Segregation of Triazine-Resistant Chloroplasts after Introduction into Winter Type Oilseed Rape (*Brassica napus* L.) by Protoplast Fusion

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Triazine-resistant chloroplasts of the Canadian spring oilseed rape variety OAC Triton were transferred into four German winter oilseed rape lines and two cultivars of double-low quality by means of protoplast fusion. X-irradiation has been used to reduce the amount of nuclear DNA of the spring type cultivar and to promote cybrid formation. RFLP-analysis showed that some regenerants and their progeny carried both types of chloroplasts. In some instances regenerants and progeny containing mixtures of both chloroplasts not kept under selective conditions lost their triazine-resistant chloroplasts completely during further plant growth. Preliminary results of greenhouse and field experiments indicate that volunteer plants can be eliminated by application of 150–300 g/ha metribuzin (Sencor^R, Bayer AG) in a stand of triazine-resistant oilseed rape of double-low quality.

Introduction

The introduction of new winter oilseed rape varieties with double-low quality (high in yield, seeds low in erucic acid and glucosinolate content) is connected with the problem of volunteer oilseed rape plants containing high levels of glucosinolate. Seeds of previously grown old oilseed rape varieties are still able to germinate even after 10 years in soil. Thus, seed producer and farmers constantly face volunteer plants in the stand of double-low oilseed rape. A possible way to eliminate these volunteer plants is the use of double-low varieties resistant to a herbicide not selective in oilseed rape.

Certain forms of triazine/triazinone(tri)-resistance are cytoplasmatically inherited and encoded by chloroplast DNA [1–3]. Spring oilseed rape cultivars with tri-resistance are already available in Canada where tri-resistance was transferred from *B. campestris* to *B. napus* by cross breeding [4]. Protoplast fusion techniques offer the possibility to produce cells with mixed chloroplast populations in species where chloroplasts are maternally inherited. Genetic traits located in chloroplasts and mitochondria can be combined with a desired genome of a recipient plant by X-irradiating the unwanted nuclei of the donor plant before fusion [5–7].

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We describe the transfer and segregation of tri-resistant chloroplasts of the Canadian spring oilseed rape cultivar OAC Triton [8] into some high-yielding German winter oilseed rape lines and varieties of double-low quality by means of protoplast fusion (Fig. 1).

Materials and Methods

Isolation, fusion and regeneration of protoplasts

Hypocotyl protoplasts of winter oilseed rape and X-irradiated (15 kR) mesophyll protoplasts of the tri-resistant spring oilseed rape OAC Triton were fused according to Menczel and Wolfe [9] with slight modifications [10]. Fusion products were isolated within 24 h after fusion using a microcapillary. Approximately 50 fusion products were collected and injected into drops of 20 µl of culture medium supplemented with 0.6% agarose before solidification and a two day old hypocotyl protoplast nurse culture was added. Plants were regenerated [10] and tested for tri-resistance by leaf application with 300 g/ha of the triazinone metribuzin. "Cybrids" were identified on the basis of their resistance to metribuzin and the need to be vernalized in order to flower.

Molecular and genetic analysis of regenerants and progeny

The tri-resistant chloroplasts of the spring oilseed rape variety are mutated in the *psb*A gene coding for the 32 kDa herbicide binding protein.

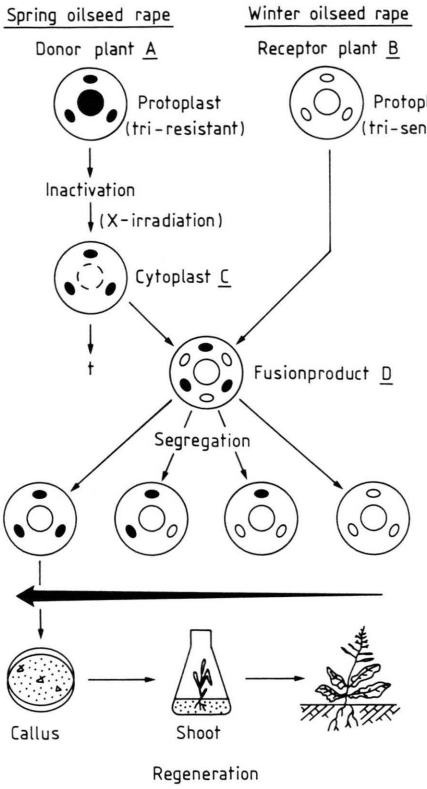

Fig. 1. Nuclei of the spring oilseed rape protoplasts (A) containing tri-resistant chloroplasts are inactivated by means of X-irradiation and the resulting cytoplasts (C) are fused with winter oilseed rape protoplasts (B) containing the tri-sensitive chloroplasts. The fusion products (D) contain the active nuclei of the tri-sensitive winter oilseed rape protoplasts and a mixture of tri-resistant and tri-sensitive chloroplasts. Chloroplast segregation takes place during the further development of fusion products *via* callus to whole plants.

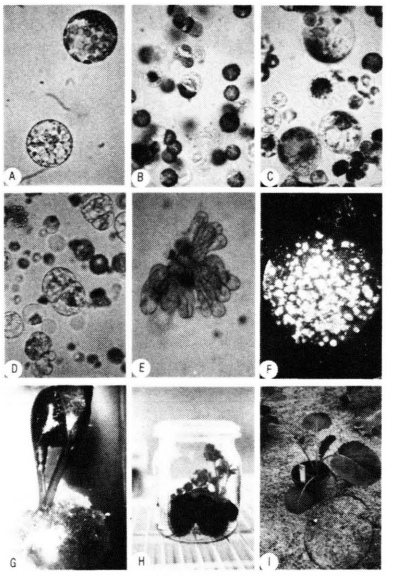

Fig. 2. A) Mesophyll protoplast of the tri-resistant spring oilseed rape OAC Triton and hypocotyl protoplast of a tri-sensitive winter oilseed variety Ceres. B–C) Formation of fusion products 5 min (B) and 1 h (C) after induction of fusion. D) Dividing fusion product after 3 days in culture. E) Microcolony forming extended cells (16 days). F) Formation of calli in an agarose droplet. G) Regeneration of shoots on SR-medium. H) Rooting and hardening-off in peat pellets placed in preserving jars. I) Regenerated oilseed rape plant.

This mutation introduces a new restriction enzyme target for the enzyme BstXI (Fig. 3), providing an ideal opportunity for the identification and differentiation of tri-resistant und tri-sensitive chloroplasts by RFLP-analysis. For RFLP-analysis [10] total plant DNA was isolated and analyzed by Southern blotting [11] using a subcloned fragment of the *psb*A gene of *Solanum nigrum*.

Results and Discussion

Protoplasts of 45 different double-low winter oilseed rape lines were regenerated to plants [10]. A remarkable difference in the regeneration capability of the 50 different genotypes was observed. Calli of 3 genotypes produced shoots at a frequency of

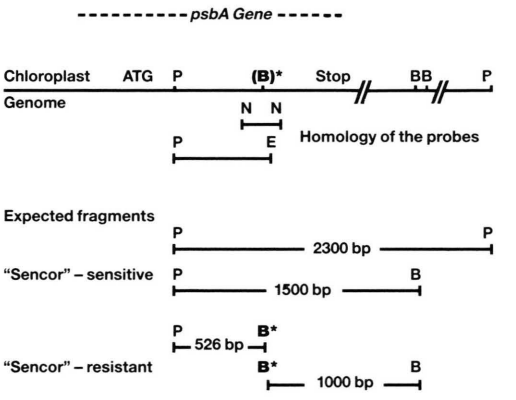

Fig. 3. Schematic map of the *psb*A gene on the chloroplast genome of *B. napus*, homology of the probe and expected restriction fragment size. P = PstI; B = BstXI; E = EcorV.

more than 20% (ex. cv. Ceres). The regeneration frequency for most linies was between 10–20%. Only a few lines tested failed to regenerate shoots or formed shoots at a very low frequency of < 3%. X-irradiation promoted cybrid formation and effectively prevented unfused protoplasts from forming microcolonies. The fusion method described by Menczel and Wolfe [9] was most convenient for the isolation of individual fusion products. Callus was easily obtained from fusion products by injecting isolated cells into agarose droplets before solidification [10].

All plants regenerated from protoplasts fusion experiments were routinely treated with 300 g/ha metribuzin. Tri-resistant plants survived a treatment rate up to 1000 g/ha metribuzin, whereas sensitive plants were killed within 10 days by rates of less than 100 g/ha under green-house conditions.

RFLP-analysis provided a very sensitive identification and differentiation of tri-resistant and tri-sensitive chloroplasts in regenerants and progeny plants. The analysis of 33 regenerants which survived the metribuzin application and possessed the winter habit demonstrated that tri-resistant chloroplasts were present in plants analyzed between metribuzin application and vernalization. Some regenerants contained a mixture of both chloroplast types (Fig. 4). In other regenerants tri-resistant chloroplasts were not detected when analyzed during late flowering stage. We assumed that these regenerants initially contained enough tri-resistant chloroplasts to survive the herbicide application but lost tri-tolerant chloroplasts during further development after vernalization. This assumption was supported by experiments with tri-resistant progeny plants, containing less than 1% tri-sensitive winter oilseed rape chloroplasts, analyzsed during further plant development. Progeny plant not kept under selection condition with metribuzine lost all resistant chloroplasts within one month after vernalization (Fig. 5). However, plants which

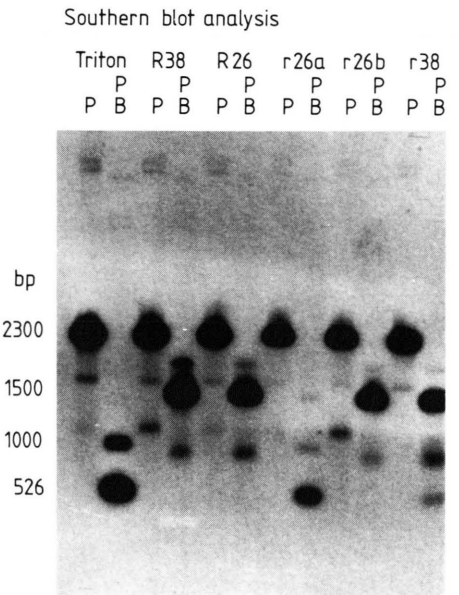

Fig. 4. Southern blot analysis of cybrid plants: Total plant DNA was digested with PstI (P) and double digested with PstI and BstXI (P/B). DNA digestion of metribuzin resistant Triton (control) generated two fragments of about 1 kb and 526 bp whereas only one fragment of about 1.5 kb was generated with metribuzin sensitive winter oilseed lines R 38 and R 26 (controls). Regenerant r26a generated two fragments (resistant) while r26b generated only one fragment (sensitive). A strong fragment of 1.5 kb and weaker fragments of 1 kb and 526 bp were generated with DNA of regenerant r38.

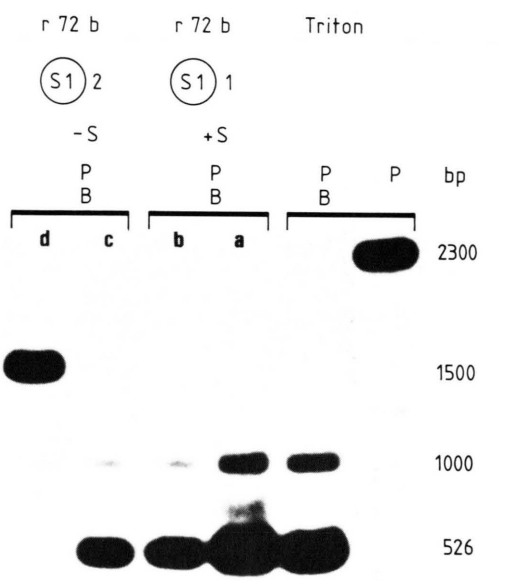

Fig. 5. Southern-blot analysis of progeny plants: Progeny plant r72b S1 1 was kept under permanent metribuzin selection (+S) and analyzsed during vernalization (a) and 1 to 3 months later (b). In both cases to fragments of 1 kb and 526 bp were generated indicating the presence of resistant chloroplasts. DNA of progeny plant r72b S 1.2 (–S = no metribuzin selection) was analyzsed in parallel. DNA isolated during vernalization (c) generated two fragments (1 kb and 526 bp) representing resistant chloroplasts. However, only sensitive chloroplasts were present within the same plant one month later indicated by the 1.5 kb fragment.

were constantly watered with 5×10^{-7} M metribuzin kept the tri-resistant chloroplasts during further plant development (Fig. 5).

Our results indicate that both types of chloroplasts can still be present in regenerants and their progeny. In regenerants containing a mixture of both types of chloroplasts few winter-type chloroplasts can still outcompete tri-resistant summer-type chloroplasts during further plant development when no selection pressure is applied. A homogenous population of tri-resistant chloroplasts was necessary to obtain stable tri-resistant winter oilseed rape lines. Meanwhile we succeeded in obtained stable tri-resistant winter oilseed lines and cultivars of double-low quality. Results of greenhouse and preliminary field experiments indicate that volunteer plants of tri-sensitive *B. napus* can be completely eliminated in a stand of double-low oilseed rape by the application of metribuzin.

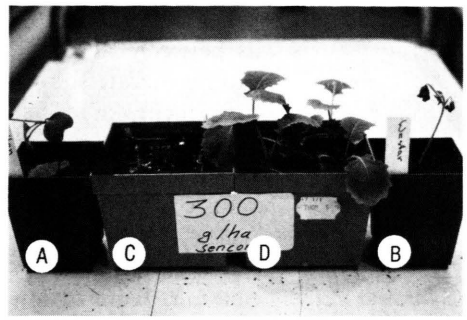

Fig. 6. Seven days after leaf application of 300 g/ha metribuzin. A) Tri-resistant spring oilseed rape variety Triton (control). B) Tri-sensitive spring oilseed rape variety Westar (control). C) Tri-sensitive winter oilseed rape variety Ceres. D) Winter oilseed rape variety Ceres after introduction of tri-resistant chloroplasts of spring oilseed rape Triton.

[1] V. Souza Machado, in: Herbicide resistance in plants (H. Le Baron, J. Gressel, eds.), pp. 185–214, Wiley/New York 1982.
[2] C. J. Arntzen and J. H. Duesing, in: Advances in gene technology, Molecular genetics of plants and animals (K. Downey, R. W. Voellmy, F. Ahmad, J. Schultz, eds.), pp. 273–294, Academic Press, New York 1983.
[3] M. Reith and N. A. Straus, Theor. Appl. Genet. **73,** 357–363 (1987).
[4] W. D. Beversdorf, J. Weiss-Lerman, L. R. Erickson, and V. Souza Machado, Can. J. Genet. Cytol. **22,** 167–172 (1980).
[5] D. Aviv and E. Galun, J. Hered. **76,** 135–136 (1985).
[6] T. Kumashiro and T. Kubo, Jpn. J. Breed. **36,** 39–48 (1986).
[7] J. Immamura, M. W. Saul, and I. Potrykus, Theor. Appl. Genet. **74,** 445–450 (1987).
[8] W. D. Beversdorf and D. J. Hume, Can. J. Plant. Sci. **64,** 1007–1009 (1984).
[9] L. Menczel and K. Wolfe, Plant. Cell. Rep. **3,** 196–198 (1984).
[10] J. E. Thomzik and R. Hain, Theor. Appl. Genet. **76,** 165–171 (1988).
[11] E. M. Southern, J. Mol. Biol. **98,** 503–517 (1975).