TRANSFORMATION OF CEREALS

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INTRODUCTION

In recent years, considerable progress has been made in genetic engineering of monocotyledonous crop plants (reviewed in references 1-7). It has now become possible to genetically engineer all important cereal species. Some of the approaches have been used with much routine, especially in rice, wheat and corn, and resulted in hundreds of independent transgenic lines.

An ideal method for plant transformation should be inexpensive, efficient, applicable to any desired genotype, and provide plants with predictable integration and expression patterns of the foreign genes. Such a method does not exist yet. There is a persistent need to improve cereal transformation technology. One of the remaining bottlenecks is the regeneration of fertile plants from a single transformed cell. Another bottleneck is the predictability and reliability of foreign gene expression.

In this review, we first provide an overview on the currently used methods to generate transgenic cereals. We then discuss in more detail the core problems of transformation experiments, i.e., the biology and manipulation of plant regeneration from single cells. Finally, expression signals used for cereal transformation are presented and discussed. Even though we tried to cover most of the published work, some omissions were unavoidable due to the broad spectrum of this review.

METHODS FOR CEREAL TRANSFORMATION

Key factors to successful generation of any transgenic plant are the i) identification and culture of regenerable (totipotent) cells, ii) efficient delivery of foreign DNA molecules to these cells, and iii) a selective agent/marker gene system that allows one to discriminate between the rare transgenic clones and the nontransformed background. In general, it is not the delivery of the foreign DNA that is considered as the true hurdle, but the recovery of the transformed cells. The choice of methods and techniques available for

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cereal transformation has constantly been extended during the past 10 years. The "one and only" method for cereal transformation does not yet exist. Any decision on which of the methods to use will depend on the experiences and technical possibilities in a laboratory (tissue culture and greenhouse facilities, DNA delivery techniques, availability of transformation vectors, etc.), and the experimental conditions have to be optimized for every genotype.

Here we attempt an evaluation of the cereal transformation technology on the basis of published reports. We focus on the work that was designed to improve the transformation technology and resulted in the regeneration of fertile, transgenic plants. Emphasis is put on the reproducibility of the methods, their efficiencies, and the structure of the newly-introduced DNA after integration into the cereal genome. An overview on the selective agents that were employed to recover the transformed tissues is also provided.

Routine Methods

Among the many approaches to generate transgenic cereals, delivery of the foreign DNA to tissues of primary zygotic embryos, as well as to callus and derived suspension cultures, has been employed most routinely. Ballistic DNA delivery methods have been and still are the methods of choice in many laboratories. However, *Agrobacterium*-mediated gene transfer now also shows a lot of promise in cereal transformation (Table 1).

Target Tissues for DNA Transfer

Primary Embryos. Histological evidence indicates that somatic embryogenesis and/or primary embryogenic callus formation arises from epidermal (8) or sub-epidermal cell clusters (9) in scutellar tissue of immature or mature embryos, rendering these tissues amenable to genetic engineering and transgenic plant regeneration. In *Triticum* species, high-frequency somatic embryogenesis from scutellar cells was observed after removal of the embryo axis (10, 11). Transgenic wheat, maize, barley, rice (references in Table 1) and Triticale (12) plants have been obtained with immature or mature zygotic embryos as the target tissue for DNA delivery. In most of these cases, DNA was delivered by ballistic methods. In recent years, an increasing number of *Agrobacterium*-mediated transformation protocols have been established (references in Table 1). In addition, the transforming DNA could also be delivered *via* electroporation into organized embryogenic tissue of maize (13, 14) and rice (15).

One major advantage in using primary embryo tissue as a starting material to generate transgenic cereals lies in the relatively short *in vitro* culture periods required for the regeneration of plants. Unless used for DNA transfer directly after isolation (e.g., 8, 16, 17), the explants are cultured on 2,4-D-containing media for just a few days prior to delivery of the transforming DNA (e.g., 18-21). Occasionally, the primary calli that formed during this period were also removed from the scutellar tissue and used as targets for gene transfer (22). Through the exploitation of these techniques, fertile transgenic wheat plants, for example, could be obtained within three (20) to five (19) months after DNA delivery, making it possible to obtain third-generation homozygous plants within one year. These relatively short culture periods also minimize somaclonal variability, resulting in a high rate of regenerated plants of normal-looking phenotype (e.g., 8, 17, 23). In addition, induction of somatic embryogenesis on primary embryos seems to be less genotype-dependent than other regeneration protocols. In rice (e.g., 16), barley (24, 25), and maize (21), the transformation protocols could already be adapted to numerous genotypes. It is expected that through systematic testing of a range of key variables including explant type,

Table 1. Routine Methods to Produce Transgenic Cereals

Target Tissue	Species	Method of transformation	
		Microprojectile-mediated gene transfer	*Agrobacterium*-mediated gene transfer
primary embryos	Barley	24, 25, 113, 114[a)]	71
	Wheat	6[b)],10, 17, 18, 19, 20, 22, 115, 116	69
	Corn	8, 21	67
	Rice	16, 23, 74, 117	62, 65, 80, 118
embryo-derived callus	Barley	114	-
	Wheat	18, 22, 38	69
	Corn	46[c)], 119, 120	-
	Rice	30, 45, 76, 75, 121	62, 66, 68, 72
suspension cells	Barley	-	-
	Wheat	-	-
	Corn	34, 35, 36, 73	-
	Rice	45, 59, 60, 122	62

[a)] including also microspore-derived somatic embryos
[b)] *Triticum durum*
[c)] anther culture-derived Type I-callus

medium composition and culture conditions, the rates of somatic embryo induction and plant regeneration in more species and elite breeding lines will be improved to become sufficient for transformation experiments (26-29).

One of the drawbacks of using zygotic embryos is the fact that the permanent supply of high quality embryos - particularly of immature embryos - is rather labour- and time-intensive. Greenhouse facilities are often required to control the quality and homogeneity of the zygotic embryos, because these traits are affected by the culture conditions during growth of the donor plants.

Calli and Suspension cells. Embryogenic callus cultures can be induced from mature or immature zygotic embryos, microspores or anthers and serve as sources of large amounts of totipotent cells. For instance, thousands of embryogenic indica-type rice calli were produced from 50 mature embryos in 10 weeks (30). In a further step, increased amounts of starting material can be obtained after the initiation of suspension cultures from such calli. Using these tissues as targets for gene delivery, simple, efficient and reproducible transformation protocols that are independent of the constant supply of immature embryos as a source of target explants have been developed. Transgenic barley, wheat, rice, maize (references in Table 1), rye (31) and oat (32, 33) plants have been obtained in this way.

However, the establishment of embryogenic cultures can be difficult. Identification of rare embryogenic callus types [e.g., the highly embryogenic type2 callus in corn (34-37), "type C" callus in wheat (38)] can be difficult and time-consuming. Somaclonal variations (39) induced by prolonged tissue culture can lead to a high level of phenotypic variation in regenerated plants (40-44). Regeneration of fertile transgenic plants after transformation of calli cultured for a prolonged time period was therefore rather poor in the pioneering approaches (38). Many of these problems may be avoidable if suspension and callus cultures are re-initiated frequently (45). In corn, type1 calli initiated from anther cultures were highly regeneratable (46), indicating that the more genotype-dependent initiation of type2 calli might be circumvented. In barley, further progress in plant regeneration from embryogenic callus cultures has been made (47), rendering this type of explant suitable for transformation of a broader range of commercial cultivars.

Calli and suspension cells are usually transformed by ballistic methods (Table 1), but *Agrobacterium* has also been employed reproducibly, particularly for DNA transfer to rice calli. Transgenic maize has also been recovered after electroporation- (37) and silicon-whiskers- (48) mediated transformation of suspension cells.

DNA Delivery Methods

Particle Bombardment. Ballistic gene delivery (microprojectile bombardment, biolistics, particle acceleration) is to date the most successfully-employed method to produce transgenic cereals in many laboratories around the world (Table 1). The strength of ballistic methods lies in their versatility. Ballistic methods allow the delivery of any desirable DNA fragment into a broad spectrum of organized or non-organized tissue and cell types of virtually any species (reviewed in references 49-51). Ballistic methods that are frequently used for cereal transformation are the original gun powder-driven particle delivery system (52, 53) or a helium-driven version of it (54), the particle inflow gun based on direct acceleration of the microprojectiles in a gas flow (55), and devices based on particle acceleration by electric discharge (56).

A range of physical and biological parameters affect the efficiency of the ballistic gene transfer process and might even be essential for transgenic plant recovery (e.g., 57). The particle size and the extent of accelerating forces both determine the penetration forces of the particles and are therefore important parameters to be modified if a particular, e.g., subepidermal cell type, needs to be reached by the particles (58). In order to reduce deleterious effects of the bombardment process on the target tissue, the turgor pressure in target cells is often reduced by incubation of the tissue under hyperosmotic culture conditions (36). Also the type of particles (59) and the physical configuration of the transforming DNA (60) were shown to have an impact on the transformation efficiencies.

Agrobacterium. For many years, cereals have not been considered to be within the host range of *Agrobacterium tumefaciens* (61). This view now has to be revised. Since the first unequivocal proof of *Agrobacterium*-mediated gene transfer to rice (62), different laboratories have reported transgenic rice (japonica and indica varieties), wheat, barley and corn (Table 1). The factors that have mainly contributed to this important addition to the cereal transformation methods have been reviewed by Smith and Hood (63).

As experienced with a wealth of transgenic dicot species obtained *via* *Agrobacterium*-mediated gene transfer, this method of genetic engineering usually leads to a more predictable pattern of foreign DNA integration (i.e., defined insert ends, little rearrangement and low copy number of the inserted DNA) when compared to ballistic methods. It also allows the delivery of large pieces of DNA, and the efficiencies of transgenic plant regeneration after *Agrobacterium*-mediated gene transfer are among the greatest obtained.

Agrobacterium-mediated gene transfer to cereals - especially to rice (reviewed in reference 64) - is now sufficiently routine to allow first evaluations of the above issues. Transgene copy numbers range in the great majority of the analyzed cases from 1 to 3 (62, 65-70), but at least 10 copies have also been observed (71). Transformation of rice with a 14 kb-long stretch of foreign DNA resulted in only a little rearrangement of this comparably long insert, as clarified in a very detailed analysis (66). Some rearrangements of the T-DNA after integration have also been described in other cases (68, 72). Ishida et al. (67) estimated that 5% of the transgene copies were rearranged in corn. In other instances (65), no rearrangements could be observed with the analytical strategy employed. These results have to be compared to ballistic transformations, where transgene copy numbers range from 1 to more than 10 (73-76), with about one-fourth of the plants having a single

copy or two copies, and occasionally with significant rearrangement of the introduced DNA (77-79).

Several factors were found to have an impact on the efficiency of *Agrobacterium*-mediated transformation, including Ti plasmid type (62, 70), bacterial strains with broad host spectra (62, 65, 66), culture conditions prior to and during inoculation (65), the induction of *Agrobacterium* by exogenously added acetosyringone (65, 69, 72) and the presence of a surfactant during inoculation (69). Pre-culture of the immature embryos on a hyper-osmotic medium improved transformation rates in a japonica-type rice (80). The type of target tissue was found to be crucial for efficient transformations in rice (62) and maize (67), while no significant differences were observed in wheat (70). Strongest influence on the transformation frequency is exerted by the plant genetic background in indica-type rice varieties (65, 73), which had not been reported for three different japonica-type cultivars (62).

Alternative Approaches

Besides the well-established approaches described above, there are a number of additional methods that are successfully used to generate transgenic cereals, based on DNA delivery to primary explants and protoplasts.

Primary Explants other than Embryos. Isolated microspores are an attractive target for ballistic gene delivery because they are unicellular and the time required for the production of homozygous transgenic plants is very short. The dihaploid stage of the regenerated plants allows direct exclusion of plants carrying lethal mutations resulting from the transformation process. Despite these advantages, efficient microspore regeneration and transformation protocols have so far only been reported for barley (81, 82).

In an attempt to shorten the culture periods and to render the transformation technology fully genotype-independent, several approaches have been taken towards transformation of germline cells. The early work, involving pipetting of *Agrobacterium* into the spikelets of wheat (83), could not be reproduced (84). Ballistic gene transfer to rice shoot apices resulted in the regeneration of chimeric plants (85).

Transgenic rice was regenerated following the inoculation of isolated shoot apices with *Agrobacterium tumefaciens* (86). Transgenic Tritordeum plants were regenerated after bombardment of inflorescence tissue (87). Transgenic plants of two maize cultivars were obtained after ballistic gene delivery to apical meristems in maize immature embryos, which increased the likelihood of germline transformation (88). In order to reduce the input of labor, maize shoot tips were also proliferated *in vitro* prior to transformation (89), a procedure that had been established for a variety of maize genotyes (90). It remains to be shown in how far such approaches of germline transformation can extend the range of transformable genotypes in other species.

Protoplasts. Direct gene transfer to cereal protoplasts (reviewed in reference 91) has been the only method available for cereal transformation before the development of ballistic DNA delivery systems. The foreign DNA is transferred by co-incubation of the protoplasts in a solution containing a high concentration of polyethylene glycol (e.g., 92-94) or by electroporation (95, 96), and the latter method proved to be very efficient in comparative experiments (97).Transgenic plants of barley (95, 98-100), wheat (96), corn (94, 101), japonica (92, 102-104) and indica rice (93, 105-108) have been obtained in this way. Protoplasts have been isolated from microspore-(95) and immature embryo-(100) derived primary calli or from embryogenic suspension cells that were established from zygotic embryos (105-107) and from cultured microspores (93, 95, 104).

The major drawback of these approaches is the strong genotype dependence of the protoplast-to-plant regeneration systems. In addition, evidence for genomic rearrangements resulting from the prolonged culture periods have been found in rice (108).

Efficiency of Transformation in Cereals

Transformation efficiencies are often expressed as the ratio of independently-derived transgenic plants per number of treated explants (51) or per number of bombarded plates containing suspension cells. They are usually in the per mil to percent range (Table 2). Direct comparisons of frequencies obtained with different gene transfer techniques are not available. Comparative experiments might be rather difficult to be carried out: the optimal conditions (e.g., target tissue) found for one gene delivery method do not necessarily apply for another method. The current state of the art in terms of transformation efficiencies can also be illustrated by the following hypothetical comparison: depending on the expertise in a particular laboratory, the minimal effort to generate, e.g., 10 fertile transgenic indica type rice plants, would be the treatment of 2.5×10^6 protoplasts isolated from embryogenic suspension cells (e.g., 106), or the bombardment of 1 ml packed cell volume of suspension cells (e.g., ref. 59), or the incubation of 50 immature embryos with a supervirulent *Agrobacterium tumefaciens* strain (e.g., ref. 72).

Selection of Transgenic Tissue and Plants

In view of the low transformation efficiencies, transformed cells or tissues need to be recovered under selective culture conditions. A handful of marker genes is available that upon expression confer resistance against antibiotic or herbicidal compounds (Table 3). Although there is a broad spectrum of selective culture conditions that has been employed, most of the approaches focus on the herbicide phosphinothricin and the related tripeptide compound bialaphos, as well as on the antibiotic hygromycin (the latter especially in rice). Under the aspects of planned large-scale release experiments and commercialization of the transgenics, this biased dependence on a herbicide tolerance strategy could become problematic for ecological reasons. Several attempts have therefore been undertaken to discard the resistance gene once the transgenic line has been established and stabilized. Also for technical reasons (multiple-step transformations) it is desirable to have available more than one or two selective agents for transgenic plant regeneration. A major advantage of herbicides over antibiotic compounds is the fact that selection can be applied not only in the medium *in vitro*, but also by spraying whole plants either *in vitro* or in the field or greenhouse.

Problems associated with the use of particular antibiotic compounds are the lack of selectivity (73), the lack of plant regeneration or the regeneration of albino plants (109), as often encountered with kanamycin sulfate or geneticin (G418) (99, 110). A way to circumvent such problems could be the regeneration of plants from antibiotic-resistant transgenic calli on antibiotic-free medium (98), or the omission of selection pressure throughout the entire cell proliferation and plant regeneration process (95), as shown for barley. So far there is only one report describing the use of paromomycin for the selection of transformed oat (33).

Hygromycin at low concentrations (1 -10 mg/l) kills susceptible cells within days (111). This antibiotic compound does not interfere with the regeneration process (65) and root formation (10). The use of a hygromycin-based selection scheme doubled the transformation efficiency in wheat when compared to phosphinothricin; however the gene

Table 2. Examples of Transformation Efficiencies

Species (Varieties)	Method[1] of transformation	Target Tissue[2]	Selective agent[3]	Transformation frequency[4]	References
Barley					
	B	IE	HYG	0.2% - 0.7%	24
	B	IE	PPT	0.3% - 1.5%	25
	A	IE	PPT	4 %	71
Wheat					
	B	IE	PPT	1.5%	20
	B	IE, IE-derived calli	HYG, PPT	5%	22
	B	IE	G418, BXN	2%	123
	A	IE, IE-derived calli	G418	0.1% - 4%	69
Corn					
	B	IE	CSN	2%	8
	B	IE	PPT	2% - 4%	21
	A	IE	PPT	5% - 30%	67
	E	IE	G418	1%	14
	B	Anther culture-derived type1-calli	PPT	11 / 1.5 g of callus tissue	46
	B	Type2 callus-derived suspension cells	PPT	3.5 / bombarded plate	36
Rice					
div. Indica varieties	B	IE	HYG	1% - 8%	16
IR72, TCS10	A	IE	HYG	1% - 4%	65
Radon[j]	A	IE	HYG	27%	65
Taichung Native 1	B	ME-derived callus	HYG	2% - 5%	30
Gulfmont, Jefferson	A	IE-derived callus	HYG	4% - 5%	66
Basmati 370	A	ME-derived callus	HYG	22%	72
Pusa Basmati	A	ME-derived callus	HYG	13.5%	68
Pusa Basmati	B	Suspension cultures	PPT	5 / bombarded plate	59
Taipei 309[j]	B	Suspension cultures	HYG	16 / bombarded plate	60
IR43	P	Suspension-derived PP	HYG	4 / 10^6 PP	106

[1] B, ballistic methods; A, *Agrobacterium tumefaciens*; E, electroporation; P, PEG-mediated

[2] IE, immature embryos; ME, mature embryos; PP protoplasts

[3] HYG, hygromycin; PPT, phosphinothricin; BXN, bromoxynil, CSN, chlorosulfuron, G418, geneticin

[4] if not indicated otherwise, number of independent transgenic plants per treated explant

[j] Japonica varieties

Table 3. Agents Used to Select Transgenic Cereals

Selective agent [a]	Resistance gene(s) [b]	Barley	Wheat	Corn	Rice
KAN	*npt*2 (124)			13, 88, 94	
G418	*npt*2 (124)	98, 100, 195	22, 69	14	15, 105, 118
HYG	*hph* (125)	24	123	119	16, 23, 30, 45, 62, 65, 66, 68, 72, 75, 76, 92, 93, 103, 106, 107, 121
PPT	*bar* (126) *pat* (127)	25, 44, 81, 114	10, 11, 18, 17, 19, 20, 22, 38, 96, 116	34, 35, 120, 94, 36, 48, 37, 46, 67, 89, 21	16, 23, 59, 76, 86, 122
MTX	*dhfr* (128)			101	
GLP	CP4, GOX (129)		115		
CSN	*als* (130)			8	102

[a] KAN, kanamycin; G418, geneticin; HYG, hygromycin; PPT, phosphinothricin, including Basta, glufosinate ammonium, and bialaphos; MTX, methotrexate; GLP, glyphosate; CSN, chlorosulfuron

[b] genes for: *npt*2, neomycin phosphotransferase; *hph*, hygromycin phosphotransferase, *bar*, *pat*, phosphinothricin acetyl transferase; *dhfr*, dihydrofolate reductase; CP4, enolpyruvylshikimate-phosphate synthase; GOX, glyphosate oxidoreductase; *als*, mutant acetolactate synthase

expression signals of the two employed vectors were not comparable (22). Delayed start of selective culture may lead to the formation of chimeric calli (111).

The effectiveness of four phosphinothricin-based selective agents were evaluated for use in maize transformation: glufosinate and its commercial formulation, Basta, were less effective in controlling growth of non-transgenic corn callus than the tripeptide of phosphinothricin, bialaphos, or its commercial formulation, Herbiace (112). Also in other instances, bialaphos yielded more vigorously-growing maize (73) and wheat (20) plantlets than phosphinothricin.

Regeneration

Gene transfer in biotechnology requires cells which combine three very different characters. These cells (i) must be accessible for the physical delivery of DNA, (ii) they must have the competence for stable integration of the foreign DNA fragment into their genome, and finally (iii) they must be competent to regenerate a complete plant. In addition for all seed-propagated species the regenerate needs to be fertile. The combination of these three prerequisites in one single cell was probably never an advantage in any natural and not even artificial selection. It can therefore be expected that such cells are *a priori* rare, either with regard to the species and/or the cell type. In order to develop technically more efficient transformation systems, one could increase the range of any of the three groups of cells. As an example, microprojectile bombardment increased the number of cells accessible to physical DNA delivery. Bombardment can reach cells *in situ*, still surrounded by their natural cell wall. Some of these cells are indeed regeneratable in most cereals, e.g., the cells of the immature scutellum, and have not been accessible for gene transfer before.

A natural regeneration is embryogenesis of zygotes. Most reports of 'artificial' regeneratability used for genetic transformation refer to embryogenesis. Other regeneration systems have been communicated, like direct meristem-organogenesis (88-90, 131) or regeneration from root cultures (132). Embryogenesis has the advantage of providing regeneration products which are largely derived from single cells or at least only small cell groups. Clonal transgenic regenerants make inheritance of the transgene more likely, since non-transgenic sectors in regenerants do not contribute to inheritance of the transgene.

This chapter will summarize the current knowledge in embryogenesis and also mention other regeneration techniques. We discuss the topic according to the different explant tissues. This review cannot be complete, due to the size of the field and the difference in viewpoints. For an exhaustive recent collection of references about monocot somatic embryogenesis see KrishnaRaj and Vasil (133).

Explants. Trivially, every plant species possesses regeneration potential from single cells or from small cell groups. In every seed-propagated species at least the zygote has the potential to regenerate a complete fertile plant *in situ*. The shoot apical meristem, which develops during early embryogenesis, represents a small cell group, which has the potential to regenerate a complete fertile plant at least *in situ*. In vegetatively propagated species the regeneration potential of the generative cell type zygote is less important. Instead, other cell types or groups of cells (or tissues) bear the main burden of regeneration. Again, the shoot apical meristem is among these cell groups, and in addition, often also other parts of the plant, like shoots (e.g., potato), roots (e.g., a number of weeds) or certain parts of leaves (e.g., Bryophyllum). These parts of the plant are "more somatic" than the zygote. Since in plants a germline *sensu stricto* does not exist, a clear-cut definition of 'somatic' is difficult. However, it makes sense to define as 'somatic' in plants all those

tissues or cells, which under normal, natural, non-pathogenic conditions will not contribute to gamete development.

Mesophyll Protoplasts. In dicotyledoneous plants regeneration from somatic tissue in case of wounding has been an advantage in natural selection (61). Consequently, most dicot species can be regenerated from mesophyll protoplasts or other somatic cells. In contrast, cereals do not readily regenerate from mesophyll protoplasts, due to their different wound response, which does not involve activation of cell cycle in somatic tissue. This is not a general inability of the monocots. Wound response of some members of the Liliacean and the Dioscoriacean family have been reported to be similar to dicots and, consequently, they are competent to regenerate from protoplasts of somatic cells (61). Remarkably, the phenomenon of secondary growth is restricted to these families in monocots. In cereals, including particularly wheat, all efforts have failed so far to regenerate reproducibly green, fertile plants from mesophyll protoplasts, although they can reenter the cell cycle (134, 135); however, promising results have been obtained recently with sorghum (136).

Zygote. Particularly the zygote or its early derivatives in the immature embryo, including shoot apical meristems at least *in situ,* must be able to regenerate a fertile plant. Regeneration competence of these cell types in cereals has been studied in detail (16, 24, 31, 32, 88-90, 113, 121, 131, 137-149). Regeneration from zygotes is usually clonal. Monocot zygotic embryo development *in situ* has been studied by clonal analysis (150-153). Genomic alterations during the very first cell divisions of the zygote might lead to chimeric plants (150).

A very early differentiation, e.g., in embryogenesis, is the suspensor. It does not contribute to the body of the embryoaxis, and therefore its genome is lost during further development. Mutation studies in dicots suggest that the competence of the suspensor to develop a second embryo is suppressed under natural conditions (154, 155). In somatic embryogenesis of monocots, the suspensor is mostly not prominent (146, 147) but present (142). The second cell line, which is somatic under natural conditions, is the differentiating scutellum. The shoot apical meristem is generated in parallel to the scutellum on one side of the originally globular proembryo. In cereals the whole plant body develops from this shoot apical meristem with the single exception of the transitory primary root. Morphogenesis seems auxin dependent in the globular proembryo during shoot apical meristem differentiation (156). However, the molecular causality during zygotic embryogenesis is largely unknown. The culture methods for zygotes (137-140) or for very small globular proembryos (157) and older embryos (158) might contribute to causal molecular studies of *in vitro* cereal embryogenesis. For comparison with a non-regeneratable tissue, endosperm culture (159) and genetic transformation of endosperm (160) might be useful.

Immature Scutellum. The somatic fate of the early scutellum is apparently not definite. The scutellum is competent for secondary embryogenesis. This was shown naturally *in situ* or artificially *in vitro* by a number of laboratories with immature (142-148, 161) and mature embryos (149). The immature scutellum was often used for regeneration of cereals after gene transfer with microprojectile bombardment in a variety of cereals (16, 24, 32, 33, 113, 120, 121, 141). Protoplasts have also been scutellum-derived in successful approaches with suspensions for rice and wheat regeneration experiments (162-166) and have been used for gene transfer in rice (35, 92, 93, 167-169). Immature embryos are best freshly isolated; however, sophisticated cryopreservation methods are available to store particularly useful cultures (170) in order to avoid loss of regeneratability during long-term culture (133).

Meristems. Mature embryos contain a well-developed shoot apical meristem. In some species like barley, in addition, a number of tiller meristems already develop during embryo maturation. Although these meristems are covered by the coleoptile, they are accessible for gene transfer by microprojectiles. Ritala and colleagues have used this source of tissue for bombardment and directly regenerated adult plants (109). In cereals this direct regeneration approach leads to chimeras with only transgenic sectors. Chimeras serendipitously inherit transgenes to progeny (171). In contrast, dicots can be subcultivated under the same approach as axenic shoot cultures until they are clonal (172, 173). In maize, a tissue-culture step was included between bombardment and regeneration, in which the meristems have been multiplied directly by organogenesis. This culture step allowed for selection of the transgenic cell lines (88-90, 131).

Although leaf bases in maize and other cereals are supposed to contain embryogenic cells in their first millimeter, this tissue was never used in a successful transformation system. Following the ontogenetic development of the plant further, the flower meristem is the next source tissue for regeneration. Flower meristems and immature spikes are competent for embryogenesis (174, 175) and have been used in cereals for genetic transformation (87).

Microspores. Microspores are ontogenetically very close to the highly regenerative zygotes in the development of the plant. Since the first report about androgenetic embryogenesis of haploid plants (176), a number of attempts have been made to regenerate cereals from microspores *via* anther culture (93, 104, 177-190; reviewed in reference 191). Microspores have been used for transient expression (192) and stable transformation (193). Regeneration from microspores leads to haploid plants and sometimes spontaneously, regularly after colchicine treatment, provides double haploid plants. These plants are homozygous with regard to every gene locus. In classical breeding programs the importance of double haploids is established. In modern biotechnology, gene transfer to microspores followed by regeneration of double haploids could provide homozygous transgenic plants in one single step (180, 191). Currently, limiting factors in the success of this promising approach for gene transfer are the low efficiency of embryogenesis from microspores and the low gene transfer efficiency to this cell type. In cereals successful direct transformation *via* microspore bombardment was reported only for barley (82, 191).

Physiology

Competence. No theory of regeneration was ever published. However, it can be speculated that regeneration requires cell division accompanied by histodifferentiation and organogenesis. Studies of these processes include physiological, biochemical, cell biological and molecular genetic aspects. Cell types which are closer to the generative regeneration process might have more potential and competence for mitosis, histodifferentiation and organogenesis. Each case of regeneration and early embryogenesis from zygotes or somatic cells needs at least two basic stimuli: that for cell division and that for histodifferentiation (194). Although mitosis, histodifferentiation and organogenesis are probably developmentally independent, as has been shown with *Arabidopsis* mutants (155), the stimulus for their activation might be similar or even the same. Auxin has been shown to play a role in stimulation of DNA synthesis and mitosis (195). Inhibition of auxin polar transport interferes with apical shoot meristem development, i.e., histodifferentiation and organogenesis (196). Obviously, histodifferentiation can be separated from development of a single embryo, e.g., in case of polyembryony, several embryos develop from one single

zygote (197, 198). This requires multiple similar histodifferentiation events after a number of postzygotic cell divisions.

Polyembryony in cereals occurs naturally, e.g., in rye (197). Wheat can be stimulated to polyembryony *in situ* by dipping ears in 2,4-D as an auxin treatment (198). These adventitious embryos develop from scutellum cells, i.e., from "somatic" derivatives of the zygote and not directly from the zygote itself. According to the definition, secondary embryogenesis during polyembryony is a kind of "somatic embryogenesis". However, this conclusion is questionable. A closer look at cells which are competent for embryogenesis reveals that they are in many respects still embryonic or meristematic. In electron micrographs these cells show small or undetectable vacuoles and thin, probably still completely primary, cell walls (147). Even later on, when in cereals the scutellum and the embryonic axis differentiate, the scutellum cells keep this embryogenic or meristematic appearance at light microscope resolution and contrast (142), until they start to store starch and become yellow and opaque (199). Somatic embryogenesis from scutellum cells is possible over the whole time span of embryo development; even embryos from completely mature seeds, ready to germinate, can be stimulated (200, 201). However, the efficiency is usually best in the early embryo stage, when the shoot apical meristem is established, but the coleoptile is not yet closed (148). Embryogenesis occurs in the scutellum as well as from the epiblast (148, 202). Most of the transgenic cereals have been produced with this embryogenic system (11, 16-19, 31, 32, 113, 120, 121, 141, 203). In addition, it is this developmental stage of the embryo which has been used successfully to start suspension cultures for regeneration from protoplasts and to generate embryogenic callus cultures for direct embryogenesis (34, 35, 38, 92, 114).

Later stages of development particularly provide the apical meristems at the shoot or the root with competence for cell division and organogenesis for regeneration. Alternative meristematic tissue throughout the somatic part of the shoot like the cambium, which could have the potential for regeneration from the shoot, is lacking in most monocots, including cereals. Early shoot apical meristems, still in the vegetative stage, can be used for direct organogenesis of shoot apical meristems (88-90, 131). Only late shoot apical meristems, which are already flower meristems, can undergo embryogenesis (87). In this developmental stage of the plant, the complex accumulation of meristems in the developing spike develops more and more towards gametes. The potential for regeneration and its competence for embryogenesis apparently increases in the tissue close to gamete development.

The competence for embryogenesis and regeneration seems to be limited already at the step of phytohormone perception. If auxin, and to some extent cytokinins, in the presence of auxin, are necessary to stimulate DNA duplication and mitosis, and to set organogenesis and histodifferentiation back to a zygotic level (194), then phytohormone perception is a prerequisite. This can only work if sufficient numbers of receptor molecules are ready to perceive the respective phytohormone and if the signal transduction pathway downstream is present or will be generated by the hormone stimulus all the way down to the effector molecules. *Agrobacterium* consequently often encode in their T-DNA not only genes, which increase phytohormone production in the host plant, but in addition genes which might increase phytohormone sensitivity (205). We are not aware of any report describing the correct function of these genes in a monocot. Reported *Agrobacterium*-mediated gene transfer in cereals was exclusively to cells or tissue, which are competent for embryogenesis upon exposure to external auxin (62, 67, 71, 80).

Biochemical Environment. Cells or tissues *in vitro*, outside their natural environment, require in tissue culture all compounds, which normally would be provided

by the plant. This includes not only carbohydrates, essential amino acids, vitamins, macro- and micro-elements, but also phytohormones. The stimulus for regeneration or embryogenesis is necessary in addition. Scientists who studied tissue culture and regeneration of cereals *via* embryogenesis did not know the correct medium needed by the explant. Therefore, media composition and hormone treatments have been studied extensively (206-217). This list is far from being complete, but these publications are relatively recent and contain more references to previous work. A complete collection of different media and culture conditions for embryogenesis of monocots, which includes also the cereals, can be found in KrishnaRaj and Vasil (133).

Since auxin influences DNA duplication, tissue and organ differentiation (175, 195-198), it is not surprising that particularly many cereal embryogenesis studies made use of auxin in the medium (16, 24, 31, 32, 113, 120, 121, 141, 142, 144-146, 148, 149, 161, 198, 214, 218; reviewed in reference 133). Direct regeneration of plants from young zygotic embryos or embryo rescue does not require external auxin (219). Many authors prefer lower auxin content or in addition cytokinin for regeneration of plants from zygotic (157) or preformed somatic embryos (32, 120, 121, 145, 149). Reports about effects of other phytohormones have been rare. Some gibberellic acid studies refer to direct regeneration of plants from zygotic embryos (196) or embryogenesis from microspores (185). Abscisic acid has been reported to inhibit (210) and to stimulate embryogenesis (217).

Sugar in the media is important as a carbohydrate source for the explant. The type of the sugar influences embryogenesis (213, 215), with maltose being the sugar of choice in wheat, barley and rice (106, 213, 220). Increased osmolarity is the other effect sugars can have in media. The osmotic effect should be independent of the compound which is used to adjust osmolarity. Increased embryogenesis due only to doubling the concentrations of ingredients in the media (221) might be caused by a nutritional effect in parallel to the osmolarity. Embryo development is supported by a similarly high osmolarity as the approximately 500m Osmol in the endosperm of the developing kernel during grain filling (158). Treatments with other chemicals like high molecular weight polyethylene glycol (208), acetone (212), or salt (222), might stimulate embryogenesis *via* lowering the water potential rather than by their chemical nature. Gelling agents tested for mechanical support during *in vitro* embryogenesis (211) could have in addition chemical or osmotic effects. Other chemical treatments influence hormone turnover and thus can stimulate embryogenesis and regeneration (216).

In summary, a number of culture conditions have been tested. Some basic components of protocols have been established. Though many reports have their value for the conditions described, their results are not very conclusive for other explant tissue, physical culture conditions and species or genotypes. In most cases the studies differ too much in their basic parameters and have been limited to few selected genotypes due to the available varieties and laboratory sources. The genotypes, however, probably differ very much in their response to regeneration stimuli.

Molecular Genetics

The approach of somatic embryogenesis from scutellum cells of immature embryos is widely applied (see above) and includes different species and varieties within the species (223). Although this approach works in most genotypes, its efficiency can vary considerably with the genotype (27, 185, 188, 200, 206, 224-226). Genetic parameters for regeneration have been studied in wheat (227). Genotype-dependent competence for embryogenesis can also be observed in microspore culture (184). Genotype effects might

be partially compensated for by the appropriate culture medium (228). Apparently it was possible to breed and select for embryogenesis. An inbred line of a natural polyembryonic Secale genotype showed better embryogenesis not only *in situ* but also *in vitro* (197). Inbreeding for a maize line with improved tissue culture response was reported (229). Also *in vitro* selection for improved embryogenesis led to lines which inherit improved embryogenesis (230, 231). Even different lines from interspecific crosses between wheat and barley showed in tissue culture different regeneration, which was inheritable (232).

It was suggested that several nuclear genes might be involved in regeneration (223, 230). The responsible genes might also be involved in morphogenesis of the adult plant (233). These authors reported stimulation of callus growth and regeneration by some day-length controlling genes in wheat but not in rye. This makes selection for embryogenesis in crop genotypes very difficult, since the genes could be unfavourably linked to genes interfering with important breeding traits, e.g., disease resistance (234). Moreover, embryogenesis and regeneration from preformed somatic or zygotic embryos might be controlled by different sets of gene (235). For example, it can be expected that genes involved in phytohormone perception and response would play a crucial role during embryogenesis and regeneration. Multiple, pleiotropic effects of substances such as auxins make it very likely that genetic alterations, which stimulate embryogenesis and/or regeneration, might interfere with important characters of the adult plant. Moreover, it cannot be excluded that some important genetic factors interfere with somaclonal variation (236-238), which is a limiting factor for tissue culture (239-244). Limiting factors for tissue culture are also indirectly limiting for regeneration. Somaclonal variation can be so severe that an additional mutagenic treatment shows no significant effect (245). Beneficial somaclonal variation might be selection for useful genotypes *in vitro* (246); however, most of these attempts have failed (223). Transposable elements have been reported to be activated in tissue culture and have been claimed to be responsible for somaclonal variation (247, 248); however not all of the unfavourable genetic alterations during tissue culture are due to nuclear genes but, instead, are due to mitochondria (249) or plastids (250).

In conclusion, unless genetic elements are found which directly stimulate embryogenesis and regeneration and do not interfere with characters of the adult plant, breeding for improvement of tissue culture stays difficult. Such elements might be found in legume nodule morphogenesis (251-253). It is very likely that mitogenic and morphogenic nodulins have homologues in all higher plant species including cereals (254).

Signaling Molecules

To study molecular components of embryogenesis and regeneration, at least two basic processes in development have to be distinguished: regulation of cell cycle for division (mitosis) and synchronized differentiation of cells to form tissues (histodifferentiation). Most likely organogenesis including polarity is a third independent developmental event. It has been proposed that phytohormone treatment might reset the development program, i.e., starting mitosis and histodifferentiation (194). However, how phytohormones trigger this process is an open question. To study only the approximately 20,000 genes with embryonic expression will probably not yield clues, since many of these genes are for housekeeping, without major regulatory function (255).

More concrete answers might currently be expected from homologous genes or signal molecules, which are known from other eukaryotic studies. Cells which are arrested

in G_1-phase of the cell cycle can enter S-phase and mitosis upon activation of genes like the *cdc2*, which encodes a protein kinase, mediating G_1-S-transition (256). This gene seems universal in eukaryotes and has been detected in cereals (257). Events which activate this gene stimulate mitosis. Genes which are involved in precocious germination, like the *viviparous* gene in maize (258, 259), look interesting, but they probably just inhibit germination in the seed, which could be completely independent from histodifferentiation and organogenesis. More promising are genes like *leafy cotyledon1* (260), which have morphogenic function in late embryogenesis and might be very conserved. Although the cotyledons in cereals became very distinct from dictots during evolution, the homologous gene in cereals might still be very similar and/or similarly activated. Other genes like the *knotted-1* from maize (261) are required for development and maintenance of shoot apical meristems. Homologues of this gene have been found to be expressed in soybean somatic embryos (262).

A series of genes has been described, expressed in tissue culture producing somatic embryos of carrot, among them two genes encoding for extracellular glycoproteins, *EP1* (263) and *EP3* (264). The gene product EP1 is not present in somatic embryos but in the surrounding non-embryogenic callus (263) and could have an inhibitory function to the non-embryogenic callus and a stimulating effect on the neighboring embryogenic cells. The secreted glycoprotein EP3 rescues a temperature-sensitive mutant of carrot, which is blocked in somatic embryo development. EP3 has an endochitinase activity and can be replaced by *Rhizobium* Nod factors to rescue somatic embryogenesis in the mutant carrot line (264). This indicates that low molecular weight molecules like oligoglucans, polysaccharides or other complex sugar molecules might play an important role in signaling of differentiation and organogenesis. Soluble antigens have been found in tissue culture, which bind to the surface of embryogenic cells, but not to the surface of non-embryogenic cells in the same culture; both cell types are generated from the same mother cell by unequal division in carrot suspension; the antigen is or contains a complex sugar (265, 266). These results can explain position-dependent differentiation in plant tissue also during somatic embryogenesis and regeneration. Moreover, soluble extracellular signals might help to explain the phenomenon of the embryogenesis-stimulating effect of feeder or nursing cultures (162), which was also observed in microspore culture (267). These soluble extracellular signals are not just tissue culture effects. Accordingly, the zygotic embryo *in situ* receives signals depending on the *MEDEA* gene from the maternal tissue (268) or the endosperm (269). Such signals must be soluble, since there are no plasmodesmata between the zygote and the maternal tissue.

A completely different molecular genetic aspect came into discussion recently (270). Telomere length might determine the (future) mitotic activity of eukaryotic cells. Interestingly, barley has the longest telomeres in cells of those tissues, which expose highest somatic embryogenesis (271). The telomere length seems to be genotype dependent (272). Since telomeres are synthesized by telomerase, the (transcriptional) activity of telomerase might be a limiting factor in cereal embryogenesis and regeneration (273).

Empirical studies have the merit that they developed tissue culture to a stage which allows for gene transfer to all important cereals. If we want to fulfill all the tasks ahead of us, we have to provide cereal transformation systems with comparable efficiencies to transformation of *Arabidopsis* or tobacco. Cereal transformation is far from that goal. Improvement of tissue culture is one prerequisite for increase of transformation frequency. This will probably also help to surmount genotype dependency. Empirical studies might profit from future support by causal molecular studies to improve *in vitro* regeneration.

Control of Gene Expression in Transgenic Cereals

For applied purposes proper expression of the introduced transgenes is of utmost importance. This requires that expression occurs in the right tissues or under the right induction conditions and in proper quantities, but also that this type of expression is stably inherited over many generations. Expression of endogenous genes can be regulated at many different levels including transcription initiation, elongation and termination, polyadenylation of mRNA, mRNA splicing, RNA transport from the nucleus to the cytoplasm and within the cytoplasm, RNA stability, translation initiation and elongation, and protein stability, targeting and modification. All of these processes have been more intensely studied with non-plant eukaryotic systems like yeast, *Xenopus* oocytes or mammalian cell cultures. For plants very little is known about most of these processes and signals influencing them are characterized only poorly. Recent reviews summarize the current knowledge on plant promoters (274), transcription factors (275, 276), RNA splicing (277, 278), RNA 3′-end formation (279), RNA stability (280), translation (281, 282), and protein targeting (283), folding (284) and degradation (285, 286). For gene expression of transgenes so far most emphasis has been on the choice and characterization of promoter fragments useful for constitutive or specific expression. These promoters are derived from plant genes or from genes of plant pathogens. To be useful for genetic engineering, they have to function as isolated fragments, i.e., the respective control sequences have to be present in a not too extended region upstream of the protein coding sequence. Promoter functionality is usually tested by fusion of the respective DNA fragments to a coding sequence for a reporter protein like β-glucuronidase (GUS) (287), firefly luciferase (288), green fluorescent protein (GFP) (289), or anthocyanin synthesis inducing transcription factors (290). Such reporter genes are introduced into single cells and expressed transiently from multiple extrachromosomal gene copies, or into transgenic plants, where expression of one or a few chromosomal gene copies can be monitored in specific tissues and over many generations. Due to the difficulties with transformation of cereals, until recently most promoter analyses with transgenic plants have been performed with dicots like tobacco, petunia or *Arabidopsis*. These studies have been valuable, but the data obtained - like those from studies using only transient expression systems - may not completely reveal the regulatory properties that a given control element may have in transgenic cereals. At present, rice is the most widely-used cereal model system for such analyses (291). Detection of promoter activity is most frequently done by staining for GUS activity only; since the staining procedure thus may determine the detection limit, absence of staining in certain cells or tissues may not necessarily mean complete absence of expression. This has to be considered in approaches where absolute specificity of transgene expression is required.

Constitutive Promoters

So far, most transgenes in cereals have been expressed under the control of only a few constitutive promoters with ubiquitous activity (for review see references 292, 293). In early work, mainly the 35S promoter of cauliflower mosaic virus (CaMV) has been used for expression of selectable markers and GUS reporter genes but also for genes conferring a novel property of agronomic interest to the plant. Later, promoters of the rice actin1 gene (294) and the maize ubiquitin1 gene (295) have been isolated and incorporated into gene expression vectors for transgenes (296-298). The relative strength of these promoters varies

greatly between different assay systems or different gene expression constructs as was shown for slightly different variants of the CaMV 35S promoter in transfected rice seedlings (299). In monocot-derived suspension cells or protoplasts from such cell cultures, the ubiquitin promoter tends to be the strongest of the three, followed by the actin promoter, which in turn is stronger than an unmodified CaMV 35S promoter (e.g., 76, 104, 294, 295, 300-304). Similar results have been obtained in cells transfected by particle bombardment of wheat (305) and of a number of other monocots including rice (306). The rice actin and the maize ubiquitin promoter both have only very low activities in transfected dicot cells (294, 295). However, a dicot (sunflower) ubiquitin promoter had a higher activity in bombarded wheat cells than the CaMV 35S promoter (305) and therefore might be useful for genetic engineering of cereals. The strong rice actin and maize ubiquitin promoters are usually about tenfold more active than the CaMV 35S promoter in these assay systems. Other supposedly constitutive promoters that have been tested in transient expression or in stable transformed monocot cell lines include the rice *GOS2* promoter and the mannopine synthase 1′2′ promoter of *Agrobacterium tumefaciens* (307), the former having about the same activity as the CaMV 35S promoter and the latter a lower one. The promoter of a chlorella virus adenine methyltransferase gene was found to be stronger than the CaMV 35S promoter in transfected maize and sorghum cells and in a number of transgenic dicot plants (308, 309).

Quantitative expression levels in transiently transfected cells may not really reflect promoter strength in an integrated transgene. For some promoters, association with histones seems to be very specific and can be required for proper activity (310, 311). Furthermore, transient expression can be severely influenced by the methylation status of the introduced DNA. The bacterium-typical dam methylation increased expression up to 50-fold for the promoters discussed here in wheat (312). Similar observations have been made for barley by Rogers and Rogers (313); we found that this effect is very variable in different test systems but can affect all promoters tested so far (unpublished data). Higo and Higo (314) observed that the activity of a rice catalase promoter in transfected rice protoplasts depended entirely on dam methylation of two sites downstream of the transcription initiation site. It is probably safe to assume that this type of "regulation" does not reflect natural regulatory properties of plant promoters and therefore will not contribute to expression from integrated gene copies, which have lost the bacterium-specific methylation pattern.

In transgenic plants, the actin, ubiquitin and CaMV 35S promoters direct a constitutive expression in all or most plant tissues in monocots with quantitative differences in different tissues. The rice *actin1* promoter was found to produce very strong expression in all tissues, including the pollen in transgenic rice (294, 315-317), maize, where about 3% of the total soluble protein was produced from an actin1-GUS fusion gene (318), and wheat (17, 319). The promoter was used to express high levels of selectable marker genes, particularly for wheat and barley transformation, and, e.g., insecticidal proteins (320-322) or a LEA protein from barley (323) in rice.

The CaMV 35S promoter was active in most rice tissues with a preference for the vascular tissue, lower expression in the epidermis and possibly no expression in pollen (45, 105, 316, 324, 325), but showed lower activity than the actin promoter (317). Nevertheless its activity is strong enough for expression of sufficient levels of antibiotic- or herbicide-inactivating proteins or proteins with insecticidal (69, 326, 327), antiviral (328, 329), antifungal (167), or nematocidal (330) activity in rice or maize (120). A 35S-GFP fusion gene produced sufficient GFP in transgenic rice to allow visual selection (331).

The maize *ubiquitin1* promoter was also active in most tissues with a preference for dividing cells in rice (332) and barley (82); it seems to be heat-inducible and cell cycle controlled to some extent (332-334). The promoter was useful for expression of selectable marker genes (e.g., 335), insecticidal proteins in rice (69, 336) and overexpression of the maize *kn1* gene in barley (337). In a comparatively large set of independent lines, ubiquitin 1 promoter controlled Bt toxin genes produced about tenfold more toxin than those under control of the CaMV 35S promoter in transgenic rice (69). In dry seeds of transgenic maize harboring an avidin gene under control of the ubiquitin promoter very high avidin protein levels could be obtained (338).

A constitutive promoter with ubiquitous activity, directing particularly high expression in cells of the vascular system and of leaf mesophyll and in the root tip in transgenic rice and tobacco, was isolated from the plant pararetrovirus Cassava vein mosaic virus (CVMV; 339). It may represent an alternative to the CaMV 35S promoter but has so far not been used for applied purposes.

The promoter of a rice α-amylase gene also conferred constitutive activity in all tissues of mature plant, however, not in very young leaves (118). The rice triose isomerase promoter could be a useful alternative for constitutively expressed transgenes (340).

Modified Constitutive Promoters

Improved derivatives of the CaMV 35S promoter or the maize *Adh1* promoter have been developed. The latter by itself has a similar activity as the CaMV 35S promoter in protoplasts from maize (341, 342) or wheat (304, 343, 344) but was much stronger in bombarded maize (345) or wheat (305) plant cells. The core promoter region of the *Adh1* promoter plus the gene's first intron were linked to multiple copies of the Anaerobic Response Element of this promoter and of the ocs element from the octopine synthase gene from *Agrobacterium tumefaciens* to create the pEmu promoter, which in a number of different monocot protoplast systems was 10- to 100-fold more active than the CaMV 35S promoter (76, 104, 300, 306, 346, 347). A *bar* gene under control of the pEmu promoter allowed selection of phosphinothricin-resistant rice (348) and wheat (116); however, reduced activity of the promoter with increasing plant age and in further generations was observed (116). Due to the many repeated sequences in this promoter, it showed instability in *E.coli* (346) and it is unclear whether reduction of promoter activity in transgenic plants is also related to such an instability.

The CaMV 35S promoter could be enhanced by insertion of an intron into the transcribed region (see below), and to some extent by duplication of its enhancer region (347). A promoter with doubled enhancer was useful to express oat arginine decarboxylase in transgenic rice (349). Intron-enhanced CaMV 35S promoters have been used successfully to drive expression of selectable marker genes or genes of agronomic interest like a coat protein of rice stripe virus in transgenic rice (350) or insecticidal Bt toxin (322, 351). The duplicated enhancer region of the CaMV promoter was also combined with a truncated wheat α-amylase promoter and found to direct strong, ubiquitous expression in maize protoplasts and transgenic plants (94).

Specific Promoters

While selectable marker genes have to be expressed in all plant tissues at least early in the regeneration process, most transgenes designed to confer new desired properties to

the plant need to be expressed only under special developmental or environmental circumstances. For this purpose specific expression signals are required. Theoretically it should be possible to construct such signals by combining suitable transcription factor binding sites; however, our present knowledge of such sites in plant promoters and the interaction of the respective binding factors is very far from allowing such an approach. Most work performed with specific promoters in transgenic cereals still aims at their characterization and only very few have been used for applied purposes.

Seed Specific Promoters. A number of monocot-derived, endosperm-specific promoters have been tested in transgenic plants or in transient expression assays in homologous or at least closely-related plants: the promoters of the maize zein gene *zmZ27* and of the rice small subunit ADP-glucose pyrophosphorylase both conferred endosperm specificity in transgenic maize (352); the promoter of the rice glutelin 1 gene was endosperm-specific in transgenic rice (353, 354) and maize (352). Other such promoters have only been tested in transgenic or transfected dicots, mainly tobacco; these include promoters of different rice glutelins (355-359), an oat globulin (360), maize zein (361, 362), wheat glutenin (363) and barley hordein (364). In these tests in heterologous systems, the promoter activity often appeared to be lower than expected (361) and was sometimes also detectable in vegetative organs (357, 362), suggesting that either the regulatory sequences and factors differ between monocots and dicots or that some sequence elements required for proper regulation are located outside of the promoter fragments used in these studies.

Complete genes for a high molecular weight (HMW) glutenin of wheat have been introduced into wheat varieties lacking that specific gene and were found to be correctly expressed in the endosperm (203, 204, 365). The promoter of the HMW glutenin and also that of wheat α-gliadin had only a low activity in transfected maize endosperm suspension cell protoplasts when compared to the maize *Adh1* promoter or the CaMV 35S promoter (366). The rice glutelin 1 promoter has been used to express a phytoene desaturase (367) and a pea legumin (368) in transgenic rice. The promoter of the maize waxy gene specified activity in the endosperm and in pollen in transgenic maize (352), and the corresponding rice promoter showed the same specificity in transgenic rice (369). The latter promoter was active only in pollen but not in the endosperm of transgenic *Petunia*, suggesting that the regulatory elements differ (369).

Genes under control of the promoter of the rice pI α-amylase or the wheat α-amylase could be transiently expressed in rice aleurone (370) or oat aleurone protoplasts (371), respectively. The promoter of the barley lipid transfer protein 2 directed aleurone-specific expression in transgenic rice (372). The promoter of a subunit of barley α-amylase inhibitor was inactive in transgenic tobacco but specific for the outer cell layer of the endosperm in bombarded immature maize kernels (373). The rice *rab16* gene promoter conferred constitutive expression in the embryo and anthers in transgenic rice (374), and the maize *rab17* gene promoter was shown to be active late during embryo development in transient expression experiments (375); both promoters are additionally induced by abscisic acid or osmotic stress in other tissues.

The wheat germin promoter showed the same tissue specificity in transgenic tobacco as was observed for the complete gene in wheat, which includes activity in the developing seed but also in young seedlings and after induction by a number of metal ions (376). Also the maize zein zE19 tandem promoter displayed the same activity in seeds and anthers in a transgenic *Petunia* as observed for the gene in maize (377), although in transient expression in tobacco and maize only one of the two tandem promoters was active

(378). In these cases, it is to be expected that the same promoter fragments will also be functional in cereals.

Pollen- and Tapetum-Specific Promoters. Several of the above-described promoters also have an activity in the pollen. Pollen specificity was also reported for the maize Zm13 promoter in transgenic tobacco and bombarded pollen from maize and *Tradescantia* (379, 380). Promoters with specificity for the tapetum have been isolated from maize and rice (381) but have not been well described. Also the promoter of the rice *osG6B* gene was found to be very specific for the tapetum in transgenic rice (382) and *osG6B* and *osC4* and *6* also were tapetum-specific in tobacco (383). Tapetum-specific promoters are being mainly used for the genetic engineering of male sterility by expressing Barnase, a cytotoxic RNase, (381) or endo β-1,3-glucanase (383). Pollen-specific promoters were used to engineer expression of *Bacillus thuringiensis* toxins in the pollen of transgenic maize (120, 384).

Expression in the Vascular Tissue. Predominantly phloem-specific activity has been described for the promoters of the maize sucrose synthase 1 gene in transgenic tobacco (385) and transgenic potato (386), of the rice sucrose synthase 1 in transgenic tobacco (387), and of the monocot plant pararetroviruses Commelina yellow mottle virus (CoYMV) in transgenic tobacco (388) and rice tungro bacilliform virus (RTBV) in transgenic rice (389, 390). At least the viral promoters are also active in other cell types including the axial parenchyma and anthers and tapetum for CoYMV (388) and cells of the vascular system. Depending on which sequences are included, the RTBV promoter can also be active in mesophyll cells and the epidermis of young leaves (391). In transgenic rice specificity for vascular tissue was observed for the *rolC* promoter of *Agrobacterium rhizogenes* (392) and the *mas1'2'* promoters of *Agrobacterium tumefaciens* (393). The promoter of the maize caffeic acid O-methyltransferase conferred activity in the xylem and other lignifying cells in transgenic maize and tobacco (394).

Expression in Green Tissue. Monocot plant-derived promoters with predominant activity in green plant tissue, i.e., regulated by light and/or development, have been identified in analyses with transgenic rice, maize or tobacco or transient assays with protoplasts or bombarded tissue. As for endosperm-specific promoters, subtle differences in regulatory properties between dicots and monocots became apparent (395, 396) and for some promoters possibly also between C3 and C4 plants (397). Promoter fragments have been derived from *cab* genes of maize (398, 399), rice (396, 400, 401) and wheat (402); *rbcS* genes of maize (395, 397), wheat (395) and rice (403); wheat chloroplast fructose-1,6-bisphosphatase (404); PEP carboxylases of maize (397) and sorghum (405); maize or rice pyruvate, orthophosphate dikinase (PPDK, 406-408); oat phytochrome (409); rice GOS5 (307); and rice aldolase P (410) and glutamine synthase (411). The maize *rbcS* promoter was mainly active in bundle-sheath cells (412), while the maize *cab-m1* promoter was mainly active in mesophyll cells and only weakly in bundle sheaths in transient assays with maize (398, 399). The rice *cab* promoter in transgenic tobacco was additionally active in the vascular system and to some extent even in roots (400). In transgenic rice, this root activity was not observed and strong activity was restricted to leaves, stems and floral organs (401).

It is noteworthy that dicot-derived *rbcS* promoters were inactive or less active in monocot test systems than their monocot-derived counterparts (395, 403) and that the oat phytochrome promoter was inactive in dicot protoplasts (409). For this type of promoter the choice of the DNA sequence used may be particularly important. For a number of dicot light-regulated genes, important regulatory sequences have been located within the transcribed region or even within the respective protein coding region (see below).

Omission of such sequences may cause altered or suboptimal expression patterns.

Most of the work discussed here has been performed to dissect the complex, light- and development-dependent, plant specific control mechanisms. However, promoters with activity mainly in green tissue have also been used in applied work to express insecticidal proteins in maize (120) and rice (322), or antisense RNA to downregulate rice rbcS RNA (413).

Meristem Specific Expression. Promoters from histone genes of maize (414) and wheat (415-420) have been characterized in transient expression systems (418-420), transgenic tobacco (414, 416, 417, 420) and rice (417). In single cells, they display S phase specificity and in transgenic plants they are predominantly active in meristems. [Histone gene promoters can, however, also be expressed replication independent as exemplified by the maize histone H4C7 promoter in tobacco mesophyll protoplasts (421)].

Another promoter with predominant activity in meristems in transgenic tobacco was derived from the rice gene for the proliferating cell nuclear antigen (PCNA, 422). Also the promoter for a maize hydroxyproline-rich glycoprotein gene was mainly active in cells with enhanced cell-wall production, which includes the meristems (423); a promoter of a related rice gene (*Osgrp1*), however, was inactive in meristems but restricted to expanding cells (424).

So far, analyses have mainly aimed at characterization of promoter function. We are not aware of use of these promoters for applied purposes.

Inducible promoters. Many of the previously-mentioned promoters contain elements that make them susceptible to induction by environmental or developmental cues (e.g., the maize ubiquitin promoter or the promoters active in green tissue). Some monocot-derived promoters have been studied or applied specifically because of their induction characteristics. Most of this work is relevant for the basic understanding of regulation of gene expression and development, but most of the results have so far not been exploited in applied genetic engineering projects.

The maize *Adh1* promoter has a constitutive activity in root caps, anthers, root and shoot meristems and some parts of the embryo, but it can be strongly enhanced by anaerobic conditions or 2,4-D in roots and shoots of transgenic rice (425, 426) or in maize protoplasts (341). At locations where it is active, it reaches a similar or even higher activity as the CaMV 35S promoter (305, 342-344). Also the maize promoter for cytosolic glyceraldehyde-3-phosphate dehydrogenase was inducible by anaerobiosis in maize suspension cells (427) and the promoter of the maize *GapC4* gene in transgenic tobacco (428).

In transgenic tobacco, abscisic acid was found to induce a barley heat-shock protein gene (*Hvhsp17*) (429), the maize *Glb1* promoter (430) and the maize *rab17* promoter (431). The latter was also activated by osmotic stress (375) similar to the ABA-induced rice *rab16* promoter in transgenic rice (374).

Negative regulation by sucrose has been observed for the maize shrunken 1 promoter in maize protoplasts (432) and the rice α-amylase promoter in cells from rice, tobacco and potato (433). Interestingly, the respective regulatory elements in the maize *sh1* promoter seem to reside exclusively at or downstream of the transcription start site (432). This may be of relevance in gene expression constructs, in which the downstream region of the shrunken 1 gene is used to enhance the CaMV 35S promoter (see below; 434).

Promoter induction by metal ions has been described for the barley *Hvhsp17* promoter (429) and the wheat germin promoter (376). This induction can be very specific for specific ions.

Osmotic stress induced the maize *rab17* promoter (431), the rice *pwsi18* promoter (435) and the wheat *Em* promoter (436). The response to heat-shock has been verified for the barley *Hvhsp17* promoter in transgenic tobacco (429) and in transient expression in maize and rice for two soybean heat-shock promoters (437, 438). The induced *Gmhsp17.5-E* promoter was tenfold more active than a CaMV 35S promoter (437). An analysis of heat induction of the endogenous rice heat-shock genes *hsp70* and *hsp82* revealed very different induction characteristics of these two genes (439).

The maize promoters A1 and bronze1 and 2 depend on the maize C1 and R transcription factors (440-442).

For purposes of genetically-engineered resistance to insects, nematodes or microbial pathogens, promoters that are induced after wounding or pathogen infection could be useful. The promoter of a maize hydroxyproline-rich glycoprotein was found to be wound-inducible in bombarded maize cells (443) and that of the basic rice chitinase RC24 in transgenic rice (444); the potato *pin2* promoter directed strong, wound-inducible activity in transgenic rice, when the first intron of the rice *actin1* gene was present downstream of it. With such a construct sufficient PIN2 protein could be expressed to confer insect resistance (445). The promoter of the grapevine stilbene synthase was found to be induced by wounding or fungal infection in transgenic rice (446) and barley (447), and a maize α-tubulin promoter was induced by *Gigaspora margerita* in the root cortex of transgenic tobacco (448). It still remains to be seen whether the induction characteristics of any of these promoters can be exploited for applied purposes.

A number of artificial promoter induction systems have been developed and tested in dicot plants (reviewed in reference 449); there is no reason why these systems should not work also in monocot plants or cells, but for practical purposes, their induction characteristics still require improvements.

Enhancement of Expression by Sequences Downstream of the Transcription Start Site

It has been noted that inclusion of an intron into an expression cassette usually enhances expression. In most experiments, introns are transferred together with surrounding exon sequences and it is often not clear which feature of the transferred sequences is actually responsible for the expression enhancement. The rice *actin1* and the maize *ubiquitin1* promoter are always used together with the first exon and intron of the gene and are active only in this configuration (296). Other promoters, which require the presence of the first exon/intron region for activity in monocots, are the rice *tpi* promoter (340), the maize *GapA1* promoter (450, 451) and possibly the oat *phyA3* promoter (452). It is likely that so stringently-required sequence regions contain transcriptional control elements, even though the effect of these regions is usually position dependent. A transcriptional component has been implicated in the enhancing effect of the maize *shrunken1* first exon/intron region (432, 453) and may be indicated by the position dependence of the effect of the rice *tpi* intron (454). Transcription control regions downstream of the start site have also been reported for the promoters of RTBV (391, 455) and a number of dicot genes (e.g., 456-460). Downstream sequences of the barley lipoxygenase 1 (*Lox1*) gene contain qualitative and quantitative control sequences for embryo-specific expression (461). It is, however, likely that non-transcriptional effects at least contribute to the enhancement effect of the presence of an intron (462). Intron-containing downstream regions from a number of genes have been used to enhance

expression of heterologous promoters, mainly the CaMV 35S promoter, and positive effects have been mainly observed in monocot cells. Stimulation of the CaMV 35S promoter in monocots has been found with several introns from the maize *Adh1* (34, 301, 463-465), *ubi1* (466) and shrunken 1 (434, 453) genes, the rice *actin1* (301) and *waxy* (467) genes, the castor bean catalase gene (468), chalcone synthase (466) and from RTBV (391). Often, enhancement is in the range of 2- to six-fold, but dependent on the construct and the assay system, up to 1000-fold enhancement of expression could be obtained. All these experiments have been performed in transient expression systems and the compared constructs often varied by several features. In extreme cases, introns acting positively in one construct or one expression system can become inhibitory in another. As an example, the *Adh1* intron was inhibitory in sugarcane (347) or its stimulatory or inhibitory activity depended on the promoter context and varied between different monocot cells (346). Recently it has also been shown that the enhancing effect can be cell-type specific (342) and reporter-gene specific (469, 470). It seems that the presence of intronic sequences affects one or more steps of gene expression that is not well understood, but is certainly worth much further investigation. Predictive generalizations about intron effects, particularly in transgenic plants, are quite impossible at present, but it appears that the presence of an intron in a transgene expression cassette is rather advantageous, provided, of course, that the intron is spliced properly in the intended expression system; consequently, introns have been incorporated in many gene expression cassettes for monocot transformation (see above and reference 471).

Polyadenylation Signals

Transcription termination and polyadenylation signals (terminators) are used to create well-defined, polyadenylated RNA 3′ ends. In plants, the process has been analyzed only in a few cases (reviewed in 279) and only very rarely in monocot systems (472-474). It appears that dicot signals work in monocots and *vice versa;* however, also differences in functionality have been reported (475). Besides being required for mRNA 3′-end formation, terminators contribute RNA sequences to the 3′ untranslated region and thus might affect RNA stability and also translation (476). In dicots, different terminators led to up to 100-fold differences in expression levels, particularly with stable integrated transgenes (477, 478). To our knowledge, no comparative analyses of terminators have so far been performed with transgenic cereals. It remains unknown whether terminators other than the most commonly used ones (derived from CaMV or from the agrobacterial *nos* gene) could lead to higher expression levels. Some such terminators have been incorporated into gene expression cassettes (297, 303).

Transgene Sequence

In the course of Bt gene expression in transgenic plants it has been observed that alteration of the coding sequence can lead to drastically-improved expression levels (reviewed in 479). Alterations are being made particularly for genes derived from bacteria, but might also be useful for some viral genes, which are, like the bacterial ones, not selected for proper expression in the nucleus of a eukaryotic cell. In the course of such alterations, usually the protein coding sequence is made more GC-rich. This leads to (I) reduction of the numbers of rarely used codons, (II) removal of potential polyadenylation signals, (III) reduction of an eventually intronic (i.e., AT-rich) character, and (IV) removal

of possible RNA instability determinants. There may also be other, less obvious, effects. It is unclear which of these effects is the most important. The modifications are sometimes termed "adaptation to the codon usage of plants". This reflects the fact that the codon usage in plants shows a certain bias for GC-rich codons over AT-rich codons and it is assumed that reading frames with rare codons are translated less efficiently. However, in plants this bias is not very pronounced and in monocots, highly-expressed genes are not distinguished by lack of rare codons (480). Improvement of translation by reducing the numbers of rare codons is probably not very significant, since even in organisms with much more pronounced codon biases than in plants, translation is only slightly affected by the presence of rare codons as long as they do not appear in larger clusters (for review see 282). In contrast, removal of cryptic splice sites has been shown to increase expression drastically in plant cells (e.g., of GFP, 481). It has also been shown that AU-rich sequence motifs can lead to unexpected polyadenylation events in maize cells (482). Such cryptic splice or polyadenylation sites are probably present in many RNAs that normally do not encounter the nuclear RNA maturation machinery.

General RNA instability determinants so far have not been well studied for plants. In the absence of this knowledge, the general increase of the GC content remains probably the best strategy to improve expression of a non-eukaryotic coding region in transgenic plants.

Translation

Translation of a transgene-derived mRNA can be influenced at various levels. The competitivity of mRNA for ribosomes seems to be determined mainly by the RNA 5′-end. Some plant viral RNA 5′ untranslated sequences (5′UTR) have been shown to enhance expression; in dicot systems up to 80-fold enhancement has been reported by the tobacco mosaic virus 5′ UTR (Ω) (reviewed in 281, 282, 479). Even though the effects observed in monocot cells have been much smaller (342), Ω or the leader sequences of alfalfa mosaic virus RNA 4 are sometimes used in gene expression constructs. Even if these sequences do not contain features actively recruiting ribosomes to the specific RNA, they will provide a relatively open structured mRNA 5′ end and can solely for that reason be more effective than sequences derived from polylinkers often introduced into expression constructs during the cloning procedures. For the CaMV 35S promoter it was shown that inclusion of more of the authentic viral downstream sequences drastically improved expression in maize (483, 484). Some positive effects could also be obtained with the UTRs of maize glutelin, PEP carboxylase and *rbcS* genes, while the TMV or AMV sequences were rather inhibitory (484). In case of the most effective CaMV sequences, direct effects on promoter activity are possible in addition to translational effects.

Besides being attractive for ribosomes and translation factors, 5′ UTRs of well-expressed transgenes have to be devoid of features inhibiting the scanning process of the ribosomal subunit from the capped 5′ end to the ATG start codon. Such features are strong secondary structure and possible translational start sites (282).

The frequency with which the start codon of an open reading frame is recognized by scanning ribosomes depends to some extent on its sequence context. An optimal context (G /ANNATGG) has been deduced from statistical analysis of plant gene sequences and verified in some cases by mutation analysis (485-489). All the work was performed with transient expression systems. In transgenic tobacco two in-frame start codons were found to be differentially used in leaves and roots (490), an effect completely unpredicted by the

current knowledge of initiation of translation and illustrating that this knowledge is still incomplete.

Modulation of translation at the level of elongation has been suggested for some genes, including two oat seed protein genes (491). The elongation rate can be of importance for proper folding or targeting of a protein (for review see 282); however, features regulating translation elongation or termination have not been characterized.

Scaffold Attachment Regions (SAR) or Matrix Associated Regions (MAR)

Gene expression from a given construct can vary drastically between independently transformed plants (e.g., 492, 493). SARs or MARs are AT-rich sequences that bind to the nuclear scaffold. Fragments of at least 300 base pairs in length (but usually much longer fragments are used), flanking a transgene, have been shown to homogenize gene expression levels in transgenic dicot plants, apparently by reducing the effects on expression of the chromosomal regions surrounding an integrated transgene (494-496). Mechanistically, the function of MARs is still unclear and several different properties of MAR fragments may be involved (reviewed by Holmes-Davis and Comai, 497). Discussed functions of MARs are the induction or maintenance of an open chromatin structure or the direct inhibition of transcription from the outside chromosome by termination within the AT-rich fragment (497); this is reminiscent of the finding that transgenes flanked by real terminator fragments are also to some extent shielded against effects of the integration site (498). MAR effects have been observed with several promoters but always with GUS genes; somewhat unexpectedly, in constructs with tandem *nptII* and GUS genes flanked by MARs, only expression of GUS was normalized by the MAR (499), and insensitivity of an *nptII* gene to MAR effects was also inferred by Allen et al. (495).

In several experiments, MARs resulted in a better correlation of expression to the copy number, at least for lower copy numbers, and it was suggested that MARs could also inhibit gene silencing, thought to be the cause for the often-observed low transgene expression per gene copy in lines with high copy numbers (e.g., 495; reviewed in reference 497). No comparative studies with MARs have been reported for transgenic cereals, but it is likely that the effects would be similar since the functions of MARs are very well conserved among eukaryotes (497). MAR fragments are often detected near genes. Some of the longer promoter fragments used for gene expression control may contain MARs; e.g., a MAR was detected in the upstream region of the maize *Adh1* promoter (500, 501).

In most experiments, when MARs were used as transgene flanking sequences, identical fragments were used on both sides. It might have been expected that these very long exact sequence duplications may enhance the probability of transgene loss through recombination; however, MAR-flanked transgenes were found to be meiotically as stable as others (502).

Stability of Transgene Expression

In many publications and contributions to scientific conferences, transgenic rice and maize lines with unstable gene expression, unusual inheritance patterns, or even with physical loss of transgene sequences have been reported (e.g., 64, 73, 77, 78, 119, 503). These phenomena, however, have in most cases not been studied systematically. Only recently have two such cases been investigated closely and were found to represent cases of gene silencing. In one case, a homologous endogenous gene was turned off, probably by post-transcriptional gene silencing (504). In the other case, loss of expression in a rice line

with multiple copies of an integrated transgene was correlated with DNA methylation and was effective at the transcriptional level (505); triggering of gene silencing occurred stochastically in individuals of subsequent generations (506). These examples make clear that the different types of gene silencing described so well for dicot plants (reviewed in 507-513) also exist in monocot plants. It is unclear how often silencing is observed in transgenic cereals. With a large set of transgenic rice plants generated for various purposes, we observe expression abnormalities in about 30% of the lines in which expression was followed for a longer period. The stochastic nature of the process poses a great problem for any application of engineered plants and requires a thorough observation of plant lines over many generations before a release for application. These long-term tests also have to involve plants grown under field conditions, since in some cases inactivation of transgenes occurred under environmental stress (514, 515).

Gene inactivation can occur early during transformation and lead to reduced efficiency. Inhibition of methylation-dependent silencing by treatment with the methylation inhibitor 5-azacytidine (5-AC) during the transformation process resulted in higher transformation frequencies in tobacco (516); however, this may not be a usable method to increase transformation efficiencies with monocots since a single 5-AC treatment of rice seedlings induced heritable dwarfism (517).

It is at present argued that the transformation by particle bombardment may lead to particularly unstable transgenic lines because several or many copies of the transgene integrate and sequence repetitions may be the trigger for gene silencing. Therefore new emphasis is given to *Agrobacterium*-mediated transformation, which results in more precise integration of fewer copies. However, abnormalities have also been observed in rice transformed by *Agrobacterium* (64), and it should be remembered that most of the many dicot plants, in which the different forms of gene silencing are studied, have been obtained by *Agrobacterium*-mediated transformation. Before clearer data are available, it may be premature to abandon a well-established transformation protocol based on particle bombardment for *Agrobacterium*-mediated transformation.

Engineering of Integrated Expression Pathways

So far, the challenge in cereal transformation has been to obtain transgenic plants that express foreign genes at all. All efforts were made to reach this goal. Transgenes were constructed from different components and it appeared sufficient that these genes were expressed in some plants at reasonable levels. In this type of research, plants containing genes but not expressing them, large variations of expression levels among independent lines, observation of unusual inheritance, expression of unexpected RNAs or proteins, unexpected control patterns, etc., were only inconvenient. For applied purposes and for development of more efficient transformation protocols, an understanding of the molecular basis of these "exceptions" might be crucial. From studies in non-plant eukaryotic systems it becomes more and more evident that gene expression involves a well-organized pathway, in which the transcription process and RNA maturation and transport are linked (518, 519), and in which the nuclear history of the RNA can influence its translation (520). Translation itself can affect protein folding (521) and targeting (522) and RNA stability (523, 524). Alterations of protein sequences can lead to drastically-reduced stability or unintended mistargeting (for review see reference 283). If imperfect expression only resulted in some loss of product expression, it would be tolerable in genetically-engineered organisms. However, surveillance mechanisms may exist in cells to identify aberrant gene products and as a consequence to shut off the genes or induce stress reactions, as was found for

genes producing improperly folded proteins (e.g., 525, 526). Such a surveillance is also suggested in some models as the basis of gene silencing (510, 512). Aberrant RNAs could be produced from rearranged genes, but also from premature transcription termination due to interference of gene sequences with transcription elongation or to assembly of incomplete transcription complexes on some promoter fragments (391). Alternatively RNAs may be improperly processed due to the presence of cryptic processing sites (see above). The often dense organization of transgenes in transferred gene cassettes or after integration of multiple copies of such cassettes in one chromosome locus (527, 528) also may lead to problems of transcriptional competition or interference. It is not known, whether transcription in plants really stops at the terminator or proceeds much further, which, in a tight gene organization, would lead to transcription of adjacent gene fragments in a sense or antisense direction. Aberrant RNAs could also result from transcription starting in the bacterial plasmid region surrounding a transgene cassette. Such plasmid regions are usually also transferred to the plant. Effects of plasmid sequences on transgene expression have been observed in animal cells (529), and indirect evidence has been reported for transcription in plant cells extending from the commonly-used pUC plasmid backbone into the commonly-used cloning region of this plasmid (530).

Aberrant RNAs could also be generated as a consequence of aberrant translation, e.g., if translation initiates at a wrong start codon. This could be due to leakiness of the right one, resulting in premature translation termination and subsequent RNA destabilization (524).

In a number of analyses, it has been noted that the correlation between RNA levels and ensuing protein products can be variable even for identical RNAs in the same plant species (391, 490); some of these studies have been performed in connection with gene silencing (reviewed in 512). Such findings suggest that mRNA produced is not necessarily translated efficiently, but no knowledge exists as to the possible reasons. A more detailed analysis of expression patterns, which also considers byproducts of expression and precise localization of expression products, would be required to optimize transgene design.

CONCLUSIONS

All major cereals have been transformed at least with marker genes. Many cereals like rice, wheat or maize, are transformed in many labs routinely with agronomically useful genes. However, many questions are still open with regard to frequency and quality of transformation. It is not always easy to transform the desired elite lines due to genotype-dependent regeneration. It is often difficult to obtain lines with stable expression of transgenes inherited over many generations. It is still impossible to direct transgene integration to specific sites by homologous recombination in nuclei. Plastids have not been transformed. Consequently, some of the remaining challenges in gene transfer in cereals are the control of integrated copy number, gene targeting, plastid transformation, inheritance of stable expression over many generations, inducible and tissue-specific expression, quality and persistence of selective marker genes, and still in most cereal species the low transformation frequency. This low frequency is largely due to the limiting regeneration potential. In order to improve the situation, we have to interfere with the system in a directed manner. This requires that we understand the causalities on a molecular level. To acquire the respective knowledge will be the main challenge for the future.

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