Common functional elements of *Drosophila melanogaster* seminal peptides involved in reproduction of *Drosophila melanogaster* and *Helicoverpa armigera* females

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Received 31 October 1999; received in revised form 31 December 1999; accepted 25 January 2000

Abstract

Sex peptide (SP) and *Ductus ejaculatorius* peptide (Dup) 99B are synthesized in the retrogonadal complex of adult male *Drosophila melanogaster*, and are transferred in the male seminal fluid to the female genital tract during mating. They have been sequenced and shown to exhibit a high degree of homology in the C-terminal region. Both affect subsequent mating and oviposition by female *D. melanogaster*. SP also increases in vitro juvenile hormone (JH) biosynthesis in excised corpora allata (CA) of *D. melanogaster* and *Helicoverpa armigera*. We herein report that the partial C-terminal peptides SP 8–36 and SP 21–36 of *D. melanogaster*, and the truncated N-terminal SP 6–20 do not stimulate JH biosynthesis in vitro in CA of both species. Both of these C-terminal peptides reduce JH-III biosynthesis significantly. Dup99B, with no appreciable homology to SP in the N-terminal region, similarly lacks an effect on JH production by *H. armigera* CA. In contrast, the N-terminal peptides — SP 1–11 and SP 1–22 — do significantly activate JH biosynthesis of both species in vitro. We conclude that the first five N-terminal amino acid residues at the least, are essential for allatal stimulation in these disparate insect species. We have previously shown that the full-length SP 1–36 depresses pheromone biosynthesis in *H. armigera* in vivo and in vitro. We now show that full-length Dup99B and the C-terminal partial sequence SP 8–36 at low concentrations strongly depress (in the range of 90% inhibition) PBAN-stimulated pheromone biosynthesis of *H. armigera*. In addition, the N-terminal peptide SP 1–22, the shorter N-terminal peptide SP 1–11 and the truncated N-terminal SP 6–20 strongly inhibit pheromone biosynthesis at higher concentrations. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Sex peptide; Dup99B; JH biosynthesis; PBAN; Pheromone production; Calling

1. Introduction

Reproductive maturation in female adult insects is composed of developmental and behavioral components. In many cases, newly-eclosed adult female insects bear underdeveloped ovaries. Ovarial maturation then requires yolk protein synthesis and its subsequent uptake into the developing oocytes. Both synthesis and uptake are apparently regulated in Lepidoptera by JH alone. This is concluded from studies of several noctuid moths belonging to the genera *Helicoverpa*, *Heliothis* and *Pseudaletia* (Ramaswamy et al., 1990; Ramaswamy and Cohen, 1991; Satyanarayana et al., 1991, 1992; Cusson et al., 1994; Zeng et al., 1996 and also summarized by Ramaswamy et al., 1997). Following ovarial maturation, reproductively mature female moths synthesize and release sex pheromones, signaling their receptivity to mating. Pheromone production in several moths is under neurohormonal control by Pheromone Biosynthesis Activating Neurohormone (PBAN) (Raina, 1993; Raina and Rafaeli, 1995). A correlation between the biosynthetic maturation of corpora allata (CA) and the development of responsiveness of *H. armigera* sex pheromone glands to PBAN has recently been reported (Fan et al., 1999b). Juvenile hormone (JH) II, which is the predominant
homolog produced by the CA in *H. armigera*, may play a primer role in the initiation of sex pheromone production by newly emerged adult females.

During mating, seminal fluid, containing a variety of peptides and proteins derived from the male retrogonadal complex, is transferred to the female genital tract (Chen, 1984, 1991). In *D. melanogaster*, post-mating responses have been shown hitherto to be induced by three components: sex peptide (SP), derived from the male accessory glands; Dup99B, derived from the male ejaculatory duct; and a high molecular weight protein, Acp28Aa. SP and Dup99B induce female non-receptivity and ovulation by female *D. melanogaster* (Chen et al., 1988; Saudan, Hauck and Kubli, unpublished). Acp28Aa stimulates oviposition (Herndon and Wolfrn, 1995). SP and Dup99B have been sequenced, synthesized and characterized. They exhibit a high degree of homology in the C-terminal region (Chen et al., 1988; Schmidt et al., 1993; Saudan, Hauck and Kubli, unpublished). The sequence of Acp28Aa has been determined. It has a region of sequence similarity to the egg-laying hormone of *Aplysia* (Herndon and Wolfrn, 1995) and is proteolytically processed in the female genital tract (Park and Wolfrn, 1995). In accessory glands of male *H. zea* moths, a 57 amino acid peptide has been identified and proposed as a suppressive factor of female sex pheromone production (Kingan et al., 1995).

We have previously shown that *D. melanogaster* SP stimulates the biosynthesis of juvenile hormone (JH) in vitro by the corpora allata (CA) of the adult *D. melanogaster* female and of the noctuid moth *Helicoverpa armigera* female (Fan et al., 1999a). SP also activates vitellogenin uptake by maturing oocytes in *D. melanogaster* (Soller et al., 1999). Mated females bear more oocytes of the vitellogenic stage 10 in their ovaries than can be found in virgin females. Injection of SP into virgin females elicits similar oocyte maturation, as does application of the juvenile hormone analogue methoprene. Taken together, these observations appear to be consistent with a primer effect of SP on allatal maturation and of JH-mediated oocyte maturation.

This present report deals with the functional elements of the *D. melanogaster* SP and Dup99B, requisite for regulation of allatal activity in these two disparate insect species and for pheromone production and calling behavior in *H. armigera*.

2. Materials and methods

2.1. Insect culture

Larvae of the noctuid moth *H. armigera* were reared on an artificial diet (Rafaeli and Soroker, 1989) in the laboratory under a constant temperature of 26°C, 80% relative humidity and a 14 h/10 h (light/dark) non-diapause photoperiod. Pupae were sexed and males and females were allowed to emerge separately. Emerging moths were fed with 10% sugar water. *D. melanogaster* wild-type Canton-S strain was reared on a standard diet at 25°C. Adults were collected at eclosion, and separated by sex.

2.2. *D. melanogaster* male retrogonadal synthetic peptides and partial sequences

The partial SP sequences of the *D. melanogaster* male retrogonadal peptides evaluated in this present study were synthesized in the facilities of the Zoological Institute, University of Zurich-Irchel. They fall into two categories: those with truncated N-terminus, and those with intact N-terminus, but lacking the major part of the C-terminal residues (Fig. 1).

2.3. JH biosynthesis — in vitro radiochemical assay (RCA)

The RCA for JH biosynthesis in vitro of adult virgin female *H. armigera* CA was adapted from Pratt and Tobe (1974) as described by Fan et al. (1999a,b). Briefly, pairs of excised CA were preincubated for 1 h in methionine-free physiological medium supplemented with Ficoll 400TM and a JH-esterase inhibitor (Hammock et al., 1984), and incubated for an additional 2 h in medium supplemented with 5 µCi of L-[3H-methyl]-methionine (specific activity of 80 Ci/mmol, final concentration in the medium 1.2 µM), in the absence or presence of synthetic *D. melanogaster* peptides or partial sequences (Fig. 1). At the end of the incubation period, the medium and tissue together were extracted with 150 µl ice-cold hexane. A 50 µl aliquot of hexane-soluble extract was evaporated under N2 and the radiolabeled methyl moiety incorporated into total hexane-soluble extract was counted using a β-counter (LKB).

The RCA for JH biosynthesis in vitro of adult virgin female *D. melanogaster* CA was modified from Pratt and Tobe (1974) and is based on the incorporation of the methyl moiety of [3H-methyl]-methionine into JHB3 (Richard et al., 1989; Altaratz et al., 1991). In this case, preincubation was not performed, and the excised *D. melanogaster* CA were incubated for 2–4 h.

2.4. TLC for separation of JHB3

Aliquots of extracts were chromatographed on silica gel plates (Polygram SIL G/UV254; Macherey-Nagel). JHB3 was separated from JH-III and other JH homologs by development of the plates in hexane:ethyl acetate (2:1), identified by short UV fluorescence of marker compounds (Rf=0.37) and counted using a β-counter. Values of incorporation are expressed as cpm/h/gland JHB3 biosynthesis±standard error.
2.5. Reversed phase C\textsubscript{18} HPLC

Chromatography was performed as previously described (Fan et al., 1999b). Briefly, evaporated extracts of \textit{H. armigera} CA incubation media were resuspended in 40% acetonitrile and chromatographed on a 5 mm LiChrospher Merck C\textsubscript{18} reversed phase HPLC column (Darmstadt, Germany) (100 Å pore, diameter: 4 mm×12.5 cm) using a linear gradient of acetonitrile/water (40–80%) at a flow rate of 1 ml/min for 40 min. Markers were monitored in the eluant at 220 nm. One ml fractions were collected and \textsuperscript{3}H-labeled compounds counted using a \textbeta-counter (LKB). Synthetic JH-I and JH-II were kindly provided by Dr Zdenek Wimmer, of the Institute of Organic Chemistry and Biochemistry, Prague, The Czech Republic. JH-III was purchased from Sigma. All three non-radioactive JHs were used as standards to identify radioactively-labeled JHs produced in vitro by \textit{H. armigera} CA. The major JH homolog produced in vitro by \textit{H. armigera} CA is JH-II (Fan et al., 1999b). Results are herein reported as femtomol JH-II produced per hour by pairs of female moth CA.

2.6. In vitro bioassay of pheromone production

A radiochemical bioassay was used to monitor de novo pheromone production according to the method of Rafaeli and Gileadi (1995). Briefly, intersegmental membranes (hereafter referred to as pheromone glands) between the eighth and ninth abdominal segments were removed. After 1 h preincubation in Pipes buffered incubation medium, pheromone glands were dried on tissue paper and then transferred individually to 10 ml incubation medium containing 0.25 \textmu Ci \textsuperscript{1-\textsuperscript{14}C}-acetate (56 mCi/mmmole, NEN, Boston, USA) in the presence or absence of \textit{Hez}-PBAN (Peninsula Labs., Belmont, CA, USA) and in the presence or absence of synthetic \textit{D. melanogaster} regulatory peptides or partial sequences (Fig. 1). Incubations were performed for 3 h at room temperature. In order to measure the incorporation of \textsuperscript{1-\textsuperscript{14}C}-acetate, glands were extracted in 200 \textmu l hexane for 0.5 h at room temperature. Radioactivity in a 100 \textmu l aliquot of the upper hexane phase was determined using a \textbeta-counter. Based on TLC and GC separations, we have previously shown that the total incorporation levels depict relative levels of incorporation into the pheromone component where the majority of the label was found to co-elute with the main pheromone component of \textit{H. armigera}, Z11-hexadecenal (Rafaeli and Gileadi, 1995).

2.7. In vivo pheromone production and calling behavior

In vivo sex pheromone production by female moths was determined in 2-day-old females (D2) which were decapitated during the photophase of D2 and subsequently maintained for an additional 24 h, after which they were injected with either physiological saline (control) or 5 pmol/moth PBAN in saline. Full length SP, SP partial sequences, or Dup99B were injected 1 h later. Ovipositor tips were removed 2 h after injection and extracted for 30 min in hexane, containing 25 ng tridecanyl acetate (Sigma) as internal standard. The hexane extract was concentrated to 1–2 \textmu l final volume under a slow stream of \textit{N \textsubscript{2}} and chromatographed on a Carlo Erba GC-6000 gas chromatographic system (Italy) using a 30 m SE-54 fused silica capillary column (internal diameter 0.32 mm) (Alltech, USA). The results were analyzed using a Barspec Chrom-A-Set (Israel). The following conditions were used: initial temperature of 120°C was held for 0.4 min then increased to 270°C at 10°C/min, and kept for 15 min at the final temperature. The detector temperature was held at 300°C, column inlet at 280°C. Helium was used as a carrier at a flow pressure of 22 psi. Z11-hexadecenal, the main pheromone product of \textit{H. armigera} (Dunkelblum et al.,
1980), was quantified using the internal standard quantification methods as described previously (Soroker and Rafaeli, 1989; Rafaeli, 1994).

“Calling” in *H. armigera* was defined as the extrusion of the ovipositor and exposure of pheromone-producing abdominal integumental pheromone gland and was monitored by observations in a dark room, using a dim red light as reported previously (Rafaeli and Soroker, 1989).

2.8. Statistics

Differences between treatments were compared by ANOVA.

3. Results

3.1. Effect of Dup99B, SP and partial sequences on JH biosynthesis in vitro

Full-length *D. melanogaster* SP1–36 stimulates JH biosynthesis of CA excised from *D. melanogaster* (Moshitzky et al., 1996) and similarly stimulates the CA of the noctuid moth *H. armigera* (Fan et al., 1999a). In contrast, truncated N-terminal partial sequences of SP, lacking the first five amino acid residues, do not stimulate in the *D. melanogaster* CA system in vitro (Fig. 2).

**Fig. 2.** The effects of SP, and partial SP sequence peptides on CA activity of *D. melanogaster*. A: JHB III biosynthesis; B: JH-III biosynthesis. Values with different letters indicate significant differences (*P*<0.05). Numbers below the error bars indicate the number of replicates.

SP partial sequences, containing only the full C-terminal, are similarly inactive. This suggests that the first five amino acid residues of SP, at the least, are essential for allatal activation. The longer N-terminal peptide with 22 amino acid residues is more active than the shorter N-terminal peptide with the first 11 N-terminal amino acid residues.

JHB3 normally comprises about 95% of the total JH (Richard et al., 1989). We observed differential effects on JHB3 and JH-III, the two *D. melanogaster* JH homologs produced in vitro: the full-length SP and SP1–22 significantly stimulated JHB3 synthesis but had no effect on JH-III synthesis. The truncated N-terminal sequence SP6–20 had no effect on either JHB3, or JH-III biosynthesis. The shorter N-terminal SP1–11 slightly stimulated JHB3 synthesis but had no effect on JH-III synthesis. The C-terminal sequences SP8–36 and SP21–36 had no significant effect on JHB3 synthesis, but did significantly inhibit the synthesis of JH-III.

Similarly, peptides lacking the N-terminal of SP had no effect on JH-II biosynthesis by excised *H. armigera* CA (Table 1). The N-terminal peptide SP1–22 stimulated at 10 pmol per incubation medium, whereas a 10-fold higher titer (100 pmol per incubation medium) of the shorter N-terminal sequence SP1–11 was necessary for eliciting significant stimulation. The full length Dup99B, with high homology to SP in the C-terminal region, but no real homology in the N-terminal region, was also inefficient in stimulating JH-II biosynthesis, confirming the hypothesis that the N-terminal of SP may be necessary for allatal stimulation in the *H. armigera* system as well.

3.2. Inhibition of H. armigera female sex pheromone biosynthesis by SP and partial sequences in vitro

We have previously shown that full length SP depresses pheromone biosynthesis in *H. armigera* in vivo and in vitro (Fan et al., 1999a). We herein examined the relative contribution of SP partial sequences to the inhibition of PBAN-stimulated pheromone production in vitro.

The C-terminal partial sequence SP8–36, at low titers, strongly depressed about 80–90% of PBAN-stimulated pheromone biosynthesis of *H. armigera*. Other partial peptides depressed PBAN-stimulated pheromone biosynthesis to different extents (Fig. 3). To attain similar levels of inhibition (72.8±5.7%; *n*=15) caused by 1 pmole of SP8–36, 10-fold higher amounts (10 pmoles) of the short N-terminal peptide SP1–11 were found to be necessary, reaching levels of 78.8±5.9% (*n*=8) inhibition (data not shown).

3.3. Pheromone biosynthesis and calling in decapitated female moths

Dup99B, SP8–36 and SP21–36 strongly inhibited sex pheromone biosynthesis after PBAN-injection in decapi-
Table 1
The effect of Dup99B, SP and partial sequences on in vitro JH-II biosynthesis by excised female adult *H. armigera* CA

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Treatment</th>
<th>2 h incubation</th>
<th>3 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>26,526±2245 (n=10) a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP (10 pmol)</td>
<td>41,398±2470 (n=9) b</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dup99B (10 pmol)</td>
<td>27,340±2413 (n=8) a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{21-36} (10 pmol)</td>
<td>23,539±5151 (n=4) a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{8-36} (10 pmol)</td>
<td>28,860±1840 (n=9) a</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>28,381±3113 (n=8) A</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{6-26} (10 pmol)</td>
<td>28,068±1780 (n=8) A</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>Control</td>
<td>32,515±2396 (n=9) B</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{1-11} (10 pmol)</td>
<td>30,136±3896 (n=9) B</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{1-11} (100 pmol)</td>
<td>42,259±1491 (n=9) C</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>Control</td>
<td>26,012±5009 (n=7) D</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{1-22} (10 pmol)</td>
<td>35,016±3580 (n=8) E</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SP_{1-22} (100 pmol)</td>
<td>41,359±2100 (n=9) F</td>
<td>-</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the number of replicates.
* Values with different letters indicate significant differences for each experiment separately (*P*≤0.05).

Fig. 3. The inhibition of *H. armigera* pheromone production in vitro as a result of SP, Dup99B and partial SP sequence peptides. Values with different letters indicate statistically significant differences (*P*≤0.05).

4. Discussion

The immediate post-mating response of gravid females of *D. melanogaster* and *H. armigera* to mating is to terminate reproductive behavior. This is done in *D. melanogaster* by actively rejecting male courtship. In *H. armigera* females, cessation of pheromone production and of active calling serves the same purpose of temporarily preventing subsequent mating. Behavioral non-receptivity can be induced in *D. melanogaster* by SP and
Dup99B, both bioactive seminal fluid peptides (Chen et al., 1988; Schmidt et al., 1993; Saudan, Hauck and Kubli, unpublished).

The results obtained with partial sequences with intact N-terminus of SP, and in contrast, with truncated N-terminal partial sequences, are consistent with the hypothesis that the N-terminus is responsible for CA activation in Drosophila and Helicoverpa, leading to oogenesis and perhaps oviposition in Drosophila (Soller et al., 1999). In Helicoverpa, the C-terminus is responsible for control of pheromone production (regulating reproductive behavior) and in Drosophila for reduction of receptivity and increase of oviposition. This conclusion is strongly supported by the lack of allatal stimulation obtained with Dup99B, which is very dissimilar to SP in its N-terminal amino acid residues (see Fig. 1) on Drosophila CA (Moshitzky, Kubli and Applebaum, unpublished and on Helicoverpa CA (Table 1)).

The physiological significance of inhibition of D. melanogaster JH-III biosynthesis by the short and long C-terminals (SP21–36 and SP8–36) is unclear at this time, as no distinctive function has hitherto been attributed to this minor component, nor for that matter to JHB3, the major homolog produced by D. melanogaster CA. In principle, partially degraded peptides could serve to suppress the initial physiological action of the full-length peptide, as part of a regulatory system for terminating hormonal effects subsequent to initial activation. Endogenous proteolytic processing of SP in D. melanogaster is not examined in the present study.

Pheromone production in noctuid moths is dependent on the action of pheromone biosynthesis activating neuropeptide (PBAN), which regulates the synthesis and release of sex pheromone from abdominal integumental glands (Raina, 1993; Raina and Rafaeli, 1995). PBAN has been shown to act directly on the pheromone producing glands in many moth species (Soroker and Rafaeli, 1989; Rafaeli et al., 1990; Arima et al., 1991; Jurenka et al., 1991; Jacquin et al., 1994; Matsumoto et al., 1995; Ramaswamy et al., 1995). The action of D. melanogaster SP and partial derivatives in vitro suggests that the pheromone gland is a direct target for putative moth SP-like peptides. It is inappropriate to draw physiological conclusions from cross-reactivity of a D. melanogaster partial peptide in the heterologous H. armigera system. Nevertheless, the 10-fold higher concentration required for the shorter N-terminal sequence SP1–11 to depress PBAN-stimulated activity suggests that at least pharmacologically, its effect is less pronounced.

The differential effects of D. melanogaster SP, Dup99B and partial peptides on in vitro pheromone production by pheromonal glands of H. armigera suggest the existence of multiple binding sites of the presumptive PBAN-receptor in the pheromone glands towards several regions of SP. The possibility of more than one population of receptors or receptor-sites has been previously suggested on the basis of structure-function and dose-response studies (Raina and Kempe, 1992; Rafaeli, 1994; Rafaeli and Gileadi, 1996).

The maximal inhibition of H. armigera pheromone biosynthesis in vivo in decapitated photophase females by Dup99B and SP C-terminal partial sequences is less than that obtained in vitro. This could simply be due to the fact that the peptides are diluted when injected into the hemolymph, and may also be more accessible to proteolytic degradation. These peptides are from a heterologous system; factors endogenous to the male moth, unidentified as yet, may act in the regulation of pheromone production in H. armigera.

Calling behavior, a major characteristic of female receptivity in H. armigera, expedites the dispersal of volatile sex pheromones after secretion by the gland tissue. It is not terminated after SP injection in vivo, although pheromone production is significantly reduced (Fan et al., 1999b). Dup99B, with high sequence homology to SP in the C-terminal region, also has no effect on calling behavior.

Pheromone biosynthesis and its dispersal by calling are normally coordinated, but are apparently distinct activities. Pheromone production has been shown to be regulated endogenously in H. armigera by PBAN, and is an exocrine process. Calling is a behavioral trait, unaffected by PBAN. Indeed, photophase females synthesize pheromone if injected with PBAN, reaching titres

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### Table 2
Effect of SP partial-sequence peptides (10 pmol/female) on pheromone production in vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Inhibition of mean pheromone production by decapitated females</th>
<th>% Inhibition of mean pheromone production by females during the scotophase</th>
<th>% Females calling during the scotophase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>63±24.0 (n=15)</td>
</tr>
<tr>
<td>SP8–36</td>
<td>55.5±11.1 (n=10)</td>
<td>73.3±7.7 (n=12)</td>
<td>Not tested</td>
</tr>
<tr>
<td>SP21–36</td>
<td>56.2±7.9 (n=16)</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>Dup99B</td>
<td>56.7±10.6 (n=9)</td>
<td>63.1±8.8 (n=17)</td>
<td>58±18.5 (n=20)</td>
</tr>
</tbody>
</table>

a Stimulated to synthesize pheromone by an injection of 5 pmol PBAN/female: mean production of main pheromone component (Z11-hexadecenal) by decapitated females amounted to 5.1±7 ng/female (n=31).
b Mean production of Z11-hexadecenal during the scotophase amounted to 35.7±6.7 ng/female (n=22).
attained during scotophase (Rafaeli et al., 1997), but do not exhibit calling behavior. Similarly, octopamine can inhibit pheromone biosynthesis (Rafaeli et al., 1997) but calling behavior is unaffected.

We are presently studying retrogonadal secretory peptides of *H. armigera* which affect post-mating female receptivity. By distinguishing between these two functions with the heterologous peptides SP and Dup99B, we hope to define moth-derived retrogonadal peptides which depress calling behavior and pheromone production.

**Acknowledgements**

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG) to SWA, from the Swiss National Science Foundation (grant no. 31-52440.97) and Hescheler-Stiftung to EK, and the Israel Academy of Sciences and Humanities to AR. This contribution is acknowledged as No. 418 (1999 series) of AR from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

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