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Plant regeneration and somaclonal variation from cultured immature embryos of sister lines of rye and triticale differing in their content of heterochromatin

1. Morphogenetic response

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Summary. Plants were regenerated from cultured immature embryos of two pairs of sister lines of triticale (× *Triticosecale*) cvs Rosner and Drira and five sister lines of rye (*Secale cereale*). The triticale lines differ in heterochromatic content of a particular rye chromosome (6R or 7R), while the rye lines differ in only one heterochromatic band. Variation in morphogenetic response was present between the triticale cultivars and between the rye lines. One of the rye lines (7RL + +) showed a distinctive superior response in terms of somatic embryogenesis. These findings are discussed in relation to factors affecting morphogenetic response and genetic stability in culture.

Key words: Cultured immature embryos – Triticale – Morphogenetic response – Somatic embryogenesis – Heterochromatin

Introduction

The term 'somaclonal variation' was adopted to describe the phenomenon of variability arising through the culture of plant cells, tissues, explants and organs, and their subsequent regeneration, via callus, into whole plants (Larkin and Scowcroft 1981). In the Gramineae, rice is among the few cereals in which routine regeneration has been achieved from cultured protoplasts (Fujimara et al. 1985). For many other cereals, regeneration from cultured immature embryos is the favoured explant system. Examples where successful regeneration has been achieved in this way include oats (McCoy et al. 1982), wheat (Ozias-Akins and Vasil 1982; Maddock et al. 1983), barley (Goldstein and Kronstad 1986), pearl millet (Vasil and Vasil 1981), rye (Linacero and Vazquez 1986) and triticale (Nakamura and Keller 1982).

Somaclonal variation has been described in these systems to varying degrees. Examples where variation has been observed in both quantitative and qualitative genetic traits include reports in wheat (Larkin et al. 1984; Maddock et al. 1985), maize (Edallo et al. 1981), rice (Sun et al. 1983), barley (Breiman et al. 1987; Karp et al. 1987) and triticale (Jordan and Larter 1985). In the present study, attention is focused on only one aspect of somaclonal variation, namely chromosome stability and its relation to morphogenetic response.

Chromosome variation has been observed among regenerants from cultured immature embryos of wheat (Karp and Maddock 1984), oats (McCoy et al. 1982), maize (McCoy and Phillips 1982) and triticale (Armstrong et al. 1984). Conversely, little or no chromosome instability was reported in regenerants of barley (Breiman et al. 1987; Karp et al. 1987), pearl millet (Swedlund and Vasil 1985) and *Panicum maximum* (Hanna et al. 1984). The origins and causes of somaclonal variation are the subject of much debate (Karp and Bright 1985), but a number of factors can be identified that affect the degree of chromosome instability. These include the ploidy, genotype and source of the plant cells, aspects of the procedure, such as time in culture and components of the culture medium, such as hormones. A study of how these factors operate is important not only from the viewpoint of understanding how the variation arises, or how it may be controlled, but also because it has been recognised for a long time that there is a relationship between chromosome instability and morphogenetic response (Murashige and Nakano 1967; Zagorska et al. 1974; Singh 1986).

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Recent evidence suggests one aspect of the genotype that may play a role in determining instability is the presence of heterochromatin. Changes in heterochromatic content have been observed in anther-culture derived doubled-haploids of tobacco and *N. sylvestris* (De Paepe et al. 1981, 1982) and such changes have been correlated with reduction in vigour (Dhillon et al. 1983, 1984). Furthermore, in wheat-rye hybrids, the position of break-points in structural rearrangements were found to be mostly located in or near heterochromatin (Lapitan et al. 1984), while in maize, Lee and Phillips (1987) described evidence relating chromosome breakage with the presence of heterochromatic regions.

In order to investigate the possible role of heterochromatin in both morphogenetic response and chromosome stability, we have regenerated rye and triticale plants from cultured immature embryos of sister lines differing in their heterochromatic content. The triticale lines differ in the presence of a telomeric heterochromatin band on a particular rye chromosome (6R or 7R), while the rye lines differ in only one heterochromatic band. In this paper, we describe the morphogenetic response of the different triticale and rye lines and discuss the relationship between response in culture and genotype.

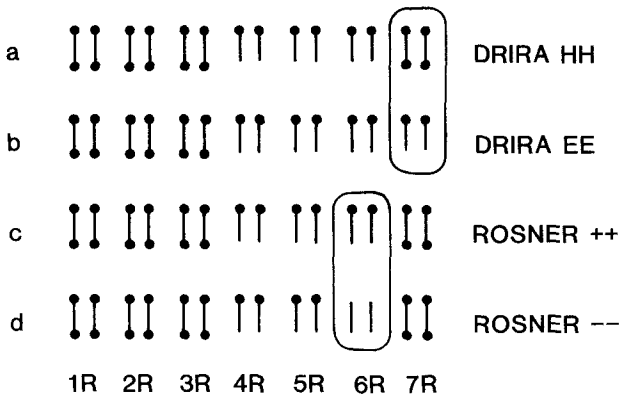

Fig. 1. The rye chromosome complement in the triticale lines: (*a*) D7R++ (*b*) D7R-- (*c*) R6R++ (*d*) R6R-- with respect to the telomeric heterochromatin (*black circles* on the end of the chromosome arms). The previous nomenclature of the lines is given on the *right*. The position of other heterochromatic bands is not shown, but all the rye chromosomes can be identified by their C-banding pattern

Materials and methods

Materials

Immature embryos for culturing were dissected from inflorescences of two pairs of sister lines of hexaploid spring triticale (× *Triticosecale*) cv Drira (Merker 1976) and cv Rosner (Roupakias and Kaltsikes 1976, 1977). The sister lines differ in the presence (denoted by symbols ++) or absence (denoted by symbols --) of heterochromatic blocks on specific rye chromosomes. The karyotype of the rye chromosomes in the triticale lines with respect to the heterochromatic telomeric blocks is shown in Fig. 1.

Drira HH carries a large block of terminal heterochromatin in the long arm of chromosome 7R (Fig. 1a) while in the sister Drira EE line, most (but not all) of this heterochromatic block has been deleted (Fig. 1b). In this paper, these lines will be designated as D7R++ and D7R--, respectively. Rosner++ carries the standard Rosner triticale karyotype in terms of heterochromatin (Fig. 1c). The sister line Rosner-- has a deletion of the heterochromatin in the short arm of 6R (Fig. 1d). In this paper, these lines will designated as R6R++ and R6R--, respectively.

Immature embryos were also dissected from inflorescences of five sister lines of rye (*Secale cereale*), kindly supplied by Dr. A. Lukaszewski of the University of Missouri-Columbia. These lines originated from a cross between a Spanish inbred line (E), which is practically devoid of telomeric heterochromatin (Giraldez et al. 1979) and a commercial variety, Dankowskie Zlote from Poland (or line H), which has considerable amounts of heterochromatin in 11 of its 14 telomeres (Lukaszewski and Gustafson 1983). The F_1 was backcrossed three times to the E line and in every generation selection was made for the lowest possible number of heterochromatic bands. After the third backcross, plants were selected with one or two bands and selfed and ++ and -- genotypes were identified. The immature embryos used in this study represent the F_4 from these selected genotypes.

The only clear telomeric band in the original E line is on the short arm of chromosome 5 (5RS). This band is very small – only half the normal size for rye. Since the E line was the 'basal' line for the rye genotypes, all five lines used contained the band on 5RS. In 5ROO some of the band was deleted and this line therefore contains very little heterochromatin. In this paper, 5ROO is designated as 5R-- (Fig. 2a). Line 7RL has, in addition to the small band on 5RS, a major telomeric band in the long arm of 7R (Fig. 2c). This line is designated as 7RL++. In the rye line 7RLOO, the band on 7RL is missing (Fig. 2b) and this is therefore designated as 7RL--. In line 7RS, in addition to the small band on 5RS, there is a major telomeric band on the short arm of 7R (Fig. 2d) and this line is designated as 7RS++. Finally, in the rye line 6RS, designated as 6RS++, the additional major telomeric band is on the short arm of 6R (Fig. 2e).

Tissue culture and regeneration

Inflorescences of the four triticale and five rye lines (DR7R++, DR7R--, R6R++, R6R--, 7RL++, 7RL--, 7RS++, 6RS++ and 5R--) were bagged to ensure self-pollination. Translucent embryos were obtained from green seeds (except in the case of cv Rosner where the seeds were green-white in colour) and both culture and regeneration were achieved using the method described by Maddock et al. (1983) with some minor modifications. The same method was used for all nine lines except for the size of immature embryos, which differed between rye and triticale. In triticale, embryos of circa 1.5 mm were cultured, while in rye embryos of circa 1 mm length were found to respond better. Only embryos with scutellar tissue were selected.

The basal culture medium used consisted of Murashige and Skoog (1962) nutrients (minus hormones) plus 3% w/v sucrose at pH 5.8, solidified with 0.6% w/v agar-agar (Fisons Ltd.), as described by Maddock et al. (1983), but coconut milk was not

added to the medium. The basal medium was supplemented with 1.0 mg l^{-1} 2,4-D. Instead of 5 embryos/dish (Maddock et al. 1983), 16–20 embryos were cultured per petri dish, scutellar side uppermost. Petri dishes were incubated at 25 °C under diffused light (16 h day ~5,000 lux) and plant regeneration was achieved exactly as described by Maddock and coworkers (1983).

Results

General features of callus growth and regeneration

The first change observed after the embryo was placed into culture was a slow increase in size. Proliferation from the scutellum took place within 10–15 days, depending on the line. Within 3 weeks, some of the callus had a watery, soft appearance. Elsewhere, a more compact callus was formed that was white or pale yellow in colour and surrounded by soft friable callus. Both types of callus could be produced by the same cultured embryo, but only the compact form was associated with shoot formation.

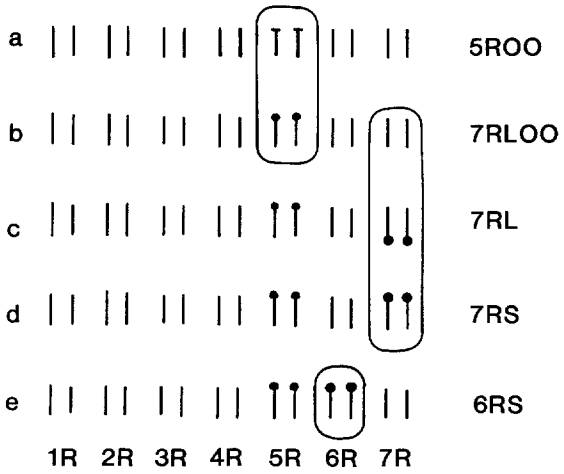

Fig. 2. The rye chromosome complement in the rye lines: (*a*) 5R––; (*b*) 7RL––; (*c*) 7RL++; (*d*) 7RS++; (*e*) 6RS++ with respect to the telomeric heterochromatin (*black circles* on the end of the chromosome arms). The previous nomenclature of the lines is given on the *right*. The position of other bands is not shown

Callus growth and shoot formation in the triticale lines

The morphogenetic characteristics of the triticale lines are shown in Table 1. The Drira lines (DR7++ and DR7––) showed no special type of callus and both lines behaved similarly in culture. The callus formed was not fragile as in Rosner, but yellow and compact, with numerous sites of both organogenesis and somatic embryogenesis (Fig. 3a, c). The percentage of somatic embryoids was slightly higher in D7R++ (Table 1), but the type of embryoid was the same in both lines. The appearance of the embryoids (Fig. 3b) was very different from the rye lines, and they were more easily distinguished from the main callus mass than in the case of cv Rosner because they formed at the edge of the callus and had a very distinctive shape.

Shoot formation occurred in a high frequency of cultures (on average 79%). In most cases, the shoots consisted of short, broad leaf-like structures with trichomes (Fig. 3a, c). After 1 month of culture, most embryos had no more than two or three shoots, although occasionally more than ten shoots could be seen. Although D7R++ had a higher percentage of cultures forming shoots, in line D7R–– there was a greater tendency for multiple shoot formation. Root production occurred quite frequently on the Drira calluses and on average 44% of cultures had roots. Over 55% of the calluses (on average) were "hairy" in appearance (Fig. 3a), which was a higher frequency than observed in cv Rosner. Albino and variegated shoots were observed in a low percentage of calluses in DR7++, but not in DR7R–– (Table 1).

The Rosner lines (R6R++ and R6R––) showed a different morphogenetic response than the Drira lines. Both lines behaved similarly, with a higher frequency of embryoids present in R6R–– (Table 1). In these lines, the compact callus had a white opaque appearance. During the initial stages of callus formation,

Table 1. Morphogenetic response in the triticale lines

Line	No. of embryos	% yellow compact callus	% embryoids	% shoot forming cultures	% callus with roots	% callus with 'hairyness'	% callus with variegated shoots	% callus with albino shoots	% callus with leaf clusters
D7R++	53	96.2	35.8	88.0	56.6	66.0	5.6	3.7	24.5
D7––	109	89.0	31.2	70.0	31.2	45.0	0.9	–	42.2
R6R++	58	93	37.9	87.0	56.8	29.3	12.0	–	82.8
R6R––	62	88.7	46.8	86.0	30.6	11.3	9.7	–	85.0

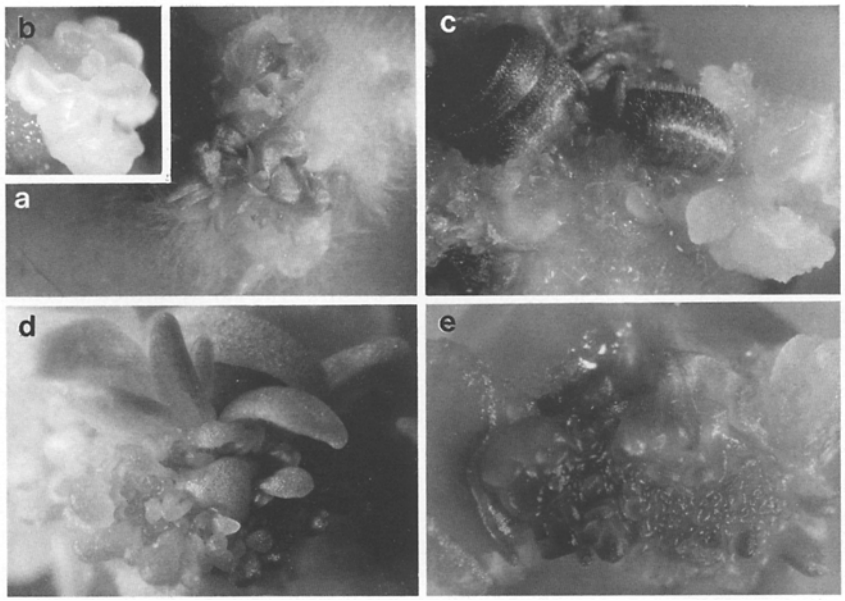

Fig. 3a–e. Callus formation in the triticale lines: **a** DR7R + +; **b** typical Drira embryoid; **c** DR7R−−; **d** R6R + +; **e** R6R−−

a white soft texture appeared and there was no clear formation of compact callus. Later, a white hard-textured callus formed and the usual glossy callus was rarely found.

The Rosner lines showed the best response of all the triticale and rye lines in terms of shoot formation. Although the percentage of shoot forming cultures was equal to that of D7R + + (Table 1), the Rosner lines showed a very strong tendency for multiple shoot formation (Fig. 3d). Over 80% of cultures in both lines formed multiple shoots in dense clusters. These clusters originated from compact green callus with a foamy texture. Small, rounded translucent constructions formed on the green callus (Fig. 3e), which later developed into thick green leaves. In most cases, the individual leaves were curled and pale-green/white in colour when they were formed on the callus, but over time they became very hairy and thick and dark green in colour.

Only a small percentage of the calluses appeared hairy (on average 20%). The Rosner line R6R + + showed higher frequencies of root production and hairy calluses than line R6R−− (see Table 1). After 2 months the callus shape had changed in some of the cultures. The callus growth had lifted the culture away from the medium. This structure was not observed in any of the other lines. Calluses producing variegated shoots were found at high frequencies (on average 11%) in both Rosner lines (Table 1). Albino shoots were not observed in appreciable frequency, although occasional albino shoots have been seen in old cultures of R6R + +.

Callus growth and shoot formation in the rye lines

The rye lines varied greatly in their tissue culture response (Fig. 4). The 6RS + + line showed the poorest response in culture (Fig. 4b). This line was characterised by precocious germination and the formation of brown watery callus. Smaller embryos were selected in an attempt to overcome the problem of precocious germination. In total, 390 embryos were cultured and 26% of them turned brown immediately. After 6 weeks of culture, a few calluses showed yellow-green callus formation. Shoot formation was relatively sparse and shoots were seen on only 16% of the cultures. Similarly, there was only 7% embryoid production (Table 2). Calluses with variegated and albino shoots were seen at low frequencies and very few of the calluses were hairy or produced roots (Table 2).

Line 7RL−− also had a poor morphogenetic response (Table 2). After 1 month in culture the callus was friable with white spotting and soft watery areas. Even the pale yellow callus that formed around the watery areas gave rise to poor shoot formation (only 19%) and in some cases the callus was not compact. There was a tendency for root formation (Table 2) and for a hairy appearance of the callus (Fig. 4c). Precocious germination was also a problem in this line. The few shoots formed did not include albino or variegated forms (Table 2).

In contrast to 7RL−−, the rye line 7RL + + showed an exceptional morphogenetic response. Within 2 weeks most of the cultures had formed nodules and distinct embryoid structures. Within 4 weeks, a 'special'

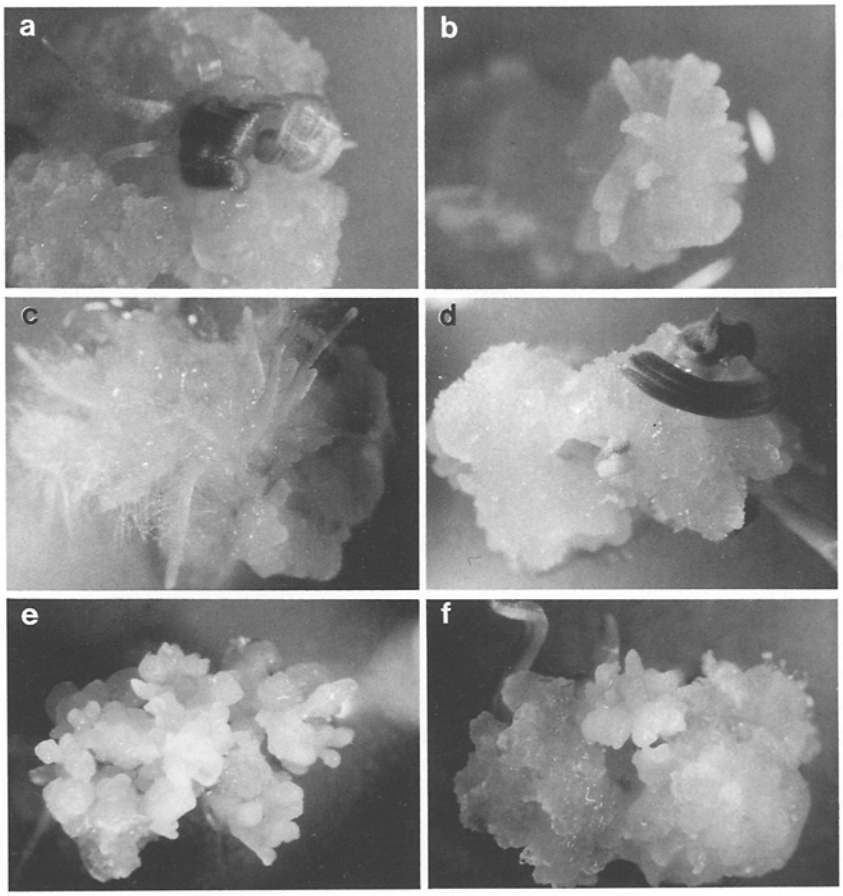

Fig. 4a–f. Callus formation in the rye lines: **a** 5R––; **b** 6RS++; **c** 7RS++; **d** 7RL––; **e** 7RL++ showing special yellow callus with prolific somatic embryogenesis; **f** 7RL++ showing yellow compact callus and normal callus

Table 2. Morphogenetic response of the rye lines. Drira cultures initiated at the same time and cultured over the same period are added for comparison

Line	No. of embryos	% yellow compact callus	% embryoids	% shoot forming cultures	% callus with roots	% callus with hairyness	% callus with variegated shoots	% callus with albino shoots	% special callus
6RS++	390	37	7.0	16.0	17.0	20.3	1.8	0.3	4.6
5R––	136	82.3	27.0	44.0	50.0	55.8	8.8	1.5	11.0
7RS++	26[a]	96.2	34.6	34.6	7.7	–	3.8	3.8	19.2
7RL++	185	90.3	68.2	75.5	10.0	18.9	10.3	3.0	67.0
7RL––	162	53.7	10.5	19.7	42.5	47.5	–	–	–
D7R++	121	92.5	26.4	85.0	25.6	51.3	2.4	1.6	–
D7R––	126	93.7	26.9	70.0	44.4	61.1	1.6	–	–

[a] Small sample size due to low fertility of donor plants

bright yellow callus formed that was almost completely embryogenic (Fig. 4e). There were only small areas of white transparent callus around the bright yellow areas. Some cultures (16%) formed only the special yellow callus, while in the majority of cultures (41%) a mixture of bright yellow and clear watery areas was observed (Fig. 4f). Only 21% of the cultures formed the normal yellow compact callus seen in the other lines. The special callus was very fragile and often fragmented when subculturing. It also grew very rapidly.

The 7RL++ line showed the highest frequency (75%) of shoot formation (Table 2). Shoot production was prolific, not only in terms of the number of calluses producing shoots, but also in terms of the number of

shoots per embryo, which could be as high as 17–24 shoots within 2 months of culture. Only a very small proportion of the calluses were hairy (18%) or underwent root formation (10%) (Table 2). A relatively high percentage of calluses, however, produced variegated or albino shoots (in total, 13%). Sometimes all the shoots arising from the same callus were albino. White leaves with areas of red pigmentation were also seen.

Line 7RS+ + responded well in terms of compact callus formation and shoot morphogenesis (Fig. 4d), although after several months in culture some of the calluses turned brown and the shoots on these calluses turned yellow. The normal yellow compact callus was seen at high frequency (96.2% of callus formed) and the frequency of embryoid production ranked second after the 7RL+ + line (Table 2). The 'special' yellow callus was not seen in the high frequency of the 7RL+ + line, but almost 20% of the compact callus was of the special form. None of the calluses had a hairy appearance and root production only occurred in a low percentage (~8%). Callus with variegated or albino shoots were observed at a low (3.8%) but equal frequency.

The 5R-- line showed better response in terms of shoot production than all the other rye lines with the exception of 7RL+ +. The callus appearance was quite watery and soft in some cases (Fig. 4a), and it appeared that shoots developed from watery foamy areas. However, removal of the foamy covering layer revealed compact callus from which embryoids were produced. The percentage of somatic embryogenesis (27%) was lower than for 7RL+ + and 7RS+ +, although shoot formation occurred at a higher frequency (44% of cultures) than for 7RS+ +. A large proportion of calluses were hairy (55.8%) or produced roots (50%) and some of the calluses were similar to those in the 7RL-- line. Only a few calluses produced albino shoots, while more callus gave rise to variegated shoots (Table 2).

Discussion

Plant regeneration systems from cultured immature embryos of triticale have been developed by numerous workers and examples where such systems are described include Nakamura and Keller (1982), Armstrong et al. (1984), Jordan and Larter (1985) and Brettel et al. (1986). Similarly, plant regeneration from cultured immature embryos of rye has been reported by Rybczynski (1979), Eapen and Rao (1982) and Lu et al. (1984). In this report, successful regeneration was achieved from four lines of triticale and five lines of rye, thereby confirming the universal applicability of immature embryo regeneration systems in the cereals.

The earlier studies of regeneration in rye and triticale have concentrated on the assessment of factors contributing to morphogenetic response and to high incidences of somatic embryogenesis. It is clear from these studies that good morphogenetic response depends on a number of factors, including the size of the immature embryo, the auxin concentration, the media composition, the orientation of the embryo on the medium and external conditions such as light intensity. Using optimal culture conditions, high efficiency regeneration can be achieved with up to 79% of calluses giving shoots in triticale (Nakamura and Keller 1982) and up to 73% in rye (Lu et al. 1984). In this report we achieved highly efficient regeneration, with an average of 81% of triticale calluses and up to 75% of rye calluses giving shoots.

In addition to the external parameters mentioned above, it has been recognised for some time that there is a genotypic component to morphogenetic response. For example, clear differences in tissue culture response have been observed between 25 cultivars of wheat (Maddock et al. 1983) and among 39 genotypes of winter wheat (Sears and Deckard 1982). Similarly, Nakamura and Keller (1982) noted significant differences in morphogenetic response between three hexaploid cultivars of triticale, while Linacero and Vazquez (1986a) found differences between four cultivars of rye. Of interest in this context is that there is increasing evidence of a genotypic component to stability in culture (Karp and Bright 1985). Recent studies considering this aspect include a report by Galiba et al. (1985), where differences in somaclonal variation were found among three winter wheat varieties, and a study in rye, where differences in variability were observed between cultivars (Linacero and Vazquez 1986b).

In this present study, plants were regenerated from sister lines of triticale and rye to investigate the nature of the genotypic component of both morphogenetic response and genomic stability, and to further investigate the relationship between morphogenetic capacity and chromosome stability. In the first part of the study, differences in morphogenetic response were described; stability of lines will be described in following papers.

Clear differences were present between the lines studied in both triticale and rye. The triticale lines showed, in general, better morphogenetic capacity than rye. In the case of triticale the two cultivars showed different response, with cv Rosner showing greater morphogenetic capacity both in terms of somatic embryogenesis and shoot formation. In this cultivar there was a marked tendency for multiple shooting from clusters on the calluses. The R6R-- line, lacking heterochromatin on 6R, showed a slightly better response, but the differences between the sister lines were very

small. Similarly, in cv Drira, the D7R + + line was marginally better in response.

In the case of rye very large differences in morphogenetic response were observed between the lines. The line with the poorest response was 6RS+ +, while line 7RL+ + was the best responder, showing exceptional embryogenetic potential. Further studies have now been initiated to investigate the full morphogenetic potential of the 7RL+ + line.

The sister rye lines are not completely isogenic, as only three backcrosses were used in their original selection (see "Materials and methods"). The lines differ in many respects, including morphology, fertility and chromosome pairing (A. Lukaszewski, personal communication). Consequently, while it is clear that the differences in response reflect genetic differences between the lines, it is not clear whether these differences are associated with the hetrochromatin or with genes closely linked to the telomeric blocks. Of particular interest is the large difference in morphogenetic response between the 7RL+ + and 7RL-- lines. These lines are very closely related, which would suggest that the genetic constitution of 7R is an important factor in determining morphogenetic response. In this respect, it is of interest that 7RS+ + also showed a good response in culture and had appreciable levels of special callus formation. However, the 7RS+ + line was more sensitive in response and some of the calluses turned brown after prolonged culture.

Support for the observation that a specific chromosome can influence morphogenetic response can be found in recent studies in wheat, where the regeneration capacity of chromosome substitution lines has been investigated. Mathias and co-workers, studying the effect of different cytoplasms, found that regeneration capacity was affected by the cytoplasmic constitution of alloplasmic lines (Mathias et al. 1986) and by a 4B substitution of Capelle Desprez into Chinese Spring in both euplasmic and alloplasmic lines (Mathias and Fukui 1986). In triticale, Nakamura and Keller (1982) noted that cv Welsh showed the best response in tissue cultue. In their study, the lines used contained substitutions from the D genome of wheat for rye chromosomes 2R and 4R, and the authors suggest that the D genome chromosomes may have contributed to the improved response. Of direct interest to this present study is a recent report in wheat where tissue culture response of different substitution lines was studied. The results indicated that morphogenetic response was under polygenic control and that, in particular, chromosomes 7B, 7D and 1D were effective (Galiba et al. 1986). Much work is needed before the genotypic component of tissue culture response is better defined. In particular, the possible involvement of chromosome 7 in rye can be tested by use of addition and substitution lines. Such studies would greatly aid the development of efficient regeneration systems in the cereals, both from explants and protoplasts, and provide important knowledge against which studies of stability can be compared.

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