Theor Appl Genet (1988) 75: 679–684

Species-specific DNA sequences for identification of somatic hybrids between *Lycopersicon esculentum* and *Solanum acaule*

G. Schweizer, M. Ganal *, H. Ninnemann [1] and V. Hemleben **

Lehrstuhl für Genetik, Institut für Biologie II, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, Federal Republic of Germany

[1] Institut für Chemische Pflanzenphysiologie, Corrensstrasse 41, D-7400 Tübingen, Federal Republic of Germany

Received November 6, 1987; Accepted November 11, 1987
Communicated by G. Wenzel

Summary. Species-specific highly repeated DNA sequences can be used to screen the progeny of protoplast fusions combining different species. Such probes are easy to clone and can be detected by fast methods, e.g., hybridization to total genomic DNA. Furthermore, due to their high copy number, hybridization signals are strong and represent more than one locus, unlike isozymes or resistance markers. After cloning and screening for species-specific DNA sequences we characterized the highly repeated DNA sequences of the solanaceous species *Solanum acaule* and *Lycopersicon esculentum* var. "gilva". DNA sequencing and hy ridization revealed a prominent, tandemly arranged satellite DNA repeat of 162 bp in *Lycopersicon esculentum* and a different satellite repeat of 183 bp, also tandemly organized, in *Solanum acaule*. Each repeat is absent in the respective other species. Therefore, we have used these DNA repeats as markers to distinguish regenerated interspecific somatic hybrids from the respective fusion partners. These hybrids were clearly identified by Southern hybridization and dot-blot assays to the respective ^{32}P-labelled satellite DNA.

Key words: *Solanum acaule* – *Lycopersicon esculentum* var. cerasiforme – Satellite-DNA – Species-specific – Somatic hybrids

Introduction

Transfer of polygenic traits from a wild species to a related crop plant by somatic hybridization may be of importance for crop improvement, although the fertility problem must be observed (Ehlenfeldt and Helgeson 1987). The fusion of plant protoplasts and the regeneration of the fusion products to complete plants have been possible for many years (Melchers et al. 1978). However, the identification of the heterologous somatic hybrids is restricted to complementation of existing mutants (Melchers and Labib 1974), characterization of isozymes (Bavand et al. 1985), volatile substances (Ninnemann and Jüttner 1981), specific secondary substances (Roddick and Melchers 1985) or resistance markers (Pental et al. 1986), which only allow screening for a few loci. On the DNA level, the occurrence of cytoplasmic specific traits of both fusion partners can be analyzed (Kemble et al. 1986). Furthermore, specific repetitive nuclear DNA components can be used as indicators for the successful combination of different genomes (Saul and Potrykus 1984).

Species-specific highly repeated satellite DNA appears to be most useful when used to distinguish interspecific hybrids at a relatively early stage after protoplast fusion. Highly repeated DNA is present in almost all eukaryotic species. These sequences are characterized for many monocotyledonous (Bedbrook et al. 1980b; Barnes et al. 1985) and dicotyledonous plants, preferably with a repeat length of 160–180 bp (Ganal et al. 1986; Grellet et al. 1986) or 350–360 bp (Hemleben et al. 1982; Ganal and Hemleben 1986). Even closely related species often develop and amplify a certain repeat type which stays as a "footprint" in the nuclear genome with a high copy number; therefore, it is possible to detect specific repeats giving a strong hybridization signal and to use them for diagnostic purposes (Dover 1986).

We used the species-specific highly repeated DNA to prove the hybrid character of plants regenerated

* Present address: Department of Plant Breeding and Biometrie, Cornell University, Ithaca, NY 14853, USA
** To whom correspondence should be addressed

after fusion of protoplasts from *Lycopersicon esculentum* var. cerasiforme, mutant "gilva" and the wild *Solanum* species *Solanum acaule*. The fusion was carried out by Ninnemann and Melchers (Ninnemann et al. 1987) to transfer polygenically coded "useful" traits (in this case the cold tolerance of the wild species) from one plant to another. For both fusion partners, species-specific repeated sequences have been characterized and sequenced, and specific repeat types were used as hybridization probes for the identification of the somatic hybrids.

Materials and methods

Plant material

The protoplast fusion partners *Lycopersicon esculentum* and *Solanum acaule* were cultivated as shoot cultures under sterile conditions on MS-Medium (Murashige and Skoog 1962). *Solanum acaule* ($2n=4\times=48$), a wild species, was obtained from CIP (Centre International de la Papa, Lima, Peru) and *Lycopersicon esculentum* var. *cerasiforme,* mutant "gilva" ($2n=2\times=24$), a chlorophyll deficient tomato, was received from Prof. Melchers, Max-Planck Institut for Biology, Tübingen. The heterologous protoplast fusion experiments were carried out by Ninnemann and Melchers by the PEG-fusion technique. Fused protoplasts were regenerated and selected for cold tolerance as described by Ninnemann et al. (1987) and identified by peroxidase polymorphism (Bavand et al. 1985). In our experiment we characterized one regenerated cold tolerant fusion line with the lab number 392 on the DNA level.

DNA isolation and characterization

DNA was prepared from isolated nuclei according to Hemleben et al. (1982) and further purified by CsCl gradient centrifugation. Standard procedures were used as described by Maniatis et al. (1982).

Cloning

The satellite DNA of *Lycopersicon esculentum* was cloned after digestion of nuclear DNA with Hae III. A single prominent band of ca. 160 bp was eluted and cloned into pUC 19 (Yanisch-Perron et al. 1985). Clones containing repeated DNA sequences were identified by hybridization to Hae III digested ^{32}P-nicktranslated total genomic DNA of *Lycopersicon esculentum.* The repeated DNA sequences of *Solanum acaule* were cloned by a different approach because a prominent band was not detectable. Nuclear DNA was digested with Taq I and randomly cloned into pUC 19. Clones containing repeated DNA sequences were identified by colony hybridization (Grunstein and Hogness 1975) and hybridization of mini-screen preparations (Birnboim and Doly 1979) to ^{32}P-nick-translated total nuclear DNA of *S. acaule.* Only repetitive DNA (or extrachromosomal DNA) gave a hybridization signal in this system (Saul and Potrykus 1984).

Hybridization

Hybridization procedures applied are described in Ganal et al. (1986). The stringent hybridization temperature was 67 °C. Eluted inserts were used for all experiments.

Sequencing

DNA sequencing was done according to the Sanger method (Sanger et al. 1977) after cloning the respective fragments into M13 mp8 (Messing and Vieira 1982).

Results

Highly repeated DNA sequences often can be detected as prominent bands in gels after digestion of total nuclear DNA (Bedbrook et al. 1980a; Ganal and Hemleben 1986). These sequences appear to be specific for species or genera. Therefore, we have characterized species-specific sequences of *L. esculentum* and *S. acaule* for the identification of somatic hybrids regenerated from protoplast fusions.

Characterization of a L. esculentum specific repeat

DNA of *L. esculentum* was digested with several 4 bp – recognition restriction enzymes and separated on a high resolution polyacrylamide gel to detect sequences with the characteristic satellite DNA feature. A very prominent band of 162 bp occurred in the Hae III digestion (Fig. 1 A, B). In *S. acaule* only faint bands were found in the region between 1 kbp and 50 bp (Fig. 1 B). The prominent 162 bp band in the Hae III digestion of the tomato DNA was eluted and cloned into pUC 19. Those clones containing highly repeated DNA sequences were identified by hybridization of Southern blots with ^{32}P-labelled Hae III digested total nuclear DNA from tomato. The hybridization pattern of all clones containing the repeated DNA sequence was identical. Hybridization of a ^{32}P-labelled 162 bp insert to Southern blots of digested nuclear DNA of tomato resulted in a short ladder of bands typical for a tandemly arranged organization of this repeat in the genome (Fig. 1 C). Screening for species-specificity was achieved by hybridization to *S. acaule* DNA: the hybridization of the highly repeated tomato sequence showed no cross-reaction to the heterologous DNA of *S. acaule* (Fig. 1 C).

In order to characterize this sequence in more detail we have sequenced two repeats. Both have a length of 162 bp and show a sequence diversity of only 3%; their G/C-content is 46%–48% (Fig. 2). All restriction enzyme sites predicted from the DNA sequence were also present in most of the genomic repeats tested (not shown).

Characterization of a S. acaule specific satellite DNA

Since the digestion of *S. acaule* DNA with the used restriction enzymes did not result in a prominent band in the gel, we used a different approach. Total nuclear

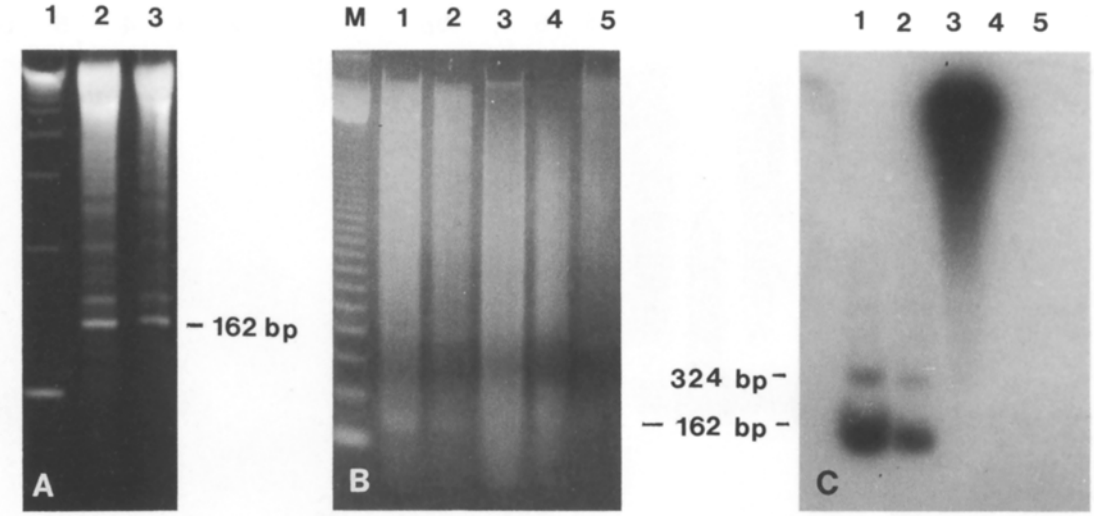

Fig. 1A–C. Characterization of species-specific highly repeated satellite DNA of *L. esculentum.* **A** Total nuclear DNA (2 µg) of *L. esculentum* "gilva" was digested with Hae III (*lanes 2* and *3*) and separated on a 5% polyacrylamide gel. The 162 bp band was eluted from the gel and cloned into pUC 19. *Lane* (*1*) contains the 123 bp ladder as marker. **B** In each case 2 µg nuclear DNA of *L. esculentum* and *S. acaule* was digested with various restriction endonucleases and separated on a 2% agarose gel. *L. esculentum* DNA was digested with Hae III (*lanes 1* and *2*) and Alu I (*3*); the *S. acaule* DNA was digested with Rsa I (*4*) and Hae III (*5*). **C** Hybridization of the digested DNA (in **B**) with ^{32}P-labelled 162 bp-insert of pLEG 15. *Lanes* are marked as in **B**

```
          1       10        20        30        40        50
pLEG 15: CCAACCGTATGCATAGACAACGTCTTGACGGAAGTCCACGAACAAATTTG
pLEG 18: ..................................C.................

                 60        70        80        90       100
         GCATTTTTGACGTCGGAATCCTGATCACCCAAAAAATAGTGTGCAATAGC
         ....................................G.............

                110       120       130       140       150
         ACACGAAAATTGTCAAAATGAGGCGTATGCTCGCTCCGGTGCTCGTTTGA
         ..........C.............................G..........

                160
         CCTTCCAAACGG
         .........T..
```

Fig. 2. Nucleotide sequences (5′–3′) of the inserts with the species-specific satellite DNA of *L. esculentum* in pLEG 15 and pLEG 18. For pLEG 18 only the positions of non-homology are indicated

```
          1       10        20        30        40        50
pSA 287: GAAAAAATATGCGGGTTCACAAAAAAATCGCCTGAAACGGACATCCGAAG

                 60        70        80        90       100
         CCCGAGTTATGGTCGTTTGAATTTCCTTTTTCCTTTCTTATCTTAATTTT

                110       120       130       140       150
         ACTATTCAAAGAATGAGTAAAAGGCATTTTTGGAAAATCGGCACACTCAA

                160       170       180
         ATCAACTTACGGTAGTTTGGTTGCGTAGATATC
```

Fig. 3. Nucleotide sequence (5′–3′) of the insert from pSA 287 containing the species-specific satellite DNA repeat of *S. acaule*

DNA was digested with Taq I and shotgun-cloned into pUC 19. About 500 clones were then probed against ^{32}P-labelled total nuclear DNA of *S. acaule* and *L. esculentum,* respectively. Clones giving a strong hybridization signal with the *S. acaule* DNA were considered to contain repeated DNA sequences of that species. Furthermore, hybridization with the *L. esculentum* DNA eliminated those clones containing repeated DNA sequences present in both species (as ribosomal DNA). Finally, several clones were characterized by hybridization to blots of DNA from both species.

One repeat was analyzed in detail by DNA sequencing. The repeat length was 183 bp with a G/C content of 36% (Fig. 3). There was a sequence homology of 10 bp (nucleotide 123–133) to the described tomato satellite. Hybridization of a nick-translated 183 bp repeat to Hae III or Taq I digested nuclear DNA of *S. acaule* also showed a ladder of bands indicating the satellite DNA character of this repeat type (Fig. 4 C). The sequenced satellite repeat does not completely reflect the majority of the genomic repeats as revealed by the hybridization pattern, since there is no Hae III site and only one Taq I site in this sequenced repeat. However, using this sufficiently representative probe it is possible to detect this satellite DNA family.

Characterization of protoplast fusions products

Since the characterization of these repeats revealed a specificity for fusion partners, they were used as probes to characterize the somatic hybrid plants. DNA was

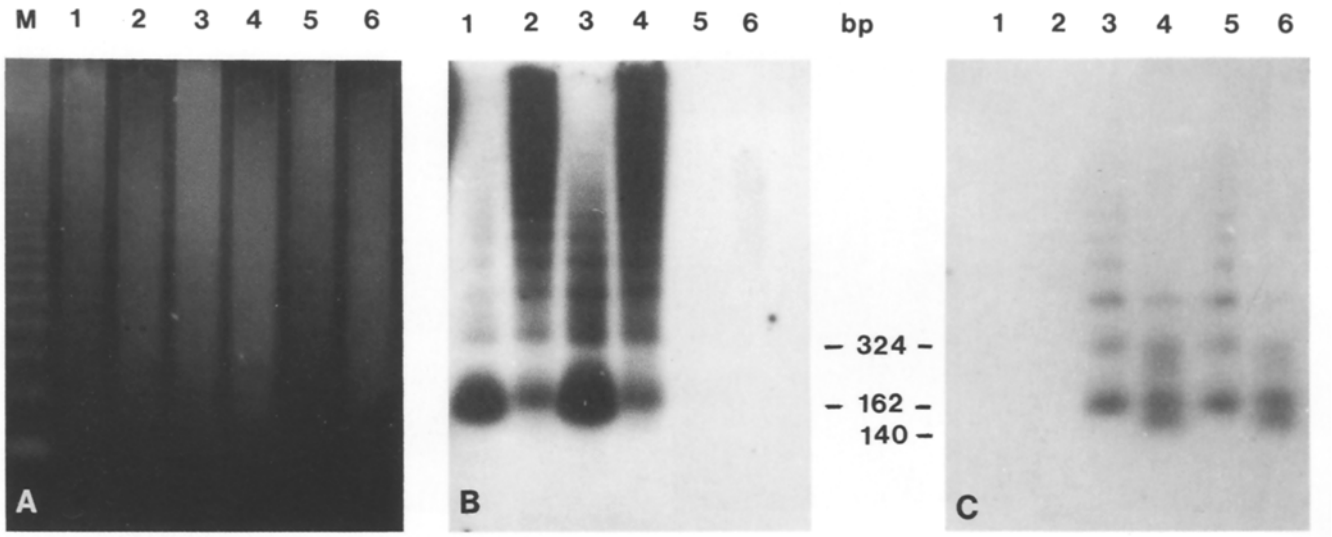

Fig. 4A–C. Identification of the somatic hybrid plant. Total nuclear DNA (2 μg each) of *L. esculentum* (*lanes 1* and *2*), of the hybrid plant No. 392 (*3* and *4*) and *S. acaule* (*5* and *6*) was digested with HaeIII (*1*, *3* and *5*) or Taq I (*2*, *4* and *6*), separated on a 2% agarose gel **A** and hybridized to the *L. esculentum* specific 162 bp repeat **B** or to the *S. acaule* specific 183 bp repeat **C**

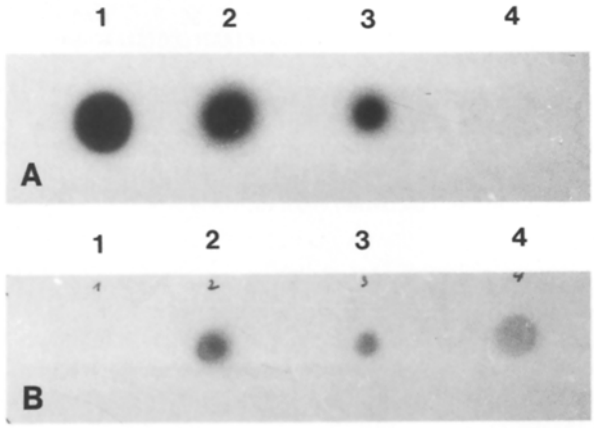

Fig. 5A, B. Dot-blot assay to identify somatic hybrid plants. Total nuclear DNA was blotted onto nitrocellulose filters (**A** and **B**) in the following order: (*1*) 1 μg of *L. esculentum* DNA; (*2*) and (*3*) 1 μg or 0.5 μg, respectively, of the somatic hybrid plant No. 392; (*4*) 1 μg of *S. acaule* DNA. Filter **A** was hybridized to the ^{32}P-labelled *L. esculentum* satellite repeat in pLEG 15 and the other filter **B** was hybridized to the *S. acaule* specific repeat in pSA 287

extracted from the regenerated protoplast fusion plant No. 392 and characterized in two different approaches. First, by digesting DNA from regenerated somatic hybrids with several restriction enzymes in comparison to the fusion partners and hybridization to the respective species-specific satellite DNA (Fig. 4), we obtained information on the quantity and organization of the repeats. In both cases we found the identical bands in nearly the same intensity as observed in the respective fusion partner, indicating that both genomes, or at least the repetitive components, are stably maintained in the somatic hybrid plants. Secondly, in a dot-blot assay the presence of the respective satellite was tested in comparison to the DNA of the fusion partners. DNA from both partners and the respective somatic hybrid was spotted on filters and hybridized with the two species-specific satellite probes. The tomato and the somatic hybrid plant gave a signal if hybridized with the *L. esculentum* specific repeat (Fig. 5A). The *S. acaule* specific repeat hybridized only to the *S. acaule* DNA and to the somatic hybrid DNA (Fig. 5B). Thus, we have a good and fast test for the identification of somatic hybrids between two closely related species.

Discussion

The fusion partners of a protoplast fusion experiment between *Lycopersicon esculentum* and the wild species *Solanum acaule* can be clearly distinguished by species-specific satellite DNA. The nucleotide sequences of these satellite repeats with 162 bp for *L. esculentum* and 183 bp for *S. acaule* show nearly no homology except for a short region of 10 bp found between nucleotide 50–59 in the tomato repeat and between nucleotide 123–132 in the *S. acaule* repeat (see Figs. 2 and 3). Both repeat types show a length in the range as found for other dicotyledonous plants investigated so far (Ganal et al. 1986; Martinez-Zapater et al. 1986).

Highly repeated satellite DNA sequences are present in almost all eukaryotic species and are characterized by their

tandemly repeated structure. These sequences can represent up to 30% of the total nuclear DNA of a plant species (Ganal et al. 1986). They are considered to be more or less neutral and can vary enormously, even in closely related species. Satellite DNA is a major part of the heterochromatin in the genome. Mostly it is found at the centromers and telomers of the chromosomes (Jones and Flavell 1982a, b) and therefore represents more than a single locus. These features enable us to use highly repeated DNA sequences as a marker to characterize species.

Highly repeated DNA sequences are easy to clone after digestion of total DNA with restriction endonucleases and the elution of the prominent bands. Sometimes it is not possible to detect such distinct bands in the gel. This problem can be bypassed by using a different assay first described by Saul and Potrykus (1984) and based on a random cloning procedure. The cloning of a certain number of random fragments should also contain repeated DNA sequences. These are detected by hybridization to total genomic DNA. Since the molarity of repeated sequences is much higher than DNA sequences of a low copy number or of unique sequences, they give a much stronger signal in colony hybridization assays. Therefore, we can find species-specific DNA repeats by hybridizing to the respective total nuclear DNA of the fusion partners. Furthermore, it is possible to screen many different individuals by a dot-blot assay using crudely purified DNA from plants or even from callus material by a modification of the colony hybridization procedure (Grunstein and Hogness 1975; Hutchinson et al. 1985). This would allow for screening for the interspecific somatic hybrids at a very early step after protoplast fusion and the regeneration procedure and selectable markers like antibiotica resistance genes would not be needed (Brunold et al. 1987). In addition, it is possible to use any improved cultivar (e.g., of economic interest) without a known marker. By in-situ hybridization it is possible to show how many chromosomes from the respective fusion partner are maintained in the somatic hybrids, because the satellite DNA often is localized in the heterochromatin of nearly all chromosomes (M. Ganal, N. Lapitan and S. T. Tanksley, personal communication). Interestingly, in the *L. esculentum/S. acaule* hybrids both highly repeated DNA components seem to be relatively stable and maintained in similar amounts as in the fusion partners.

Recently it has been shown that the 162 bp tomato satellite described here is present not only in *Lycopersicon esculentum* but also in a number of other *Lycopersicon* species and even in *Solanum lycopersicoides* (M. Ganal, N. Lapitan and S. T. Tanksley, personal communication). This observation is in contrast to the investigations on the *Cucumis* highly repeated DNA, where the *Cucumis melo* (melon) genome is characterized by a different satellite DNA than the *Cucumis sativus* (cucumber) genome (Hemleben et al. 1982; Ganal et al. 1986). Therefore, the tomato satellite repeat can be used only in heterologous fusion experiments as shown here for *Lycopersicon esculentum* and *Solanum acaule*. For the combination of different *Lycopersicon* species or even cultivars a more specific probe has to be applied.

Acknowledgements. The financial support of this work by the GFP and BMFT No.: 0318942A/A20/86-ZF is gratefully acknowledged. The responsibility for the content of this publication is with the authors.

References

Barnes S, James A, Jamieson G (1985) The organization, nucleotide sequence, and chromosomal distribution of a satellite DNA from *Allium cepa*. Chromosoma 92:185–192

Bavand M, Gekeler W, Ninnemann H (1985) Laminarströmungsmethoden zum Gießen von Microultradünnschichtgradientengelen und neue Peroxidasefärbung zur Linien- und Artauftrennung von *Solanum* und *Lycopersicon*. In: Radola BJ (ed) Proc Elektrophoreseforum. Bode, München, p 284

Bedbrook J, O'Dell M, Flavell RB (1980a) Amplification of rearranged repeated DNA sequences in cereal plants. Nature 288:133–137

Bedbrook J, Jones J, O'Dell M, Thompson RD, Flavell RB (1980b) A molecular description of telomeric heterochromatin in *Secale* species. Cell 19:545–560

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Brunold C, Krüger-Lebus S, Saul MW, Wegmüller S, Potrykus I (1987) Combination of kanamycin resistance and nitrate reductase deficiency as selectable markers in one nuclear genome provides a universal somatic hybridizer in plants. Mol Gen Genet 208:469–473

Dover G (1986) Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. Trends in Genet 2:159–165

Ehlenfeldt MK, Helgeson JP (1987) Fertility of somatic hybrids from protoplast fusions of *Solanum brevidens* and *Solanum tuberosum*. Theor Appl Genet 73:395–402

Ganal M, Hemleben V (1986) Different AT-rich satellite DNAs in *Cucurbita pepo* and *Cucurbita maxima*. Theor Appl Genet 73:129–135

Ganal M, Riede I, Hemleben V (1986) Organization and sequence analysis of two related satellite DNA in cucumber. J Mol Evol 23:23–60

Grellet F, Delcasso D, Panabieres F, Delseny M (1986) Organization and evolution of higher plant alphoid-like satellite DNA sequence. J Mol Evol 187:495–507

Grunstein M, Hogness D (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc Natl Acad Sci USA 72:3961–3965

Hemleben V, Leweke B, Roth A, Stadler I (1982) Organization of highly repeated satellite DNA of two *Cucurbitaceae* species. Nucleic Acids Res 10:631–644

Hutchinson J, Abbott A, O'Dell M, Flavell RB (1985) A rapid screening technique for the detection of repeated DNA sequences in plant tissues. Theor Appl Genet 69:329–333

Jones J, Flavell RB (1982a) The mapping of highly repeated DNA families and their relationship to C-bands in chromosomes of *Secale cereale.* Chromosoma 86:595–612

Jones J, Flavell RB (1982b) The structure, amount and chromosomal localization of defined repeated DNA sequences in species of the genus *Secale.* Chromosoma 86: 613–641

Kemble RJ, Barsby TL, Wang RSC, Shepard JF (1986) Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens.* Theor Appl Genet 72:787–793

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory

Martinez-Zapater JM, Estelle MA, Somerville CR (1986) A highly repeated DNA sequence in *Arabidopsis thaliana.* Mol Gen Genet 204:417–423

Melchers G, Labib G (1974) Somatic hybridization of plants by fusion of protoplasts. Mol Gen Genet 135:277–294

Melchers G, Sacristan MD, Holder AA (1978) Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res Commun 43:203–218

Messing J, Vieira J (1982) The pUC plasmids and M13 mp7 – derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

Ninnemann H, Jüttner F (1981) Volatile substances from tissue cultures of potato, tomato and their somatic fusion products. Comparison of gas chromatography patterns for identification of hybrids. Z Pflanzenphysiol 103:95–107

Ninnemann H, Bavand M, Melchers G (1987) Transfer of cold tolerance from *Solanum acaule* into *Lycopersicon esculentum* plants by somatic hybridization (Abstr). 7 wiss Tagung Ges Entwicklungsbiol and deutsche Sekt Int Assoc Plant Tissue Cult 1987, FU Berlin

Pental D, Hamill JD, Pirrie A, Cocking EC (1986) Somatic hybridization of *Nicotiana tabacum* and *Petunia hybrida.* Mol Gen Genet 202:342–347

Roddick JG, Melchers G (1985) Steroidal glycoalkaloid content of potato, tomato and their somatic hybrids. Theor Appl Genet 70:655–660

Sanger M, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5476

Saul M, Potrykus I (1984) Species-specific repetitive DNA used to identify interspecific somatic hybrids. Plant Cell Rep 3:65–67

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC 19 vectors. Gene 33: 103–119