Characterization of Triazine-Resistant and -Susceptible Isolines of Canola (Brassica napus L.)

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ABSTRACT

Morphometric, electrophoretic, and immunological procedures were used to probe the structural and physiological differences between triazine-resistant (R) and susceptible (S) isolines of canola (Brassica napus L.). The R biotype exhibited increased grana stacking and decreased amounts of starch compared to the S biotype. Likewise, characters associated with an increase in grana stacking (lower chlorophyll a/b ratio, increased chlorophyll a/b-light-harvesting complex, and relatively lower amounts of the P700 chlorophyll a protein and chloroplast coupling factor) were all observed in the R isoline of canola. Proteins which occur with approximately equal frequency in grana and stroma lamellae (plastocyanin, cutochrome f) or present only in the stroma (ribulose 1,5-bisphosphate carboxylase/oxygenase) were not quantitatively different in the two biotypes. Gross anatomical parameters (volume of epidermis, palisade mesophyll, spongy mesophyll, and air space) were similar in the two isolines. Thus, the triazine-resistance mutation does not confer a shade-type anatomy despite the chloroplast changes that are characteristic of shade biotypes or shade adaptations. These data indicate that the differences in chloroplast structure noted previously in comparisons of non-isonuclear R and S weed biotypes reflect differences in the triazine-resistance factor rather than characters unrelated to triazine resistance.

Results from several laboratories (4, 10, 21) have shown that chloroplasts of R weed biotypes differ structurally and biochemically from S biotypes. Compared with the S biotype, R biotypes have larger and more abundant grana lamellae, less starch, an increase in the percentage of Chl a/b light-harvesting protein, and a lower Chl a/b ratio (4, 10, 21). Similar observations have been made when plants are treated with sublethal levels of herbicides that bind to the 32 kD quinine (herbicide)-binding protein, indicating that the changes in the R biotype are consequences of the slightly lowered PSI1 activity (20), characteristic of these R biotypes (9). Most of these comparisons have centered on differences between chloroplasts of the R and S biotypes. Recently, Holt and Goffner (10) extended these comparisons to the gross anatomy of R and S biotypes of Senecio vulgaris and found striking differences in a number of anatomical parameters, as well as some of the chloroplast differences noted by others. Triazine resistance in higher plants is due to a single amino acid change in the chloroplast-encoded 32 kD quinine-binding protein (7), the binding site for triazine herbicides (18). It is unclear why a mutation in a chloroplast gene would confer anatomical changes, although it is possible that these too are secondary effects of the reduced photosynthetic yield typical of these R biotypes.

None of the studies described above have used isonuclear lines so that, despite the consistency of the chloroplast differences, these differences cannot be unequivocally linked to the mutation conferring triazine resistance, i.e. they may be characteristic of a certain ecotype that was originally selected for some character other than triazine resistance. To eliminate these other nonplas- tome influences, structural and physiological comparisons were made between a pair of near isonuclear lines of canola (Brassica napus L.) that differ in triazine susceptibility in which triazine-resistance was transferred from an R biotype of the weed Brassica campestris L. to the canola by repeated backcrosses (2). Structural and biochemical observations of these two biotypes indicate that, although the chloroplast differences noted previously were observed in these biotypes, no gross anatomical variations were noted, nor were other alterations that could not be explained as 1st or 2nd results of mutation in the 32 kD quinine-binding protein.

MATERIALS AND METHODS

Plant Material. Seeds of the susceptible canola (Brassica napus L.) cultivar 'Tower' and the near isonuclear line with a triazine-resistant plastome (from Brassica campestris) were germinated in a 1:1:1 mix of peat, perlite, and vermiculite and grown in the same mixture under greenhouse conditions for 14 d. At this point, the growth of the two biotypes is quite similar so that secondary developmental differences, as a consequence of lower photosynthetic rate in the R biotype (6, 9), are not yet obvious. Seed was the generous gift of J. Dekker, Iowa State University.

Microscopy. Small (about 1 mm2) pieces of primary leaves were fixed in 6% (w/v) glutaraldehyde in 0.05 M Pipes buffer (pH 7.4) for 2 h at room temperature. A drop of 1% (w/v) Triton X-100 was added to the fixation buffer to enhance penetration of the fixative through the waxy cuticle and to insure uniform immersion of the samples. The samples were washed with two 15 min changes of 0.10 m cacodylate (pH 7.2) at 4°C and then fixed for 2 h at 4°C in 2% (w/v) OsO4 in 0.10 m cacodylate buffer (pH 7.2). After fixation, the samples were washed with distilled H2O and stained en bloc with 2% (w/v) uranyl acetate for 1 h at 4°C. Dehydration and embedding are as described previously (21). Leaf sections were flat embedded to facilitate light microscopic morphometry.

For light microscopy, 0.35 μm sections were mounted on coated slides and stained with 1% (w/v) toluidine blue in 1% (w/v) sodium borate. Specimens were photographed with a Zeiss photomicroscope.2 For electron microscopy, sections with gold-2. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.
silver reflectance were mounted on uncoated 300 mesh copper grids, stained with lead citrate for 15 min and observed with a Zeiss EM 10CR electron microscopy operating at 60 kV. Morphometric analysis of leaf cross sections was according to Parkhurst (16) and of chloroplasts by the procedures of Vaughn and Duke (21).

Electrophoresis and Western Blotting. Primary leaves of the canola biotypes were ground in a mortar and pestle at a 1:5 (tissue:buffer) grinding ratio in the chloroplast isolation medium of Steinback et al. (18). The brei was filtered through one layer of Miracloth and centrifuged at 200g for 5 min to remove starch, whole cells and other debris. Chloroplasts were isolated by recentrifugation of the supernatant at 3000g for 15 min. The chloroplast pellet was either solubilized directly for electrophoresis (as for the thylakoids, below) or was shocked by resuspension in 5 ml of 0.15 M Tris-HCl (pH 6.8) on a Vortex mixer and the thylakoids pelleted at 13,000g for 15 min. The resultant membrane pellets were solubilized in 6% (w/v) LDS in 0.15 M Tris-HCl (pH 6.8) with 5% (w/v) sucrose and 10% (v/v) glycerol at a Chl:LDS ratio of 1:20. The solubilized membranes were centrifuged at 20,000g for 20 min; only a colorless pellet remained after this centrifugation. A portion of the supernatant was used directly for electrophoresis and another sample was heated in a boiling H2O bath for 1 min.

Electrophoresis was performed as described elsewhere (21). Several different percentage acrylamide gels (7.5–15%) were used in the analysis. Gels from the LDS runs were subsequently used for Western blotting, Coomassie blue staining, or heme staining using the procedures of Hayer-Hansen (11). Nine percent acrylamide gels were used for blotting of all of the proteins, except for plastocyanin, where 12.5% gels were used because this protein migrated at or near the front in lower percentage gel systems. Blotting and immunostaining procedures are as described previously (20). Rabbit antiserum to the chloroplast coupling factor complex, plastocyanin, the P700 Chl a protein, and RuBisCo were used as primary antisera at dilutions of 1:350 to 1:2000. The plastocyanin sera recognizes a single protein of 11 to 13 kD in Western blots. The RuBisCo sera recognizes the large subunit of RuBisCo at 55 kD. The P700 Chl a protein recognizes the 100 kD complex in mildly denaturing gels and two proteins ~60 kD that are the apoproteins of this complex in fully denaturing gels (22). The coupling factor sera, although prepared to the entire complex, recognizes only the α and β subunits of canola (both at 59 kD in 9% gels). Gels of whole chloroplast extracts were used for plastocyanin and RuBisCo detection, because plastocyanin is lost from the thylakoids upon washing and RuBisCo is a stromal component. After blotting, heme or Coomassie blue staining, negatives of the gels or blots were scanned in a Bio-Rad densitometer in the transmittance mode for relative quantitation. The results are the average of at least three repetitions of the experiment. The peak heights from the densitometer tracing were analyzed statistically using a student’s t test. The values are expressed in tabular form in terms of ‘%S’ for comparison between the two biotypes.

Chl concentrations on whole leaf samples and in thylakoid preparations were determined according to Hiscox and Israelstam (8). Protein was determined by the Bradford (3) procedure.

Radial Immunodiffusion. Extracts for radial immunodiffusion were obtained by grinding 2 g of primary leaves in 10 ml of TBS (0.10 M Tris [pH 7.6]-0.20 M NaCl) in a chilled mortar and pestle. The extracts were filtered through Miracloth and centrifuged at 20,000g for 15 min. The supernatant was applied without dilution or serial dilutions in TBS (1:1, 1:3, 1:7, 1:15), 5 μl per well, in radial diffusion plates containing antiserum to RuBisCo from spinach. After 2 d of development at room temperature in a saturated incubation chamber, the plates were washed for 2 d in cold (4°C) TBS and for 1 d in cold H2O to remove unprecipitated proteins. The plates were then stained for 30 min in Crowley’s Double Stain (Polysciences Inc., Warrington, PA) and destained in 7% (v/v) acetic acid in H2O with gentle agitation. The destained immunodiffusion plate was then dried with Whatman No. 1 filter paper and stacks of germination paper under a 1 kg weight. Diameters of the wells were read with an immunodiffusion reader (Bio-Rad Inc.) In these plates, 0.25 μg antigen will produce an immunodiffusion ring 1.0 cm in diameter. RuBisCo levels were compared on the basis of μg/g fresh weight or μg/mg protein. Either comparison gave similar results.

RESULTS AND DISCUSSION

Structural Results. The chloroplasts from the two canola biotypes showed a pattern of differences established previously for R and S biotypes of other species (4, 21). The percentage of the volume of the chloroplast of the R biotype occupied by grana was greater than chloroplasts of the S biotypes, whereas the percentage volume occupied by starch was much less (Fig. 1, Table I). Likewise, the number of thylakoids per granum was also larger in the R biotype than in the S (Fig. 2). No grana stacks containing 11 or more thylakoids were found in the S biotype, whereas 7% of the grana of the R biotype had 11 or more thylakoids/granum. Over 70% of the grana of the S biotype were in the smallest size class (2–4 thylakoids/granum). Anatomically, the two biotypes appear to be nearly identical (Fig. 3). By comparing leaf cross-sections of the R and S biotypes morphometrically, no significant differences in the contribution of a single cell type or intercellular space to the internal anatomy of the leaf was noted (Table I). Because these are isonuclear lines (or nearly so), but differ in their plastome, it can be concluded that the mutation in the 32 kD quinone-binding protein conferring triazine resistance has relatively little direct effect on the anatomy of the plant and that the anatomical differences between Senecio biotypes reported by Holt and Goffner (10) are probably due to nuclear differences between the two biotypes. Because the leaves in the Holt and Goffner study were collected at the same

![Fig. 1. Ultrastructure of chloroplasts from primary leaves of the S (A) and R (B) biotypes of canola. Note the prominent grana stacks (g) in the R biotype and the presence of large starch grains (S) in the S biotype. Bar = 1.0 μm.](image-url)
developmental stage, S biotype leaves would be older (and more developed) than R biotype leaves because the S biotype grows more quickly than the R. Therefore, it is possible that some of the differences observed by these authors were due to differences in age rather than due to the mutation or the biotype per se.

**Physiological Comparisons.** Various acrylamide concentrations (7.5–15%) were tried and all reveal a striking qualitative similarity between the thylakoid proteins of the two canola isolines. An example of a 9% acrylamide gel separation, like that used for much of the Western blotting, is shown in Figure 4A. Likewise, none of the proteins that were blotted or heme-stained revealed any mobility difference on these gels. Thus, despite the different parental plastomes in these isonuclear lines, no obvious changes in polypeptide profiles are observed. Quantitatively, several differences are obvious when equal concentrations (depending on protein load, initial grinding ratio, or Chl/lane) are the basis of comparison. All of the data presented here are comparisons of equal protein loads on the lanes of the gels or in the immunodiffusion wells.

Previously, we had shown that R biotypes of three different species had a larger percentage of total Chl present in the LHC than the S biotype (21). This same conclusion can be obtained by comparisons of the two canola isolines, either by comparisons of the densitometry of the Chl containing band at ~29 kD of unstained gels or comparing the staining of the LHC protein bands (21) after Coomassie blue staining (Table II). The LHC is found predominantly in the grana lamellae and the relative increase observed in LHC in the R biotype is consistent with the increase in grana stacking determined morphometrically. The 'state' of Q is also related to the extent of phosphorylation of the LHC (12) and, because PSI activity is altered in the R biotype, more of the LHC may be phosphorylated (and hence present in the stroma lamellae) in the S biotype. This would explain the relatively small increase in LHC (Table II) in the R biotype compared with the relatively large increase in grana stacking (Figs. 1 and 2).

Although no absolute protein quantitative determinations can be made from the Western blots unless known amounts of purified antigen have been added to the gel for calibration, the trends in thylakoid protein concentrations predicted from ultrastructural analysis are also reflected in the relative quantitative differences found in Western blots (Table II). By diluting the extracts up to 4-fold, a linear relationship between protein applied and immune reaction was noted, indicating that, in the concentration range around which the comparisons are based the differences reflect relative antigen concentration differences. Similar relative antigen level determinations were made by Mayfield and Taylor (15) in their examination of chloroplast polypeptide biosynthesis in maize leaves. Blots of the gels probed with antisera to the chloroplast coupling factor, an exclusively unstacked thylakoid constituent, and the P700 Chl a protein, a protein present relatively more in the unstacked thylakoids than the stacked (19), gave stronger immunoreactions with the S biotypes than the R (Fig. 4B and D). The α and β subunits of the canola coupling factor are not well separated in a 9% gel system and these two polypeptides appear as almost a single band after blotting. Cyt b5/6 complex and plastocyanin are dis-

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**Table 1. Morphometric Comparisons of Chloroplast and Anatomical Parameters in R and S Biotypes of Canola**

<table>
<thead>
<tr>
<th>Character</th>
<th>R (% volume)</th>
<th>S (% volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grana lamellae</td>
<td>21.88*</td>
<td>15.78</td>
</tr>
<tr>
<td>Stroma lamellae</td>
<td>7.07</td>
<td>6.86</td>
</tr>
<tr>
<td>Starch</td>
<td>0.23</td>
<td>16.70</td>
</tr>
<tr>
<td>Stroma (includes plastoglobuli)</td>
<td>70.82*</td>
<td>60.61</td>
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</tbody>
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*Significantly different from the S biotype at the 0.05 level of confidence.

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**Fig. 2.** Distribution of number of thylakoids per granum in the S (clear) and R (hatched) biotypes of canola. A, greater percentage of the grana in the S biotype are of small (2–4) thylakoids/granum group. Data from these same chloroplasts were used in morphometric determinations of relative volume (Table I).

**Fig. 3.** Light micrograph of leaf cross-sections of the R (A) and S (B) biotypes of canola. The anatomical arrangement and relative distribution of tissue volumes is similar in the two biotypes. Arrows point to guard cells. e = epidermis; p = palisade mesophyll; s = spongy mesophyll (x400).
tributed throughout the thylakoids (1) and equally intense bands from either heme staining of Cyt f or immunoblotting for plastocyanin were obtained (Table II and Fig. 4, C and E). RuBisCo, which Holt and Goffner (10) reported to be increased in activity in the R biotype of Senecio, was present in relatively equal quantities based upon either Western blots (not shown) or radial immunodiffusion (Table II). Radial immunodiffusion, unlike Western blotting, can be a direct measurement of the quantity of RuBisCo in the R and S isolines. The RuBisCo concentration in these two biotypes is virtually equal, as determined by this procedure (Table II and Fig. 5). Thus, the results of Holt and Goffner (10) on RuBisCo activity differences between R and S Senecio biotypes is probably due to factors other than alterations in the 32 kD protein.

Gressel and Ben-Sinaï (6) compared the relative efficiency of two isolines of canola (although different from the isolines described in this report) and found them greatly different in several parameters related to growth and yield, the R biotype being much less fit. Similar sorts of data were obtained by comparisons of competition (5), yield, and photosynthetic ability (9) between nonisonuclear weed biotypes that differed in triazine resistance. This report documents that chloroplast traits associated with the change to triazine resistance described previously in nonisonuclear lines (4, 10, 21) are also found in the isonuclear lines of canola.

Although the data here do not support the idea that gross anatomical changes are primary or secondary consequences of triazine-resistance mutations, they made shed some light on why the biotypes studied by Holt and Goffner (10) did differ in anatomical parameters. The Senecio biotypes are naturally occurring variants and it is likely that the R biotype of this species occurred at low frequencies in the population even before the application of triazine herbicides (13, 14). For example, certain habitats contain naturally occurring R biotypes even though triazines have not been used in these areas. These R biotypes may take advantage of the altered, more cold-tolerant, lipid constitution and are thus able to germinate earlier in the season with less risk of cold damage (17). Similarly, Holt and Goffner (10) describe a leaf anatomy for the R biotype of Senecio that is essentially a ‘shade-type’ variation of the leaf of the S biotype of this species a variation that may take advantage of the relative increase in LHC and grana found in all of the R biotypes studied to date (4, 21).

**LITERATURE CITED**

TRIAZINE-RESISTANT AND -SUSCEPTIBLE CANOLA (BRASSICA NAPUS L.)

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