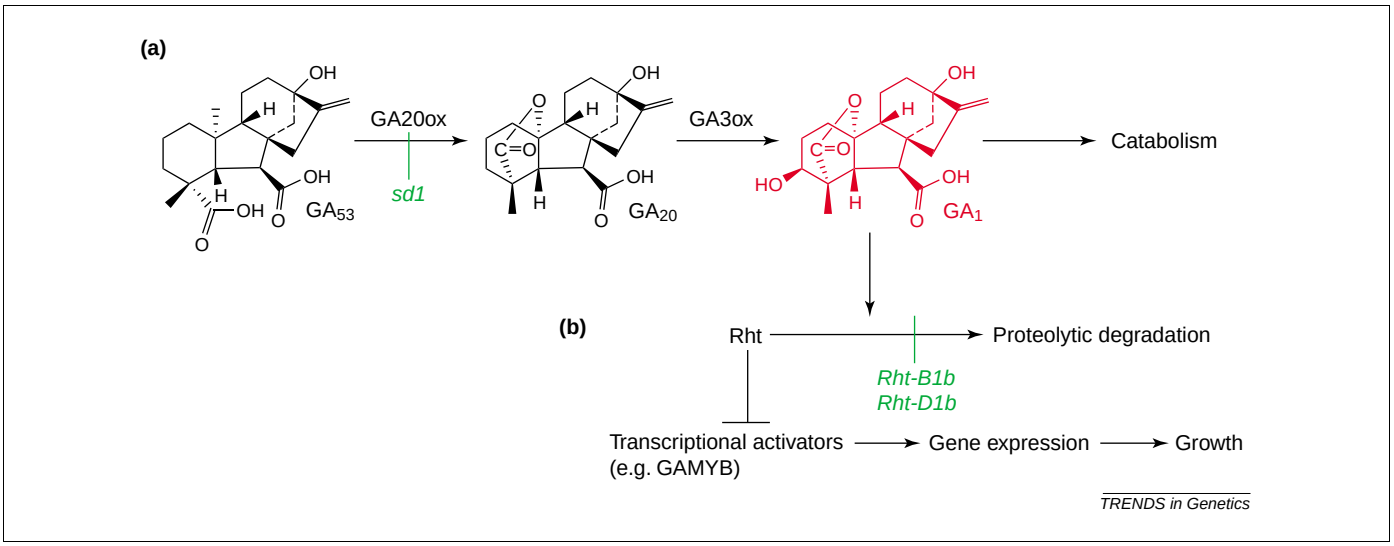

Fig. 3. Gibberellin signalling pathway. (a) *sd1* mutations affect the activity of GA 20-oxidase (GA20ox), which catalyses the conversion of GA_{53} to GA_{20} in the biosynthetic pathway to the biological active product, GA_1. GA_{20} is converted to GA_1 by the action of GA 3-oxidases (GA3ox). (b) GA action results in the degradation of Rht, which functions by suppressing expression of GA-induced genes and, hence, growth. The mutant forms of Rht (Rht-1Bb and Rht-1Db) are not susceptible to GA-induced degradation.

OsGA20ox2, however, is expressed most highly in mature leaves, and is apparently involved primarily in vegetative growth.

Conclusions

It has been shown for many species that GA concentration limits growth and that GA20ox activity is a major determinant of GA production (e.g. [25]). Therefore, it is probably no coincidence that mutations in a *GA20ox* gene have been selected in screens for semi-dwarfism in rice. In hexaploid wheat loss-of-function mutations are unlikely to be effective (unless present in all three genomes) so that dwarfs were detected from dominant (gain-of-function) mutations. Both cases highlight the important role of GAs in regulating developmental processes that are critical to agriculture. Thus, GA signalling pathways (biosynthesis and signal transduction) are prime targets for manipulation in the quest for further improvements in crop yield. The yields from present cereal cultivars appear to be approaching their limit and new strategies are needed if population is not finally to outstrip food supply. Targeted genetic modification of crop development could form a part of the next Green Revolution.

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Mammalian RNAi for the masses

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Just a couple of years ago, only biologists working with plants or *Caenorhabditis elegans* could use RNA-mediated interference (RNAi) technology to gain insight into gene function. However, the recent groundbreaking discovery that *in vitro* synthesized, 21- to 23-nucleotide, double-stranded RNAs can act as small interfering RNAs (siRNAs) to elicit gene-specific inhibition in mammalian cells has made RNAi possible in mammalian systems too. Reported only a year ago, mammalian RNAi is already changing our way of studying gene function in higher eukaryotes. And, a recent exciting advance allows delivery of siRNAs into mammalian cells by a DNA vector. In addition to providing a low-cost alternative to the chemically synthesized siRNAs, this DNA-vector-based strategy is capable of mediating stable target gene inhibition, thus allowing gene function analysis over an extended period of time.

RNA-mediated interference (RNAi), a term coined by Fire and Mello, is the inhibition of expression of specific genes by double-stranded RNAs (dsRNAs) [1]. RNAi is evolutionarily conserved among eukaryotes, and it probably has an essential role in mediating responses to exogenous RNAs (such as viruses) and in stabilizing the genome by sequestering repetitive sequences (such as transposons) [2]. RNAi is a multi-step process involving the generation of small interfering RNAs (siRNAs) *in vivo* through the action of the RNase III endonuclease Dicer. The resulting 21- to 23-nucleotide (nt) siRNAs mediate degradation of their complementary RNA. The biology and mechanisms of RNAi are reviewed in detail in Refs [2–6], so here I limit my discussion to the recent development of DNA vector-based RNAi approaches in mammals and its potential application in gene function analysis and in therapy.

Until recently, the use of RNAi as a reverse genetic tool to study gene function was restricted to plants, *Caenorhabditis elegans* and *Drosophila*, where large dsRNAs can efficiently induce gene-specific silencing [1,7–9]. A major obstacle to achieving RNAi in mammals is that dsRNAs longer than 30 nt activate an antiviral defense mechanism that includes the production of interferon, resulting in non-specific degradation of RNA transcripts and a general shutdown of host cell protein synthesis [10,11]. This obstacle has been overcome recently by using *in vitro* synthesized ~21-nt siRNAs to mediate gene-specific suppression in mammalian cells. These siRNAs are long enough to induce gene-specific suppression, but short enough to evade the host interferon response [12,13]. Following this breakthrough discovery, several reports appeared within a few months describing DNA vector-based strategies for the delivery of siRNA into mammalian cells, further expanding the utility of RNAi in mammals. The new approaches are not only cost effective, but also have unique properties that could be important for the application of the RNAi technology *in vivo*.

Plasmid vectors expressing siRNAs directed by RNA polymerase III

Biochemical analyses show that siRNAs generated by RNase III (Dicer) in *Drosophila* embryonic extracts contain 3′ overhangs of two or three nt [14–16]. This structural feature appears to be important for the *in vitro* synthesized siRNAs to inhibit gene expression effectively in cultured mammalian cells [12]. Furthermore, siRNAs with 3′ overhangs of two uridines have been found to be more efficient than those with 3′ overhangs of AA, CC or GG [15]. Recently, several laboratories have reported the success of using RNA polymerase III (Pol III) promoters to direct *in vivo* synthesis of functional siRNAs that incorporate some of these structural features [17–24]. There are several reasons for using Pol III. First, unlike RNA Pol II, Pol III normally transcribes small, noncoding transcripts that are not capped or polyadenylated at the 5′ and 3′ ends, respectively. Second, Pol III initiates transcription at defined nucleotides, and terminates transcription when it encounters a stretch of four or five thymidines [25]. Consequently, it is possible to design small RNAs synthesized by Pol III that carry 3′ overhangs of one to four uridines, a structural feature resembling that defined for siRNAs to be effective *in vitro* [16].

To date, two approaches, both using Pol III promoters, have yielded robust gene-specific inhibition.

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