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Selection for spontaneous tomato haploids using a conditional lethal marker

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Abstract. We describe a method for the isolation of spontaneous haploid tomato plants from greenhouse-grown seedlings obtained from crosses involving a transgenic parental line in which a counter-selectionable chimeric gene has been introduced. Transgenic seeds transformed with the *aux2* gene, a gene of *Agrobacterium rhizogenes* that transforms naphthalene acetamide (NAM) into naphthalene acetic acid (NAA), did not develop roots in the presence of NAM, whereas wild-type tomato seeds developed a normal rooting system in its presence. Transgenic plants homozygous for *aux2* (cv 'UC82b') were used to pollinate male-sterile (*ms322*) tomato plants (cv 'Apedice'). Using NAM as a toxic substrate to kill heterozygous diploid plants carrying *aux2*, we selected for three maternal haploid plants resulting from the development of the female nucleus without fertilization. Maternal haploid selection using the *aux2* marker was less efficient than the visual screening of haploid plants displaying recessive morphological markers of the female parent, but provided evidence for the feasibility of haploid selection in species for which no morphological markers are available.

Key words: Tomato – Haploid – Selectable marker – Indole acetamide/naphthalene acetamide

Introduction

Haploid plants are useful for the creation of inbred lines (Gallais 1986; Fouroughi-Wehr and Wenzel 1990) and for the selection of recessive mutations at the cell level (Grafe et al. 1986; Marion-Poll et al. 1988). While tomato haploid plants can be obtained by anther culture (Greshoff and Doy 1972; Sharp et al. 1972; Zagorska et al. 1982; Zamir et al. 1980, 1981), this method is not reproducible, and in most cases leads to diploid and polyploid plants regenerating from microspore and anther-wall cells. Tomato haploids can also be recovered by in vivo gynogenesis (Ecochard et al. 1969; Al Yasiri et Rogers 1971; Koornneef et al. 1989), and by this method several gynogenetic haploid plants were obtained from different genotypes (Koornneef et al. 1989). The most serious limitation to the selection of spontaneous haploid plants is the low frequency at which haploid plants occur, about 2×10^{-4} (Hille et al. 1989). Screening for spontaneous haploids has been improved by the utilization of mutant lines carrying recessive morphological markers. The restriction to lines containing such recessive markers, however, limits the method to species for which these markers are available. Moreover, screening a large number of plantlets by such a method is a fastidious enterprise. The use of a selectable marker at an early stage of development would greatly simplify the recovery of haploid plants. A dominant lethal gene, *Rac-*, present in a rootless *Nicotiana tabacum* mutant (Muller et al. 1985), could be used for haploid selection at the seedling level (Pelletier et al. 1987). Diploid plants resulting from a cross between a homozygous mutant *Rac-* used as female and a wild-type plant used as male did not develop roots and subsequently died. Normal plants were obtained at a low frequency (10^{-4}) and were shown to be androgenetic haploids. The reciprocal cross led to the selection of gynogenetic haploids. However, this method can not be transposed to tomato because such *Rac-* mutants are not available in this species.

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The introduction into tomato by gene transfer of a lethal conditional marker whose expression occurs early at the seedling stage could constitute a valuable alternative. Two genes, *tms2* and *aux2*, present in the T-DNA of *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively, encode an indolacetamide hydrolase (iaaH) that converts indole-3-acetamide (IAM) of naphtalene acetamide (NAM) into indole-3 acetic acid (IAA) and naphtalene acetic acid (NAA). Transgenic tobacco and petunia plants harboring *tms2* were able to transfrom IAM/NAM into IAA/NAA and were more sensitive to IAM/NAM than wild-type plants (Budar et al. 1986; Klee et al. 1987). This sensitivity was characterized by an inhibition of root development. In preliminary germination tests wild-type tomato seeds were germinated in the presence of IAM/NAM: no morphological changes occurred. The effects of IAM/NAM on the germination of *aux2* transgenic tomato plants were then studied. In the present report we show that *aux2* can be used as a counter-selectable marker for the selection of tomato haploid plants at the seedling level under greenhouse conditions.

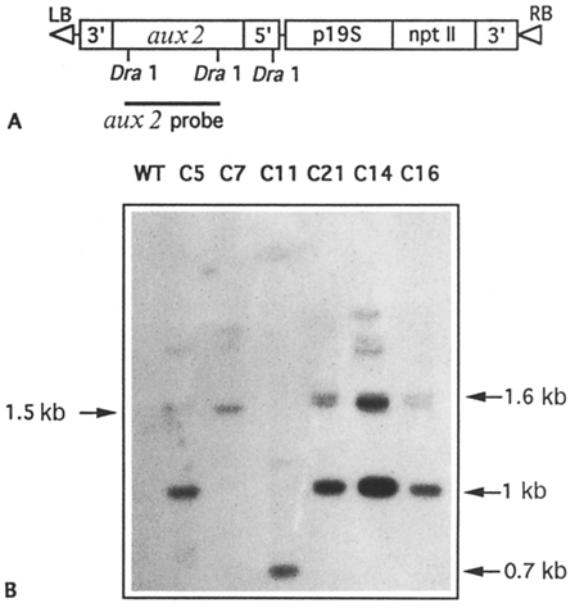

Fig. 1. **A** Physical map of the T-DNA of plasmid pCC23. The pRiA4 *Acc*I-*Bam*HI fragment containing *aux2* (nt 1156–3236 of the 6-kb *Sal*I fragment of the TR-DNA) was cloned beside a chimeric p19S-npt II-3′ CaMV gene between left (*LB*) and right (*RB*) borders of the T-DNA of the binary vector pMRK62. **B** Detection of *aux2* sequences inserted in the genome of tomato transformants. Genomic DNA of an untransformed plant (*WT*) and six transformants (C5, C7, C11, C21, C14, C16) was cut by *Dra*I and hybridized with an *aux2* internal 1-kb *Dra*I fragment

Materials and methods

Plant material

Wild-type tomato (cv 'UC82b') and a male-sterile mutant (*ms32*) homozygous for two recessive morphological mutations, without anthocyanine (a_w) and potato leaf (*c*) (cv 'Apedice'), were provided by the Station d'Amélioration des plantes maraichères (INRA, Monfavet).

Transformation vector

The *Acc*I-*Bam*HI fragment (nucleotide 1156–3236) of the *Sal*I 6-kb fragment of the pRiA4 TR-DNA (Camilleri and Jouanin 1991) contains the entire *aux2* gene with its promoter and polyadenylation signal. This *Acc*I-*Bam*HI fragment was blunt-ended by treatment with the Klenow polymerase and cloned in the blunt-ended *Xba*I site of the binary vector pMRK62 (Vilaine et al. 1987) to create plasmid pCC23 (Fig. 1A). This plasmid was introduced in the *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz and Schell 1986) according to Holsters et al. (1978), and the resulting strain was used for plant transformation.

Plant transformation

Our procedure for plant transformation was essentially that described by Fillati et al. (1987), except for the following modifications. Eight- to ten-day-old 'UC82b' cotyledons of asceptically germinated seedlings were used for co-cultivation with *Agrobacterium*. Medium for preculture and co-cultivation with *Agrobacterium* consisted of Murashige and Skoog (MS) basal salts (1962), Morel and Wetmore's vitamins (1951), 30 g/l sucrose, 7 g/l agar (Biomar), and 0.1 mg/l NAA, 1 mg/l BA. Medium for shoot induction from the tomato explants consisted of Murashige and Skoog's basal salts (1962), Linsmaier and Skoog's vitamins (1965), 0.5 g/l polyvinylpyrolidone 40 000 (Prolabo), 100 mg/l kanamycin, 400 mg/l cefotaxime, 30 g/l glucose, 5 g/l agarose (Litex), and 0.5 mg/l IAA and zeatin. Kanamycin-resistant shoots were transferred to the root-inducing medium [MS/2 salts, Morel and Wetmore's vitamins (1951), 30 g/l sucrose, 7 g/l agar, and 100 mg/l kanamycin]. The transformed plants were examined for the presence of *aux2* by molecular hybridization with an *aux2* probe (Southern 1975).

R0 transformants containing the *aux2* gene were transferred in the greenhouse and selfed. R1 and R2 seedlings were sown in vitro on tomato germination medium [MS/2 salts, Morel and Wetmore vitamins (1951), 10 g/l sucrose, 7g/l agar] supplemented with the appropriate concentration of IAM, NAM or NAA, or 100 mg/l kanamycin. The NAM (Sigma) was purified by first being dissolved in 100% ethanol. The solution was evaporated to one-third of the original volume, and the NAM was crystallized at 4 °C. The process was then repeated. IAM and purified NAM were dissolved in dimethylsulfoxide (DMSO) in order to prepare concentrated solutions of 10 and 20 mg/ml.

Crossing

Flowers of male-sterile (*ms32*) greenhouse-grown plants (cv 'Apedice') were pollinated with pollen from plants homozygous for the *aux2* transgene (C21/C21) transgenic line derived from cv 'UC82b'. The resulting plants were grown to maturity in the greenhouse, and heterozygous (C21/+) seeds from 1400 fruits were extracted by an overnight treatment with a solution of 0.05% HCl and 0.075% enzyme (Rapidase CX) and rinsed before

drying. Seeds were screened through a sieve and two classes of seeds were separated out: class 1 seeds had a diameter greater than 2.5 mm, and class 2 seeds had a diameter less than 2.5 mm. About 180 000 and 17 000 seeds of class 1 and class 2, respectively, were harvested.

Sowing

Seeds of wild-type and transgenic plants were sown in vitro independently in glass tubes or mixed together at different densities in boxes (175 × 115 mm), the distance between seeds ranging from 7 to 15 mm. In the greenhouse 400 seeds were sown in 30 × 30 cm earthenware vessels containing planting mix and watered with a nutrient solution (Coïc and Lesaint 1961) containing the appropriate concentrations of NAM until the planting medium was saturated. Earthenware vessels were placed in the greenhouse under conditions of a 16-h photoperiod, 70% humidity and 120 μE $m^{-2}s^{-1}$ lighting. Nutrient solution without NAM was supplied as the planting medium began to dried.

Chromosome counting

Chromosome counts were performed on root tips of putative haploid plants by staining with Schiff reagent after fixation in 90% acetic acid and hydrolysis by 1*N* HCl for 10 min at 65 °C.

DNA analysis

DNA of transgenic plants was isolated from young expanding leaves according to Dellaporta et al. (1983). Aliquots of 10 μg DNA were digested with restriction endonuclease *Dra*I (Bethseda Research Lab, UK), separated by electrophoresis in a 0.8% agarose gel, and then blotted to a nylon membrane (Biodyne, Pall, France). Probes were labelled using random oligonucleotide primers (Feinberg and Volgestein 1983). Hybridization were performed according to Maniatis et al. (1982).

Results

Introduction of aux2 into tomato plants

Tomato cotyledons (cv 'UC82b') inoculated with the *Agrobacterium tumefaciens* strain GV3101 harboring the plasmid pCC23 developed shoots after 4 weeks of culture on the selection medium containing kanamycin. Transgenic shoots were normal in appearance and developed roots, suggesting that the presence of *aux2* in transformed shoots of tomato does not cause auxin-induced morphological changes nor prevents rooting.

Seeds obtained by self-pollination of ten independent kanamycin-resistant transformants were sown individually in vitro on medium containing NAM (3 mg/l). Three, C5, C16, and C21, segregated approximatively 75% rootless plants to 25% normal plants on medium containing NAM, suggesting the integration of a T-DNA carrying a functional *aux2* gene at a single locus, whereas two others, C14, and C6, segregated approximatively 94% rootless plants to 6% normal plants, indicating the integration of two T-DNAs carrying a functional *aux2* gene at two independent loci. The progeny of the remaining five plants, C7, C11, C12, C8, and C3, were insensitive to NAM, suggesting that these plants did not carry a functional *aux2* gene. We confirmed by Southern blot experiments that in plants C7 and C11, the *aux2* internal 1-kb probe hybridized with a rearranged fragment (Fig. 1B), which implies a non-functional insertion of *aux2*. Conversely, for the NAM-sensitive plants, C5, C14, C16, and C21, hybridization clearly showed the presence of the 1-kb frag-

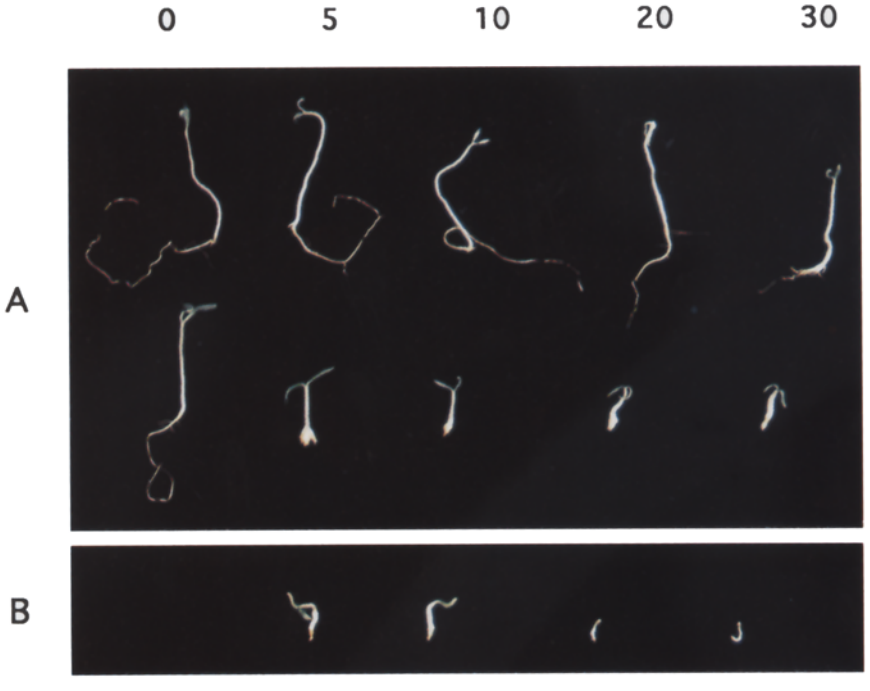

Fig. 2. Effect of NAM and NAA on wild-type and *aux2* transgenic seedlings. Seeds were sown individually in vitro on medium containing increasing concentrations of NAM (0–30 mg/l) (**A**) or NAA (5–30 mg/l) (**B**). *Lanes 1 and 3* wild-type, *lane 2 aux2* transgenic plants

ment, indicating a correct integration of the *aux2* gene (Fig. 1B).

Transformant C21, which segregated *aux2* at a single locus, was retained for further experiments. Tewnty R1 kanamycin-resistant plants obtained by self-pollination of transformant C21 were transferred into the greenhouse and selfed. All of the R2 progeny from five different R1 plants were completely rootless on medium containing NAM, indicating that these plants (C21/C21) were homozygous for the *aux2* gene.

Effect of NAM on germination and growth of wild-type and aux2 transgenic tomato plants sown in vitro

Wild-type (cv 'Apedice') and hybrid seeds (C21/+) resulting from a cross between the homozygous C21/C21 transgenic line (cv 'UC82b') and the male-sterile line (cv 'Apedice') were sown independently in vitro on media containing NAM at concentrations ranging from 5 to 30 mg/l. As shown on Fig. 2A wild-type seeds germinated and grew normally on medium containing 5 mg/l NAM, whereas C21/+ seeds germinated, but did not grow (radicles did not develop). Increasing the concentration of NAM to 20 mg/l had no significant effect on either seed germination or plant growth of the wild-type, whereas 30 mg/l NAM inhibited the growth of wild-type plants but not germination. Wild-type seeds sown on medium containing NAA concentrations ranging from 5 to 30 mg/l showed the same changes as C21/+ seeds sown on medium supplemented with NAM (Fig. 2B). This observation indicated that *aux2* is actually responsible for the transformation of NAM into NAA in transgenic tomato plants. Consequently, we tested if the inhibition of growth of *aux2* transgenic tomato plants on medium containing NAM was compatible with the development of wild-type plants sown in the same medium. Wild-type seeds were mixed with C21/+ seeds (1:9), and the mixture was sown at different densities on medium containing NAM (0–30 mg/l). When seeds were sown 15 mm from each other, wild-type seeds germinated, but did not develop roots and stopped growing, as did the C21/+ plants. This inhibition probably resulted from the release of NAA by transgenic seedlings into the medium. When IAM was used instead of NAM (assuming that excreted IAA would be less stable than NAA), wild-type plants grew, although C21/+ plants did not grow. However, when the seeds were sown 7 mm from each other, wild-type plants did not grow because of the release of IAA by neighboring C21/+ seedlings. Thus, a minimal distance of 10 mm between two seeds was required to permit the development of wild-type plants sown in the presence of C21/+ plants on medium containing IAM. Such a procedure, however, was not compatible with the selection of haploid plants, which should occur at a low frequency and require the sowing of several thousand seeds.

Effect of NAM on the germination and growth of wild-type and aux2 transgenic tomato plants sown under greenhouse conditions

Wild-type and hybrid (C21/+) seeds were sown together in a planting mix and watered with NAM at concentrations ranging from 10 to 50 mg/l. The germination rate of the wild-type seeds was not affected significantly by the proximity of C21/+ seedlings when watered with a nutrient solution containing up to 40 mg/l NAM (Fig. 3). However, a germination delay of 3 days was observed as compared to the control treatment. The death of C21/+ seedlings did not affect the survival of wild-type plants. Thus, the development of wild-type diploid plants was preserved under greenhouse conditions when up to 40 mg/l NAM was used in the nutrient solution. Since the behavior of haploid plants under such conditions was unknown, and assuming that they should be less vigorous than diploid ones, we looked for the minimal concentration of NAM required to inhibit the growth of C21/+ seedlings of both class 1 (large seed fraction) and class 2 (small seed fraction). In the presence of 20 mg/l NAM the development of approximately 98% of class 2 hybrid seeds was inhibited, whereas a concentration of 40 mg/l NAM was required to inhibit the development of approximately 98% class 1 hybrid seeds.

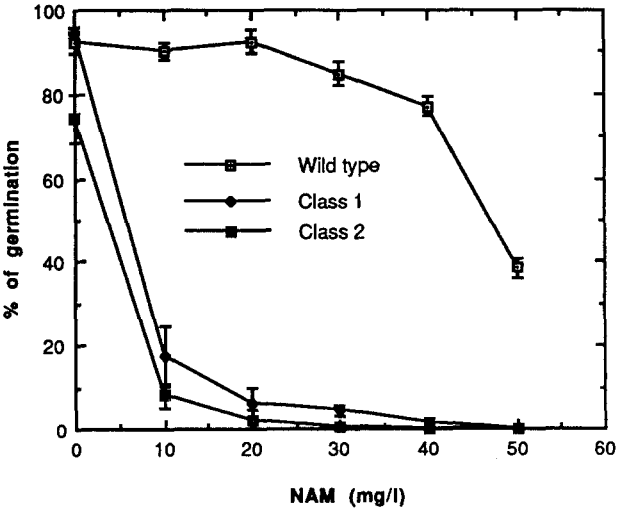

Fig. 3. Germination rate of wild-type and *aux2* transgenic seeds sown under greenhouse conditions in the presence of NAM. Wild-type seeds (13) were sown with *aux2* transgenic seeds (200) and watered with a nutrient solution containing increasing concentrations of NAM (0–50 mg/l). The number of germinating seedlings of each type was scored 30 days after sowing

Table 1. Selection of tomato haploid seedlings

Seed size[a] (mm)	Percentage germination	Morphological markers			Conditional lethal marker		
		Total number of seeds sown	Number of seedlings with maternal phenotype	Number of plants confirmed as haploid	Total number of seeds	Number of seedlings growing in presence of NAM	Number of plant confirmed as haploid
Class 1 >2.5	86	15000	4	4	26000	0	–
Class 2 <2.5	78	1500	3	3	7500	10	3

[a] Class 1 and class 2 seeds were screened for morphological markers of the female parent or treated with 20 and 40 mg/l NAM, respectively. Plants displaying the maternal phenotype had hypocotyls without anthocyanine and potato leaf morphology

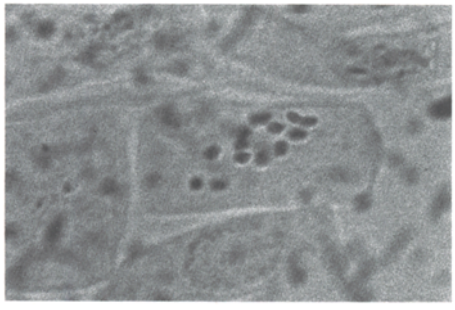

Fig. 4. Chromosome counting of haploid plants. The number of chromosomes was determined in root-tip cells at the metaphase stage

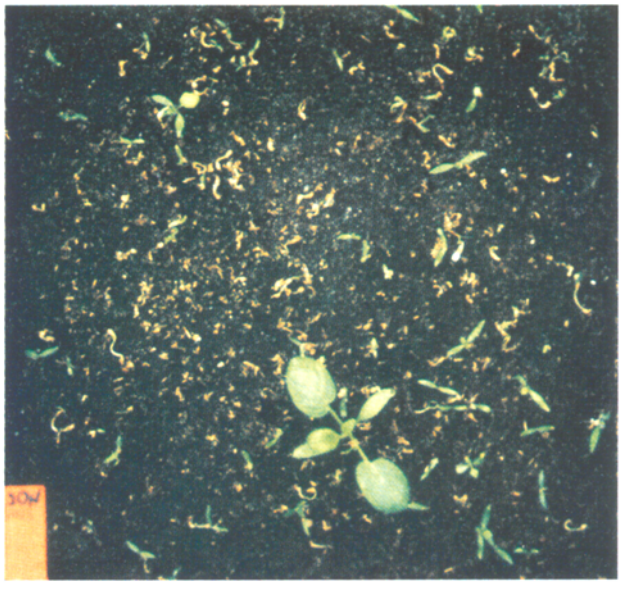

Fig. 5. Maternal haploid selection. Seeds of class 2 were sown under greenhouse conditions (400 seeds per earthenware) and watered with a nutrient solution containing 20 mg/l NAM. The photograph was taken 1 month later

Maternal haploid selection

Two types of selection were performed. The first selection was based on the visual screening of plants displaying recessive morphological markers, and the second on the counter-selection of heterozygous C21/+ seedlings. Among 15000 class 1 seeds sown in the absence of NAM, four plants were of the maternal type with respect to leaf morphology and hypocotyl pigmentation. Among 1500 class 2 seeds sown under the same conditions, three other maternal type plants were identified (Table 1). These plants are probably gynogenetic haploids. The haploidy was confirmed by chromosome counting (Fig. 4). Thus, the frequency of gynogenetic haploids was sevenfold higher using class 2 seeds than class 1 seeds (Table 1). In a second experiment, class 2 and class 1 seeds were sown and watered with a nutrient solution containing 20 mg/l and 40 mg/l NAM, respectively. From the 26000 class 1 seeds sown, not one plant developed on the selective medium; from a total of 7500 class 2 seeds sown, ten plants developed normally. Three of these exhibited the recessive morphological markers of the female parent (Fig. 5) and are presumed to be haploid plants, whereas the seven other plants displayed the morphological phenotype of the male parent and are presumed to be diploid plants that escaped the selection (Table 1). The haploid status of the three plants exhibiting the female phenotype was confirmed by chromosome counting.

Discussion

Attempts were made to select for tomato maternal haploid plants (which result from the development of the haploid female nucleus without fertilization) by

crossing a male-sterile plant with a homozygous *aux2* transgenic plant and then sowing the resulting seeds in the presence of NAM. The growth of diploid heterozygous *aux2* transgenic plants was expected to be inhibited on medium containing NAM, whereas haploid wild-type tomato plants were expected to be insensitive to NAM and to develop normally. In fact, heterozygous *aux2* transgenic seeds sown in vitro on medium containing up to 20 mg/l NAM were able to germinate but not grow, whereas wild-type seeds were able to grow. However, 30 mg/l NAM strongly inhibited wild-type plant growth in vitro, although germination was not affected. We also observed that under greenhouse conditions the development of 14-day-old wild-type plants which had germinated in the absence of NAM was strongly inhibited when NAM was present in the nutrient solution at concentrations as low as 3 mg/l, although wild-type seeds were able to germinate in the presence of NAM at concentrations up to 30 mg/l and to grow if supplied with a standard nutrient solution that no longer contained NAM (data not shown). These latter results indicated that the germination of wild-type tomato plants was not sensitive to NAM and also suggested that the expression of aspecific hydrolases able to transform NAM into NAA seemed to be turned on immediately after germination. Thus, it was decided to select for haploids at the germination level. Reconstruction experiments in which wild-type and transgenic seedlings were mixed and sown together in vitro showed that the release of NAA by transgenic plants in the agar medium inhibited the development of wild-type seedlings. The use of IAM instead of NAM overcame these cross-feeding problems when a minimal spacing of 10 mm between seeds was respected. However, selecting for haploids at this density would be difficult since a large number of seeds have to be sown. Under greenhouse conditions the use of compost avoided the cross-feeding problems observed in vitro. The diffusion of molecular compounds such as NAA produced by transgenic seedlings might be more limited in compost that in agar, and NAM or NAA might be more rapidly degraded in this kind of substrate than in agarose medium.

The identification of monoploids at the seed stage on the basis of the floating density of the relatively smaller monoploid embryos (Aalders 1958) or by X-ray radiography (Sauton et al. 1989) has been described in cucumber species. Thus, we tried to improve the recovery of tomato haploids by screening seeds through a sieve. Two classes of seeds were determined: class 1, with seeds of a diameter greater than 2.5 mm; class 2, with seeds less than 2.5 mm in diameter. The percentage of gynogenetic haploids selected by the visual screening of recessive morphological markers was sevenfold higher among small seeds (class 2) than among large seeds (class 1), suggesting that haploid plants should be easier to obtain by screening only small seeds. In fact, with NAM as a selective agent under greenhouse conditions, haploid plants were isolated from class 2, but not from class 1. Thus, the mechanical screening of small seeds should reduce the number of seeds needed to be analyzed and thus improve the efficiency of haploid selection.

The efficiency of haploid plant selection from class 2 seeds was fivefold less using *aux2* as the counter-selectable marker than that obtained by the visual screening of recessive morphological markers of the female parent. Thus, *aux2* seems to be an effective but not ideal marker for the selection of tomato haploid plants for several reasons, including the following. (1) NAM may be metabolized by endogenous hydrolases expressed at the early developmental stages of wild-type tomato plants immediately after germination. This would lead to endogenous concentrations that are not compatible with the normal development of plants (Wightmans 1962). Moreover, the toxic effect of NAA may be more pronounced on fragile haploid plants than on vigorous diploid plants for which a 3-day delay in germination has been observed when watered with a nutrient solution containing NAM. (2) The level of expression of *aux2* in transgenic plants may be insufficient to promote a high sensitivity to the low concentrations of NAM that should be required to recover normally developing wild-type plants. Indeed, in contrast to results obtained with tobacco and cabbage transgenic plants (Camilleri and Jouanin 1991; Beclin et al. 1992), all five independent transgenic tomato plants which were analyzed exhibited the same level of sensitivity to NAM, suggesting that the level of expression of *aux2* may always be low in transgenic tomato plants or that tomato cells have a lower sensitivity to NAM than tobacco and cabbage tissue. Thus, expressing the *aux2* coding sequence under the control of a strong promoter might allow one to perform haploid selection with a lower concentration of NAM. (3) Methylation may affect the expression of *aux2*, thereby allowing the development of transgenic plants in the presence of NAM. In fact, in our study diploid plants escaped from the selection by NAM at a rate of approximatively 10^{-4}. This result is in agreement with that of a previous report showing that *tms2*, the *Agrobacterium tumefaciens* gene encoding iaaH, was inactivated by methylation in the progeny of transgenic *Petunia* plants at a rate of 1.5×10^{-5} (Renckens et al. 1992).

Despite these problems, the *aux2* gene is to our knowledge the first counter-selectionable marker that allows the identification of tomato haploids. Compared to the counter-selection system based on the use of the dominant lethal marker *Rac-* in tobacco, *aux2* has several advantages: (1) it does not require the isolation of mutants (such as *Rac-*) of the species of

interest; (2) *aux2* can be transferred to several genotypes and transformation-competent species; (3) it behaves as a dominant conditional lethal marker that permits the normal development of the plant in the absence of selection pressure, whereas *Rac-* mutants need to be grafted onto wild-type stocks. Nussaume et al. (1991) showed that plants expressing a constitutive nitrate reductase gene (35S-NR) which converts chlorate into chlorite, a toxic compound, could be used as a counter-selectionable marker at the seedling stage on medium that does not contain nitrate, the inducer of the endogenous NR gene. This marker may be more useful than *aux2* for the selection of haploids in vitro, since it does not display cross-feeding problems. However, it can not be used in species in which NR is expressed constitutively at a basal level in the absence of nitrate.

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