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ORIGINAL ARTICLE

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# Use of the SSLP-based method for detection of rare apomictic events in a sexual AtSERK1 transgenic Arabidopsis population

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**Abstract** Here we present a screening method to evaluate the potential of genes to transfer aspects of apomixis into sexual crop plants. Based on the assumption that an apomictic progeny is an exact genetic replica of the mother plant we employed a set of single sequence length polymorphism (SSLP) markers to identify individuals displaying heterozygosity fixation in segregating sexual populations as an indication of rare apomictic events. Here we present the results of such a study using the *Arabidopsis thaliana SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (AtSERK1)* gene expressed under the control of the *AtLTP1* promoter in sexual *Arabidopsis* plants. In one of the three tested F2 transgenic populations expressing the *AtLTP1::AtSERK1* construct we observed two plants with heterozygosity maintenance for the full set of SSLP markers indicating a possible clonal inheritance. However, as their offspring revealed a close to binomial segregation for a number of heterozygous loci, it was concluded that these two putative apomictic plants either lost their clonal ability in the next generation or resulted from incidental recombination events displaying the genotype of the parent.

**Keywords** Apomixis · *Arabidopsis* · SSLP · Adventitious embryony · *AtSERK1*

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## Introduction

The asexual formation of a seed from the maternal tissues of the ovule is defined as apomixis (reviewed by Bicknell and Koltunow 2004). In nature apomixis occurs in more than 400 flowering plant taxa representing 40 families (Carman 1997). Apomicts can be facultative, using a combination of sexual and asexual modes or rarely they are obligate, forming exclusively apomictic seeds (Nogler 1984). Two types of apomixis are defined: sporophytic and gametophytic apomixis. In sporophytic apomixis, or adventitious embryony, embryos arise spontaneously from ovular cells late in ovule development. Gametophytic apomixis operates through the unreduced embryo sac (reviewed by Bicknell and Koltunow 2004). Endosperm development in both cases may be either spontaneous (autonomous) or fertilization-induced (pseudogamous) (reviewed by Koltunow 1993). Gametophytic apomixis is further subdivided into diplosporous and aposporous modes based on the cell type that gives rise to the unreduced embryo sac. In diplosporous types the megaspore mother cell (MMC) or a cell with apomictic potential is the progenitor cell of the unreduced embryo sac. This cell skips the reductional part of meiosis and development proceeds by mitotic division to achieve embryo sac formation (meiotic diplospory). Alternatively, the cell might undergo direct mitosis to form an unreduced embryo sac (mitotic diplospory). In aposporous apomicts, one or more somatic cells of the ovule, called aposporous initials, give rise to an unreduced embryo sac. Aposporous initials can differentiate at various times during ovule development. Aposporous embryo sacs usually coexist with a reduced meiotic embryo sac, which later degenerates (reviewed by Bicknell and Koltunow

2004). With the potential exception of meiotic diplospory, apomictic seeds originated from apospory, mitotic diplospory and adventitious embryony are clonal progeny of maternal origin because meiosis and fertilization are absent (Spillane et al. 2001).

Till now attempts to introduce apomixis into sexual species have made use of introgression of an apomictic trait from a wild apomictic relative into a recipient model or crop species (reviewed by Koltunow et al. 1995). Another proposed strategy entitles cloning of the apomictic genes and their transfer into sexual plants by transformation (reviewed by Koltunow et al. 1995; Grossniklaus et al. 1998a). So far, candidate genes for apomixis have been identified mainly by comparative gene expression studies during early stages of apomictic and sexual embryo development (Vielle-Calzada et al. 1996; Chen et al. 1999; Pessino et al. 2001; Rodrigues et al. 2003; Albertini et al. 2004, 2005). On the other hand genes that are responsible for gamete reduction, parthenogenesis or autonomous endosperm formation in sexual species are regarded as more successful candidates for 'apomixis' genes. Because sexual and apomictic development are closely interrelated, deregulating such genes in the sexual model systems such as *Arabidopsis thaliana* can result in phenotypes resembling elements of an apomictic pathway. Thus, mutations into the *fis* (*fertilization-independent seed*) genes: *fis1*/*mea* (*medea*) (Grossniklaus et al. 1998b), *fis2* (Chaudhury et al. 1997) and *fis3*/*fie* (*fertilization-independent endosperm*) (Ohad et al. 1999) induce autonomous development of the central cell to some extent. Alternatively, misexpressing genes that promote somatic embryogenesis, such as *BABY BOOM* (*BBM*) (Boutilier et al. 2002), *WUSCHEL* (*WUS*) (Zuo et al. 2002; Gross-Hardt et al. 2002; Gallois et al. 2002), *LEAFY COTYLEDON1* and *-2* (*LEC1* and *LEC2*) (Lotan et al. 1998; Stone et al. 2001) may provide a strategy to confer parthenogenesis (Spillane et al. 2001). A potential difficulty in engineering gametophytic or adventitious apomixis using genes identified in sexual species is whether parthenogenesis or adventitious embryony will occur in the complete absence of functional endosperm. To avoid this, a strategy is required where the normal sexual pathway is present without leading to parental imbalances in the endosperm caused by the fusion of an unreduced central cell with a reduced sperm (Spielman et al. 2003).

Here we further explored the potential of another embryogenesis-related gene, the *A. thaliana SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*AtSERK1*) to induce elements of parthenogenesis apomixis in *Arabidopsis*. Overexpression of *AtSERK1* can increase the embryogenic potential of cells in tissue culture (Hecht et al. 2001). In planta, expression of the *AtSERK1* gene has been detected in ovule primordia during megasporogenesis, including the functional megaspore and later in all cells of the embryo sac. After fertilization, expression could be detected in the zygotic embryo until the heart stage (Hecht et al. 2001; Kwaaitaal et al. 2005). The *C*-terminal fusion of *AtSERK1* with the green fluorescent protein (GFP) was located in developing anthers and it confirmed the absence of *AtSERK1* expression in the integuments surrounding the embryo sac prior and around the time of fertilization (Kwaaitaal et al. 2005). Whereas in the facultative apomictic species *Hieracium*, *AtSERK1* gene was expressed in both sexual and apomictic ovules (Tucker et al. 2003), a close *SERK1* homolog was found differentially expressed in sexual and apomictic plants of *Poa pratensis* (Albertini et al. 2005). This suggested that the signaling mediated by the AtSERK1 receptor is employed in both modes of reproduction and is an example of the molecular correspondence between both.

In this study we expressed the *AtSERK1* gene in *Arabidopsis* under the control of the *A. thaliana LIPID TRANSFER PROTEIN1* (*AtLTP1*) promoter (Thoma et al. 1994; Vroemen et al. 1996) aiming to induce ectopic embryogenesis in epidermal cell layers in the ovules of sexual *Arabidopsis* plants.

Introducing elements of apomixis in sexual species and also understanding the different progeny types produced by facultative apomicts creates a strong need for a robust tool to screen for rare apomictic events. Until now, apomicts were identified through morphological scoring, chromosome counting or progeny cross-methods, which were tedious and very time consuming. In *Hieracium*, a method based on the inheritance of positive and negative selectable transgenic markers have been described (Bicknell et al. 2003). This method, based on the survival of different progeny types when placed on selective media, is however limited to plant species that can be transformed genetically. Flow cytometric test in individual seeds has been shown to be particularly useful for the detection of apomicts from gametophytic origin (Matzk et al. 2000, 2001), but will not be useful to detect embryos that arise by adventitious embryony or parthenogenesis. The auxin test is commonly used to detect parthenogenesis of unfertilized egg cells in response to auxin in various *Poaceae* species (Matzk 1991a, b).

Here we present a novel screening system for apomicts that circumvents meiotic recombination, for example, adventitious embryony, mitotic diplospory or apospory. The method is based on heterozygosity fixation of single sequence length polymorphism (SSLP) markers. In *Arabidopsis* more that 50 SSLP markers have been described and they are routinely used as co-dominant genetic markers (Bell and Ecker 1994; Ponce et al. 1999). The SSLP heterozygosity screen is based on the assumption that an apomictic progeny is an exact genetic replica of the mother plant. This method is essential for determining the role of any candidate apomixis gene in eventually conferring aspects of apomixis into sexual crop species.

## Materials and methods

### Plant material

*Arabidopsis* accessions Wassilewskija (WS) and Landsberg *erecta* (L*er*) were used. Seeds were germinated

directly in soil or on half Murashige and Skoog (MS) salt medium (Murashige and Skoog 1962, DUCHEFA) supplemented with 1% (w/v) sucrose. The *Arabidopsis* seedlings were grown in soil at 22°C, with 16-h light/8-h dark periods.

### *AtSERK1* ectopic expression constructs and transformation of *Arabidopsis*

The *AtLTP1* promoter region of 1.2 kb was released from the vectors pMT121 (Toonen et al. 1997) by digestion with *Hind*III/*Bam*HI, filled in with Klenow and replaced the *Hinc*II/*Sma*I digested 35S promoter in the pRT105 vector (Hecht et al. 2001). The *AtLTP::AtSERK1::pA* fusion was PCR amplified with primers RT1(TCCCCCGGGGGAAGCTTGCATGCCTG) and RT2 (TCCCCCGGGGGACTGGATTTTGGTT) containing *Sma*I restriction sites. The PCR fragments were then subcloned into an *Sma*I site of the pMOG800 binary vector (Toonen et al. 1997). The construct was verified by sequencing and used to transform *Arabidopsis* ecotype WS plants. $T_1$ seeds were selected on half MS media containing 50 mg/l kanamycin (Km) for 10 days and transferred to soil to generate the $T_2$ and $T_3$ independent lines.

### RT-PCR and Western blot analysis

Total RNA was extracted from either flowers or 7-day-old seedlings using the Trizol protocol (Invitrogen, CA, USA). The reverse transcription (RT) and the PCR analysis were performed as described by Hecht et al. (2001). PCR products were collected after 28 cycles for the internal control, cyclophilin *ROC5* cDNA and after 31 cycles for the *AtSERK1* cDNA. Protein extraction and western blot analysis were carried out as described by Kwaaitaal et al. (2005).

### Generation of the $F_2$ and $F_3$ apomictic populations

Individual $T_3$ plants homozygous for the *AtLTP::AtSERK1* transgene were used as a male donor in outcrosses to the L*er* ecotype to generate the respective $F_1$ populations. $F_1$ plants were selfed and flower buds at stages 11 to 12 (Smith et al. 1990) were dipped in aqueous solution containing 2 μM 2,4-dichlorophenoxyacetic acid (2,4-D), supplemented with 0.04% (v/v) Triton-X100 (Vivian-Smith and Koltunow 1999). The treatment was repeated twice in a 2-day period and flowers were left to set seeds or collected for RNA isolation. Siliques containing the F2 seeds were collected individually. The resulting F2 plant populations were used for the SSLP screen. Selected individual $F_2$ plants were selfed and treated with 2,4-D to generate the $F_3$ progeny. Control experiments were carried out with $F_1$ plants generated by the cross between the WS and L*er* *Arabidopsis*. Those crosses were treated exactly as the transgenic plants and were also used for SSLP analysis.

### DNA isolation, SSLP multiplex PCR, gel electrophoresis and analysis of the PCR products

Genomic DNA was isolated as described by Ponce et al. (1999). Fourteen SSLP primer pairs that were able to amplify DNA polymorphism between Ws and Ler Arabidopsis accessions were chosen for the SSLP analysis Primer sequence, labeling and concentration used for SSLP analyses are shown in S1. The PCR reactions and the amplification conditions were as described by Ponce et al. (1999). Electrophoresis was carried out in an ABI PRISM 377 DNA sequencer with 6% acrylamide gel. GeneScan-500 (TAMRA) was used as size standard. The samples were run in the GS36C-2400 module for 2½ hours. DNA fragment analysis was performed using GeneScan 2.1 software (Applied Biosystems). The pattern was analyzed for accession-specific polymorphic bands by using Genotyper software (Applied Biosystems).

### Statistical analysis

All F2 and F3 families were tested against this theoretical distribution using the non-parametric distribution fit tool in Statistica 6.0 (StatSoft, Inc.) based on the Kolmogorov-Smirnov one-sample test.

### GUS analysis

Transgenic seeds of the Rschew ecotype, carrying an AtLTP1 promoter fused to ß-glucuronidase (GUS) gene were used (Vroemen et al. 1996). The histochemical localization of AtLTP1 GUS activity in unfertilized carpels and ovules was done as described by Hecht et al. (2001). The GUS stained material was fixed and embedded in Technovit 8100 resin (Heraeus Kulzer GmbH). Seven micrometer thick sections were cut and stained with toluidine blue solution (10 g/l toluidine blue, 10 g/l sodium tetra borate). The sections were observed using a Nikon Optiphot-2 microscope. Pictures were taken using a Nikon Coolpix 990 digital camera.

### Flow cytometry

The flow cytometry analysis was performed according to Raz et al. (2001).

## Results

### The At*LTP1* promoter is active during ovule development in *Arabidopsis*

To attempt to engineer elements of the sporophytic type of apomixis in the sexual *Arabidopsis*, we aimed to ectopically express the *AtSERK1* gene under the control of *AtLTP1* promoter. The activity of the *AtLTP1*

promoter during early ovule development was not previously documented. In situ hybridization experiments have shown that *AtLTP1* mRNA was present in very young flower buds (stages 2–4, Smith et al. 1990) and in the epidermal cells of fully developed ovules (Thoma et al. 1994). We further investigated the exact promoter activity during ovule development by looking at the *AtLTP1* promoter GUS expression (Fig. 1a). As shown in Fig. 1a (I—IV), the *AtLTP1* promoter was active at an early stage of ovule development. In young unfertilized carpels containing ovule protrusions at stages 1-II (Fig. 1a I) (Schneitz et al. 1995), the *AtLTP1* promoter was first activated in the epidermal layer surrounding the chalaza. Later at stages 2-II (Fig. 1aII), 2-IV (Fig. 1aIII) or 3-II (Fig. 1aV), the *AtLTP* expression became restricted to the nucellus and the developing integuments. In mature ovules the promoter remained active in the micropylar and in the chalazal end (Fig. 1aIV). Histological sections did not show any GUS staining in the embryo sac (Fig. 1aVI). It was concluded that *AtLTP1* expression is confined to the maternal tissues of the integuments and possibly the endothelium.

## Ectopic expression of *AtSERK1* using the *AtLTP1* promoter in *Arabidopsis*

Homozygous *AtLTP1::AtSERK1* plants carrying a single insertion of each transgene did not show any notable phenotypic changes and they all had normal

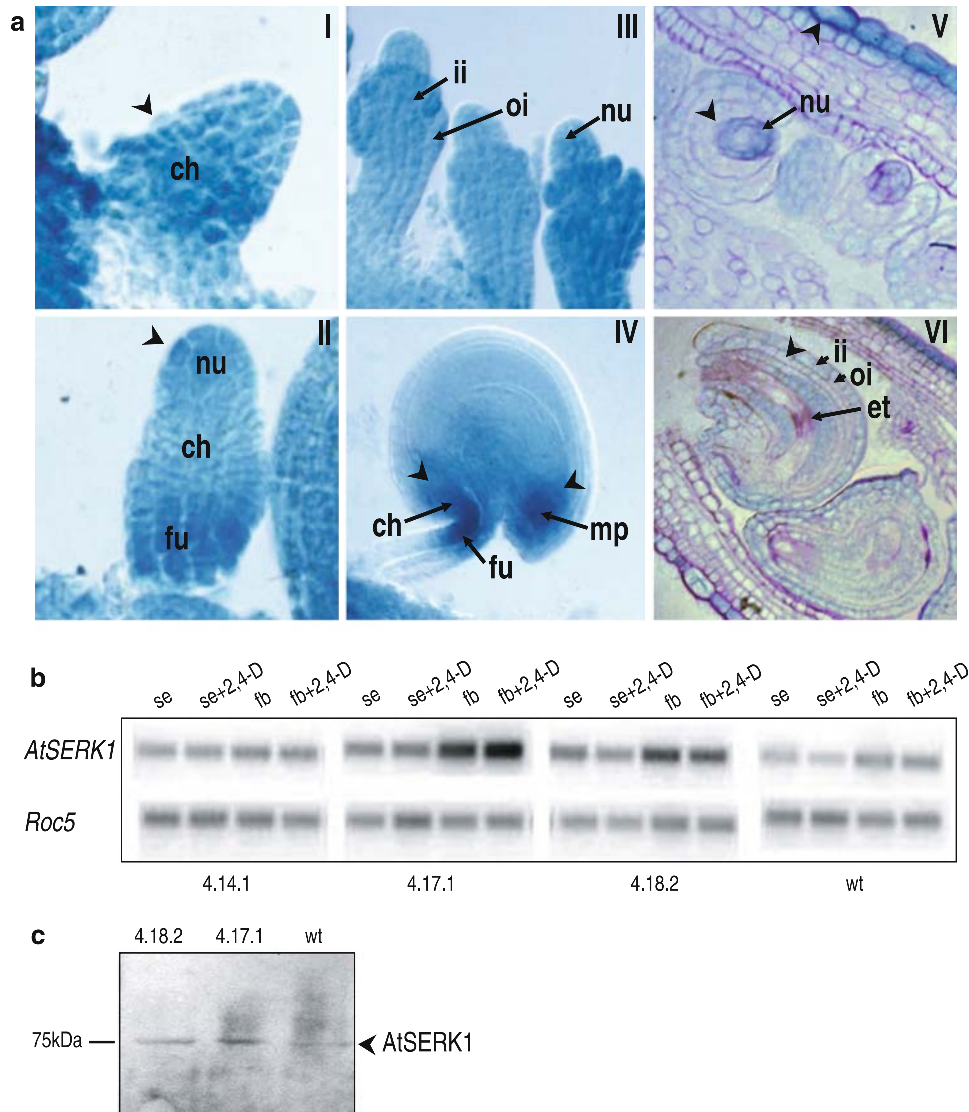



**Fig. 1** Ectopic expression of *AtSERK1* in *Arabidopsis*. (**a**) *AtLTP1::GUS* expression during ovule development in *Arabidopsis*. (**aI**), ovules at stage 1-II; (**aII**), ovules stage 2-II; (**aIII**), ovules at stage 2-IV; (**aV**), ovule at stage 3-II according to Schneitz et al. (1995). (**aIV**, **aVI**), mature ovules. *ch* chalaza; *fu* funiculus; *nu* nucellus; *oi* outer integument; *ii* inner integument; *mp* micropyle; *et* endothelium. (**b**) *AtSERK1* expression in seedling and flower buds of the transgenic and wild-type plants with and without 2,4-D treatment by RT-PCR. *se* seedlings; *fb* flower buds. (**c**) Western blot analysis of AtSERK1 protein levels in wild-type and in transgenic plants

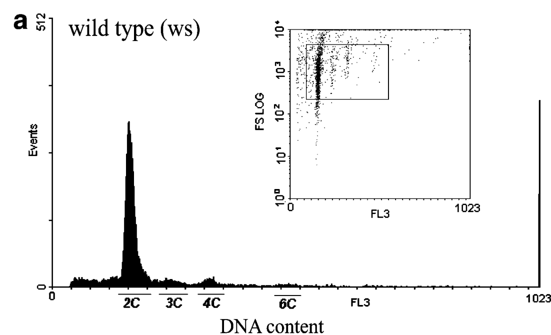
seed formation (data not shown). To select transgenic lines for further analysis we first determined the expression levels of the *AtSERK1* transgene by a semi-quantitative RT-PCR. Three independent *AtLTP1::AtSERK1* transgenic lines, numbered 4.14.1, 4.17.1 and 4.18.2, were selected and the levels of *AtSERK1* expression were compared with endogenous *AtSERK1* expression in seedlings and flower buds (Fig. 1b). In general, the expression of the *AtSERK1* gene in flower buds was higher than in seedlings, confirming previous observations (Hecht et al. 2001). The *AtLTP1::AtSERK1* lines 4.17.1 and 4.18.2 showed a higher and an intermediate *AtSERK1* expression, respectively, in flower buds and in seedlings when compared with the wild-type. In line 4.14.1 the *AtSERK1* mRNA level was close to that of the endogenous *AtSERK1* gene. The effect of 2,4-D treatment on the mRNA levels of the *AtSERK1* gene was also determined by RT-PCR in the wild-type and in the transgenic plants (Fig. 1b). The treatment with 2,4-D did not influence the *AtSERK1* mRNA level of expression suggesting that the gene is not transcriptionally regulated by the auxin. Western blot analysis performed on the *AtLTP1::AtSERK1* lines 4.17.1 and 4.18.2 showed that the overexpression of *AtSERK1* gene in those lines resulted in elevated At-SERK1 protein levels (Fig. 1c).

## Flow cytometry

We next employed flow cytometry to screen for aberrant C-values of unreduced gametes in the $T_3$ seeds obtained from the *AtLTP1::AtSERK1* transgenic lines 4.14.1, 4.18.2 and 4.17.1. Each experiment was done in replica for each transgenic line. Seeds from wild-type plants were used as control (Fig. 2a). Flow cytometry distinguishes different modes of apomixis based on the DNA content of embryo and endosperm nuclei (Matzk et al. 2000). All transgenic seed samples analyzed showed the presence of a high 2C-embryo peak and a smaller 3C-endosperm peak suggesting normal reduction of the embryo sac (Fig. 2b). No 5C peak, which is indicative for the unreduced embryo sac formation was detected. However, if *AtSERK1* overexpression causes a sporophytic type of apomixis in a sexual plant, flow cytometry cannot be used to distinguish between the C values of sexual and adventitious embryos in the presence of 3C endosperm.

## Design of the SSLP screen for apomixis in the *AtSERK1*-expressing plants

To determine whether in the *AtSERK1*-expressing plants adventitious embryony had occurred, possibly as a rare event, we devised a genotypic screen based on SSLP markers that can distinguish between plants with maternal and sexual origin. The complete procedure for generating $F_1$, $F_2$ and $F_3$ transgenic generations is summarized in Fig. 3. Individual $T_3$ plants homozygous for the *AtLTP1::AtSERK1* transgene (lines 4.14.1, 4.18.2 and 4.17.1) were used as a male donor in outcrosses to the L*er* accession. The respective $F_1$ populations were generated as follows: L*er* × line 4.14.1 gave rise to $F_1$-44; L*er* × line 4.18.2 gave rise to $F_1$-15 and L*er* × line 4.17.1 gave rise to $F_1$-45. Due to a limited amount of $F_1$ seeds the SSLP screen was performed on the $F_2$ generations obtained by the self-cross of the receptive $F_1$ plants. The $F_1$ plants were treated with 2,4-D after fertilization to increase the chance of adventitious embryony to occur. Additional genetic crosses were performed by using wild-type WS pollen to pollinate wild-type L*er* plants that yielded the control 'wild-type' $F_1$ plants. The control $F_1$ plants were selfed and two different $F_2$ control progenies were generated, one with and one without 2,4-D treatment. The control $F_2$ progenies were analyzed in the same way as the transgenic plants.




**b**

| Transgene | Line no. | C values in histograms | | | | |
|---|---|---|---|---|---|---|
| | | 2C | 3C | 4C | 5C | 6C |
| | Wild type (ws) | + | + | + | - | + |
| *AtLTP1::AtSERK1* | 4.14.1 | + | + | - | - | + |
| *AtLTP1::AtSERK1* | 4.17.1 | + | + | - | - | - |
| *AtLTP1::AtSERK1* | 4.18.2 | + | + | - | - | + |

**Fig. 2** Flow cytometric analyses. (**a**) Flow cytometry histogram of seed nuclei from sexual wild-type WS seeds with a high 2C embryo and a small 3C endosperm DNA peak. (**b**) Comparison of data from the flow cytometric screen in the transgenic *AtLTP1::AtSERK1* seeds

Fourteen SSLPs that were polymorphic for WS and L*er*, homogenously distributed throughout the *Arabidopsis* genome and unlinked (Fig. 4) were used to genotype each of the $F_1$ plants and their $F_2$ progenies (Ponce et al. 1999). The SSLP profiles of all $F_1$ transgenic and control plants were identical, always amplifying the same 28 PCR products. They corresponded to the 14 SSLP alleles in L*er* and the 14 SSLP alleles in WS ecotypes that were present in all heterozygous $F_1$ plants (data not shown). $F_2$ plants from each transgenic experiment and from the wild-type control experiment were genotyped for all 14 SSLP markers. The SSLP profile of each $F_2$ plant was then compared with the SSLP profile of the corresponding $F_1$ mother plant. The self-cross of a sexual plant produces a segregating progeny. In contrast, an apomict is expected to produce an offspring that is a maternal clone. Therefore, we examined the segregation pattern of each of the 14 SSLPs in the $F_2$ transgenic populations to determine whether the plants have a maternal origin. Plants of a maternal origin will have an SSLP profile identical to the SSLP profile of the $F_1$ mother plant indicating a lack of recombination. The segregation of the SSLP markers in the $F_2$ and $F_3$ populations was first tested for normal disomic inheritance. The selected 14 SSLP markers covered all five linkage groups (Fig. 4). None of the selected SSLP markers showed a tight linkage with its neighbor. On the basis of independent inheritance we assumed that the number of heterozygous loci followed a binomial distribution.

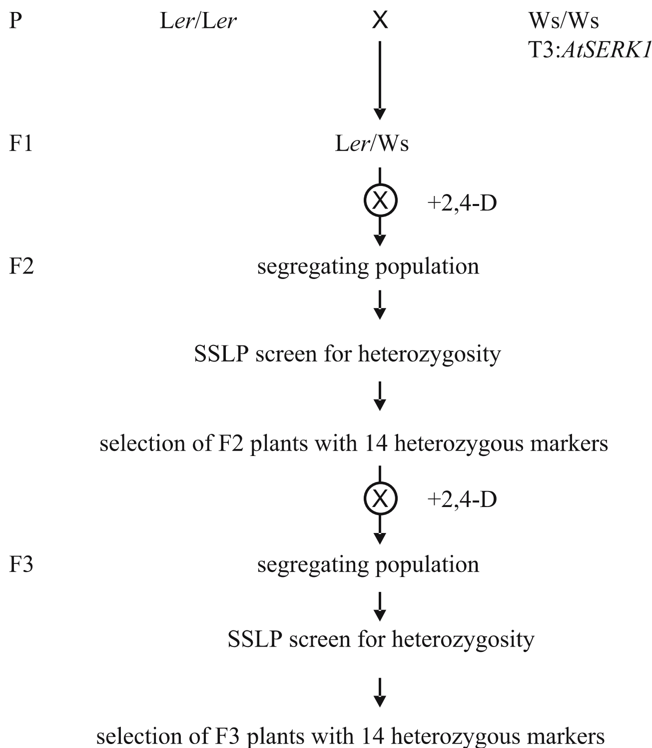



**Fig. 3** Generation of $F_1$, $F_2$ and $F_3$ populations containing the *AtSERK1* transgene. The *AtLTP1::AtSERK1* homozygous plants in WS ecotype were crossed with L*er* wild-type to generate the heterozygous hybrid ($F_1$). Self-crosses of $F_1$ were performed to produce segregating populations for SSLP analysis to detect the hybrid with heterozygous fixation from their parents, presuming apomictic progeny. The next generations of the progeny were produced by self-crosses for investigating the apomictic inheritance. *P* parents

## Segregation of the SSLP markers in the control wild-type $F_2$ population

In Fig. 5a and in S3 we show observed and expected distributions of heterozygous loci number of 196 $F_2$ plants derived from the wild-type L*er*/WS cross comprising of all 14 SSLP markers. In this population we did not find any plants heterozygous for all SSLP markers confirming that the wild-type *Arabidopsis* plants reproduce sexually. However, notable aberration of heterozygote frequency for the markers nga111, AthACS, both on chromosome 1, and nga1139 on chromosome 4, with an average of 0.474 for all markers (expected is 0.5) was observed (see S2). The tendency of reduced heterozygosity is also clearly visible in the histogram comparing observed and expected (binomial) values and was significant according to the used goodness-of-fit statistics at $P < 0.01$ (Fig. 5a).

We next investigated whether auxin treatment would affect the segregation of the SSLP markers in the wild-type population as stimulation of somatic embryogenesis in culture required 2,4-D application (Hecht et al. 2001). The segregation of the SSLP markers in the $F_2$ population generated after 2,4-D application is shown in Fig. 5b, and in S3. The results were essentially similar to the untreated control experiment, with also a slightly lower frequency of heterozygotes (0.484), and an overall significant difference for all heterozygote number classes from the expected values (Fig. 5b). Also here we observed a comparable decrease of heterozygotes for AthACS and ATHCHIB (see S2). These observations suggested that a short application of auxin does not affect the segregation of the SSLP markers and by itself is not sufficient to initiate adventitious embryony in *Arabidopsis*.

## Segregation of 14 SSLP markers in the *AtLTP1::AtSERK1* $F_2$ and $F_3$ populations

We next analyzed the distrubution of the 14 SSLP markers in the $F_2$ populations derived from three independent transgenic lines showing low, intermediate and high *AtSERK1* expression levels [4.14.1 ($F_2$-44), 4.18.2 ($F_2$-15) and 4.17.1 ($F_2$-45)], respectively. The observed segregation of the 14 SSLP markers in the three transgenic populations was significantly different ($P < 0.01$) from the expected binomial distribution (Fig. 5c–e, S3). The average frequency of heterozygosity for each of the 14 SSLPs in these populations was close to the expected values. However, when compared with the wild-type

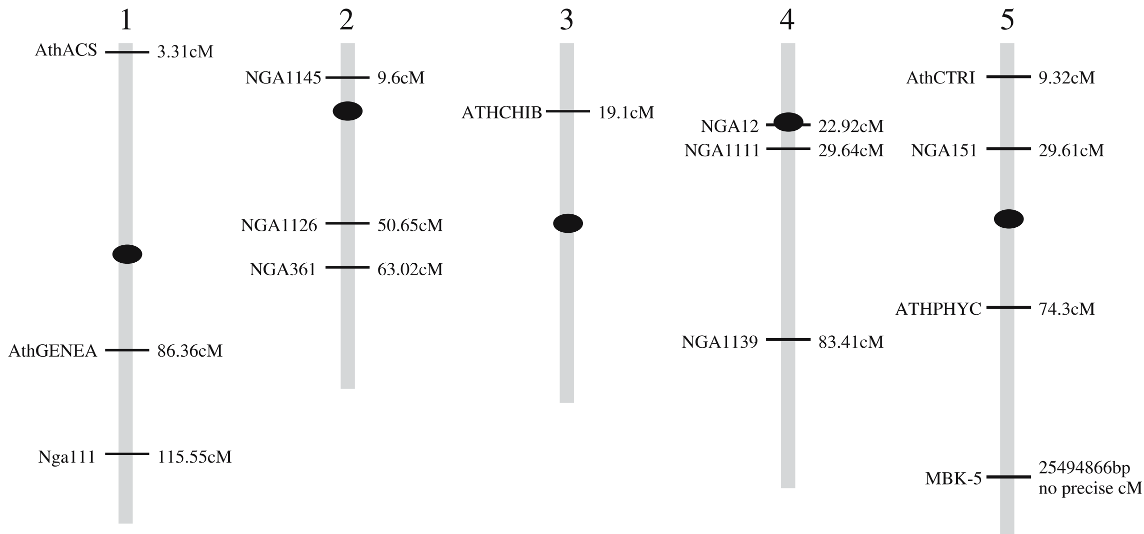



**Fig. 4** The chromosome map location of the 14 SSLP markers of *A. thaliana*

population an opposite tendency of a slight increase in the heterozygosity was observed (see S2). Two plants heterozygous for all 14 SSLP markers were identified among the $F_2$ progeny of the line with intermediate *AtSERK1* expression levels, 4.18.2 (Fig. 5c, d and S3) suggesting a heterozygosity fixation in these plants. The probability for finding two such plants on the basis of random Mendelian segregation would be as low as 0.007%. Seed contamination is an unlikely explanation because the 2.4-D-treated F1 siliques were collected individually.

In order to see if the same phenomenon will be transmitted to the next progeny we examined the SSLP segregation in the two $F_3$ populations derived from these two plants ($F_3$-15 no. 32 and $F_3$-15 no. 141). The SSLP analysis of the $F_3$ populations showed results similar to the wild-type (Fig. 5f, g and S3). This suggested that the phenomenom of heterozygosity fixation was not transmittable to the next generation.

## Discussion

Ectopic expression of genes that influence the potential of plant cells to undergo embryogenesis in somatic cells might induce a particular apomictic component such as parthenogenesis in sexual species. In this context an apomictic embryo induction in *Arabidopsis* was employed through ectopic expression of the *AtSERK1* gene under control of promoters that were active in the female gametophyte. Overexpression of *AtSERK1* using the 35S constitutive promoter increased the embryogenic potential of cells in culture. In planta *AtSERK1* was expressed in regions of the developing ovules, including the egg cell, where it might lead to initiation of embryogenesis by the appropriate signals (Hecht et al. 2001). Expressing *AtSERK1* under the protoderm-specific, *AtLTP1* (Thoma et al. 1994) promoters aimed to initiate adventitious embryony from the maternal tissues in ovules representing either the nucellus or the integuments. Endosperm development is required for embryo survival and thus the *AtSERK1* gene was introduced in sexual *Arabidopsis* plants. Initiation of adventitious embryony in a sexual background could be a rare event depending on successful fertilization of the adjacent reduced embryo sac and on the ability of the adventitious embryo to grow sufficiently to gain access to nutrient endosperm. This requested a reliable screening system that can differentiate between a rare apomictic and the sexual mode of reproduction in the progeny of the transgenic plants. So far, diverse methods for apomictic screening have been reported including flow cytometry analysis (Matzk et al. 2001), a transgenic approach (Bicknell et al. 2003), auxin induction (Matzk 1991a, b) and morphological analysis (Koltunow 1993). These methods, however, are either time consuming or they are not suitable to detect the sporophytic mode of apomixis.

In this study we developed and applied an SSLP screen to genetically identify individuals displaying heterozygosity fixation as an indication for apomixis. The screen is based on assumption that seedlings with an apomictic origin will retain the genotype of the maternal parent. *AtSERK1* transgenic WS ecotype *Arabidopsis* plants were crossed with L*er* ecotype to produce heterozygous $F_1$ plants. Auxin was applied to flower buds of the $F_1$ plants in order to initiate possible embryo development as observed in explants used for tissue culture. The SSLP-based genotypic screen was performed on both wild-type and segregating $F_2$ progenies derived from *AtLTP::AtSERK1* transgenic lines. As such, the number of heterozygous or homozygous loci for all 14 unlinked SSLP markers was determined simultaneously for all F2 plants. Alternatively a more efficient stepwise approach could be established in which

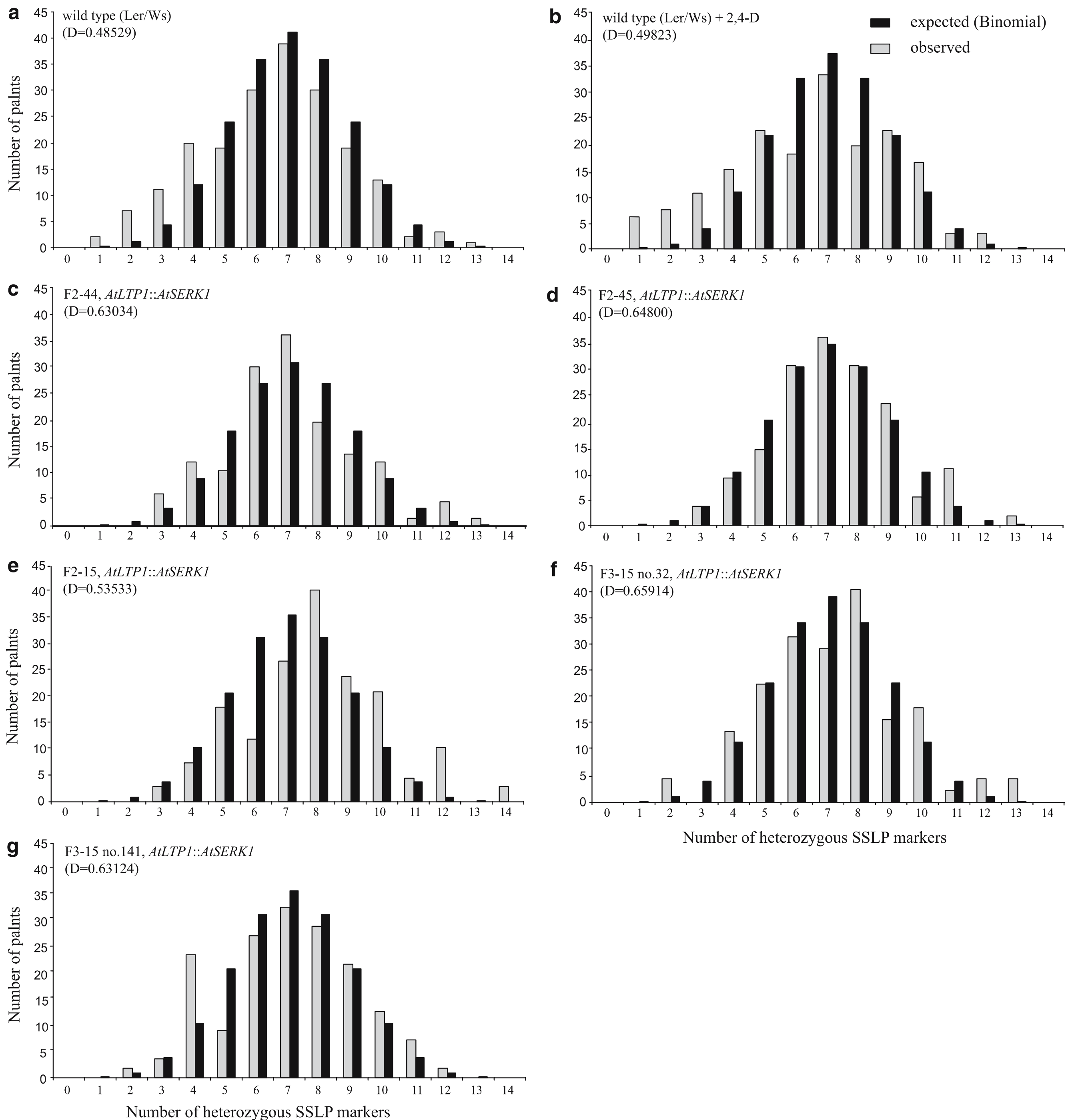



**Fig. 5** The segregation of plants with 0–14 heterozygous SSLP markers of observed (*gray bars*) and expected (*black bars*) numbers in (**a**, **b**), control populations with and without 2,4-D treatment; (**c**) $F_2$-44 *AtLTP1::AtSERK1*; (**d**) $F_2$-45 *AtLTP1::AtSERK1*; (**e**) $F_2$-15 *AtLTP1::AtSERK1*; (**f**) $F_3$-15 no. 32 *AtLTP1::AtSERK1* and (**g**) $F_3$-15 no. 141 *AtLTP1::AtSERK1*. The values within *brackets* are the *D*-statistics of the Kolmogorov-Smirnov test. All values were significant at $P < 0.01$

the distribution of the SSLP markers could be determined in groups of five. This way, the number of F2 plants and the complexity of the PCR reactions required can be significantly reduced. Such a procedure would also allow to increase the number of SSLP markers employed in the screen and so distal loci could also be tested for heterozygosity. In all $F_2$ populations, the heterozygous distribution of the 14 SSLP markers significantly differed from the expected theoretical binomial distribution. A tendency of lower heterozygosity was observed for the wild-type populations whereas the differences in the $F_2$ *AtLTP::AtSERK1* transgenic populations were random. A possible explanation for the lower heterozygosity in the wild-type population could

be the occurrence of "an outbreeding depression". Thus, alleles or their chromosome segments that originate from the same accession may collaborate better than when obtained from different ecotypes. In contrast, introducing the *AtSERK1* transgene can facilitate an increase in the recombination frequency for some of the SSLP markers. The heterozygosity frequency for individual SSLP markers showed extreme values ($< 0.4$ and $> 0.6$) in all populations to a different extent, but only AthACS was found consistently aberrant in most of the populations.

Two plants heterozygous for 14 SSLP markers were found in the *AtLTP::AtSERK1* F2 population derived from the transgenic line with intermediate *AtSERK1* expression levels, 4.18.2. Interestingly, the highest overexpression line, 4.17.1, did not produce putative apomictic progeny. Thus, the potential of *AtSERK1* to induce apomixis did not correlate with high expression levels. The finding that embryos with an entire maternal constitution were present in the transgenic population raised the question of what is their origin. Given that the flow cytometry results showed an absence of 5C-endosperm peak in all transgenic seeds analyzed we can conclude that no gametophytic type of apomixis has occurred. This leaves parthenogenesis followed by dihaploidization of the egg cell in the presence of fertilization of the central cell (pseudogamy) or adventitious embryony as possible modes of apomictic embryogenesis. Because the offspring was fully heterozygous for all markers tested it is more likely that meiosis did not take place and we can also rule out pseudogamy. This would leave adventitious embryony that was initiated normally before fertilization as the most likely model of apomixis. However none of the putative apomictic plants were able to retain complete heterozygosity in the next generation. The distribution of heterozygote frequency in the $F_3$ transgenic populations was similar to heterozygous distribution of the wild-type population, which might indicate that the influence of the transgene declined by generation.

While our observations support the prediction for the origin of the apomictic embryos further more studies will be necessary to confirm that. Although it is unlikely that these plants resulted from random meiotic recombination and segregation events we cannot exclude the possibility that other means such as suppression of recombination and non-random anaphase I segregation of the half bivalents can account for this.

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