

# Chapter 2

## Using *Arabidopsis*-Related Model Species (ARMS): Growth, Genetic Transformation, and Comparative Genomics

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### Abstract

The *Arabidopsis*-related model species (ARMS) *Thellungiella salsuginea* and *Thellungiella parvula* have generated broad interest in salt stress research. While general growth characteristics of these species are similar to *Arabidopsis*, some aspects of their life cycle require particular attention in order to obtain healthy plants, with a large production of seeds in a relatively short time. This chapter describes basic procedures for growth, maintenance, and *Agrobacterium*-mediated transformation of ARMS. Where appropriate, differences in requirements between *Thellungiella* spp. and *Arabidopsis* are highlighted, along with basic growth requirements of other less studied candidate model species. Current techniques for comparative genomics analysis between *Arabidopsis* and ARMS are also described in detail.

**Key words** *Thellungiella* spp., Halophytes, Germination, Seed handling, Vernalization, Plant care

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## 1 Introduction

Over the past few decades, a tremendous advance in our understanding of molecular and cellular responses to abiotic stresses has taken place using the model species *Arabidopsis*. Forward and reverse genetics approaches, combined with thorough functional analysis of many isolated genes, as well as biochemical characterization of key stress tolerance proteins have allowed us to characterize quite accurately many responses to salt and osmotic stresses (reviewed in refs. 1–3).

Although *Arabidopsis* has contributed to the unraveling of complex essential mechanisms that allow plants to cope with salt stress [4], it has failed to reveal the key determinants that render in natural environments some plants (halophytes) more tolerant than others (glycophytes) to saline environments. Halophytes from different families have been studied over the past decades, including

species, for example, belonging to the genera *Atriplex*, *Suaeda*, *Salicornia*, and *Mesembryanthemum*; monocotyledonous species such as *Spartina* and *Puccinellia* spp.; and mangroves belonging to the genera *Avicennia* and *Rhizophora* ([5], reviewed in ref. 6). The study of halophytic species has led to a partial understanding of the different physiological and morphological strategies used by plants to withstand harsh conditions. However, the paucity of suitable molecular genetics techniques has, to a great extent, prevented the identification of the genetic bases for salt tolerance in halophytes [6]. Genetic studies on halophytic species are very limited [7], and the potential of this resource of natural salt tolerance has remained largely unexplored [6, 8–11]. Recently, *Thellungiella salsuginea* (salt cress), previously referred to as *Thellungiella halophila*, and its close relative *Thellungiella parvula* [12, 13] have been proposed as model systems for the study of halophytic traits [14–17]. Compared to other halophytes, *Thellungiella* spp. exhibit lower levels of tolerance but may still be considered true halophytes [6]. The relatively short life cycle and other traits important to efficient experimentation together with their close relatedness to *Arabidopsis* (92 % of average sequence identity with *Arabidopsis thaliana* for *T. salsuginea*) have made them preferred species as extremophile plant model systems [11, 14, 15, 18]. Since the initial introduction of *T. salsuginea* as model system [14, 15, 18], remarkable progress has been made in the elucidation of morphological, physiological, and molecular traits that differentiate this species from the close relative *Arabidopsis* [11]. Such distinctive traits include more succulent and waxy leaves [15, 19, 20], the presence of extra layers of leaf palisade cells and root endodermis and cortex layers compared to *Arabidopsis* [19], a higher content of compatible osmolytes in both control and salt stress conditions [11, 19, 21], and a higher capability to efficiently restrict the Na<sup>+</sup> influx into the roots [11, 22, 23]. Additional distinctive mechanisms of protection from excess salt in *Thellungiella* may include a more efficient regulation of Na<sup>+</sup> fluxes at both plasma membrane [24, 25] and tonoplast levels [26]. These features indicate that *Thellungiella* is preadapted and therefore “more prepared” to efficiently tailor its response to salt stress.

The availability of these resources, coupled with the feasibility of forward and reverse genetics studies in *Thellungiella* spp. which can be compared to its close genetic relative (*Arabidopsis thaliana*), has certainly opened new avenues towards a better understanding of the fundamental mechanisms of plant salt tolerance.

### **1.1 Growth and Maintenance of *Thellungiella* spp.**

*T. salsuginea* and *T. parvula* are very similar to *Arabidopsis* in terms of growth and maintenance requirements, and they can easily be grown in growth chambers and greenhouses. Compared to *Arabidopsis*, however, the life cycle of *Thellungiella* spp. is longer and, for *T. salsuginea*, a long vernalization period is required for

flowering. Seed maturation in these species is more asynchronous than *Arabidopsis*, therefore, extra care should be taken in experimental procedures such as the recovery of transformants which may not be included in the initial wave of germination. Subheading 3.1 describes procedures of growth and maintenance of *Thellungiella* spp. that are critical for obtaining healthy plants and high-quality seeds. Less studied halophytic species can also be considered as valuable model systems [16]. For these, basic growth requirements are briefly presented in Subheading 3.2.

## **1.2 Genetic Transformation Using *Agrobacterium***

As *A. thaliana*, *Thellungiella* spp. can be efficiently transformed via *Agrobacterium*-mediated T-DNA transfer using the simple, straightforward method of the floral dip [27] or, similarly, by spraying flowers with an *Agrobacterium* suspension ([14, 15, 19], Paino D'Urzo and Bressan, unpublished). However, the prolonged asynchronous flowering process in *Thellungiella* spp. requires several repeated rounds of transformation in order to ensure a high percentage of transformants. Subheading 3.3 of this chapter describes a method for large-scale *Agrobacterium*-mediated transformation to generate collections of T-DNA insertional mutants.

## **1.3 Comparative Genomics of ARMS**

Since the completion of its genome in 2000 [28], vast amounts of genetic data have been accumulated and analyzed for *A. thaliana*. This makes the genomes and transcriptomes of *Arabidopsis* ecotypes and *Arabidopsis*-relative crucifers particularly suitable resources for comparative studies. Several comparative gene expression analyses using ESTs produced from plants exposed to various stress conditions, quantitative real-time PCRs, and different types of microarrays have confirmed [10, 21, 29–32] the presence of potentially important and distinctive paralogs that may mediate mechanisms of stress adaptation in *Thellungiella* [11].

Recent advances in next-generation sequencing technology have enabled and accelerated the sequencing and assembly of the genomes of non-model species, including crucifers. In 2011, the genomes of *Arabidopsis lyrata* [33], *T. parvula* [34], and *Brassica rapa* [35] have been published. A first draft of genome sequences of *T. salsuginea*, carried out at the Joint Genome Institute (JGI—US Department of Energy) under the coordination of Schumaker, Wing, and Mitchell-Olds, is available online (<http://www.phytozome.net/thellungiella.php#A>, [17]), and the *Capsella rubella* genome (<http://tinyurl.com/jgi-plans>) is currently being sequenced. The analysis of the *T. parvula* genome has highlighted the presence of over 3,000 predicted open reading frames (ORFs) without BLAST hits in *A. thaliana*, a portion of which may represent novel stress tolerance genes, as well as additional, i.e., duplicated, copies of *Arabidopsis* genes known to be important to stress responses such as *HKT1* and *SnRK2s* [34]. The Gene Ontology (GO) classification of the over 28,000 predicted ORFs of *T. parvula* has also

shown that subcategories of the “biological process” category were over- (“response to abiotic or biotic stimulus”) or underrepresented (“signal transduction”) in *T. parvula* compared to *Arabidopsis*, suggesting a different strategy of response to abiotic stresses in *Thellungiella* compared to *Arabidopsis* [34].

In Subheading 3.4, the tools and resources for comparative genomics in crucifers are described with the recently published *T. parvula* genome as an example [34]. *T. parvula* has a genome slightly larger than *A. thaliana* distributed in seven pairs of chromosomes [36]. With combinations of 454 and Illumina platforms, and assemblers based on different algorithms, contigs of chromosome-arm length (N50 = 5.29 Mb) were produced. The version 2.0 of the genome sequence and annotation are available through <http://thellungiella.org/>.

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## 2 Materials

### 2.1 Plant Growth

#### 2.1.1 Substrates

1. When sowing in soil, a loose and uniform soil potting type is required. Peat-based commercial mixes ensure good water retention as well as good draining properties.
2. Some specific commercial formulations have added fertilizers or bio-protectants against pathogens, such as *Bacillus subtilis*.
3. If root measures are to be performed, inert/light substrates may be preferred, such as perlite/vermiculite/light gravel. When these substrates are used, water retention may be enhanced by mixing them with coir or other fibrous substrates.

#### 2.1.2 Growing Containers

1. The use of standard 8–10 cm diameter plastic pots is frequently adequate.
2. If fine substrates are used, a thin filter on the pot bottom may avoid loss of the growth medium.
3. Plastic tubs such as 90 × 60 × 20 cm can also be used, provided they are equipped with appropriate drainage systems in order to avoid water stagnation ([www.thellungiella.org](http://www.thellungiella.org)).

#### 2.1.3 Water and Nutrients

1. Plants must be watered frequently to maintain a moist root environment and to avoid flooding in order to reduce the risk of anoxia.
2. It is emphasized that no particular watering schedule is appropriate, but must be adapted to the specific conditions present.
3. Once a week it is usually appropriate to apply a modified Hoagland nutrient solution (13.00–18.00 mM N; 0.70–1.50 mM P<sub>2</sub>O<sub>5</sub>; 3.00–5.50 mM K<sub>2</sub>O; 1.50–6.00 mM SO<sub>3</sub>; 1.25–3.50 mM Mg; 3.25–5.00 mM Ca; 10.00–40.00 μM Fe EDTA; 0.50–1.00 μM Cu; 4.00–7.00 μM Zn; 15.00–40.00 μM B; 10.00–15.00 μM Mn; 0.50–1.00 μM Mo).

#### 2.1.4 Pests

1. Bioplasts such as fungi, viruses, and especially insects are often underestimated deterrents to successful molecular manipulations. Many biological solutions (mainly biopesticides) are available and proven effective for greenhouse/growth room environments.
2. Manufacturer instructions should be strictly followed when handling pesticides, but since *Arabidopsis* and *Thellungiella* spp. are not specifically mentioned in the labels, dose test experiments might be required to establish optimal conditions, considering also that specific mutants might respond differently (*see* **Note 1**).

### 2.2 Media for Transformation Using the Floral-Dip Method

#### 2.2.1 Bacterial Growth Media for *Agrobacterium*-Mediated Transformation

1. Yeast extract peptone (YEP): Yeast extract 10 g/L, peptone 10 g/L, sodium chloride 5 g/L. Adjust pH to 7.0 with 0.1N potassium hydroxide (KOH). For plates, add agar 15 g/L. Autoclave-sterilize, typically for 20 min at 121 °C (steam at 15 psi).
2. LB medium: Tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L. Adjust pH to 7.0 with 0.1N potassium hydroxide (KOH). For plates, add agar 15 g/L. Autoclave-sterilize, typically for 20 min at 121 °C (steam at 15 psi).
3. Antibiotics and other heat-labile substances are added after medium is cooled to 55 °C in water bath, prior to pouring medium into suitable container (Petri dishes or else).
4. Antibiotic dosage: Kanamycin 50 mg/L; rifampicin 30 mg/L; gentamicin 30 mg/L; ticarcillin 30 mg/L. Use as required.

#### 2.2.2 *Agrobacterium* Infiltration Medium

MS salt (1/2×) 2.2 g/L, B5 vitamins (1×), sucrose 50 g/L, MES 0.5 g/L, N<sup>6</sup>-benzylaminopurine (BA) 0.01 mg/L, Silwet L-77 200 µL/L. Adjust to pH 5.7.

#### 2.2.3 Antibiotics Preparation

Rifampicin, kanamycin, gentamicin, and ticarcillin antibiotic stocks: these compounds are heat labile and cannot be sterilized by autoclaving.

1. Kanamycin (30 mg/mL)—(dissolve 300 mg in 10 mL H<sub>2</sub>O).
2. Ticarcillin (100 mg/mL)—(dissolve 1.0 g in 10 mL H<sub>2</sub>O).
3. Gentamicin (30 mg/mL)—(dissolve 300 mg in 10 mL H<sub>2</sub>O).
4. Rifampicin (30 mg/mL)—(dissolve 300 mg in 10 mL of methanol).
5. Filter-sterilize using a syringe and a 0.22 µm membrane filter. Aliquot into 1 mL samples and store up to 3 months at -20 °C.

### 2.3 Comparative Genomics

We list programs that aid in the comparison and viewing of genome sequences. It is, however, to be understood that the computational

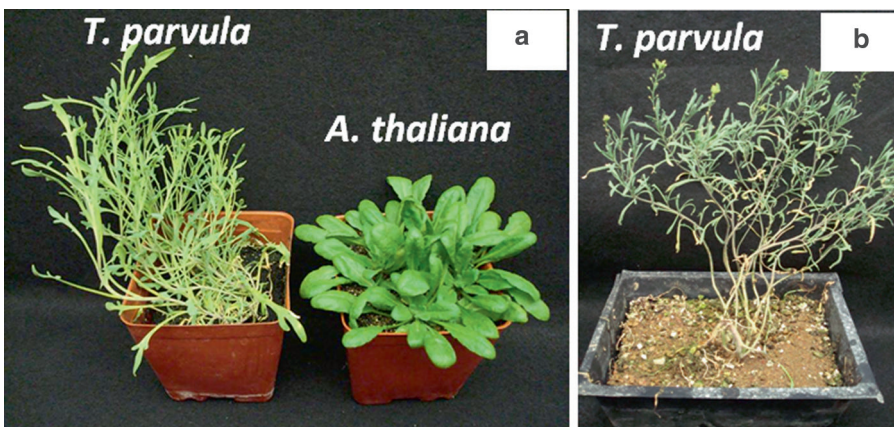
tools for the representation and analysis of genome sequences undergo rapid development and changes.

1. *Nucmer*, included in the MUMmer package [37], is the software suitable for identifying global colinearity between two genomes. For installation and documentation, see <http://mummer.sourceforge.net/>. A computer with a UNIX operating system will be required.
2. *Circos* visualization tool for comparative genomics [38]. For installation and tutorial, see <http://circos.ca/>. Also useful is the Google discussion group: <http://groups.google.com/group/circos-data-visualization>.
3. *MAUVE* [39] sequence alignment tool is suitable for identifying synteny as well as chromosome-scale inversions. For installation and documentation, see <http://gel.ahabs.wisc.edu/mauve/>.
4. Genome-wide as well as localized comparisons of sequences can be performed using the comparative genomics platform available at CoGE <http://genomevolution.org/CoGe/>.

### 3 Methods

#### 3.1 Growth and Maintenance of *Thellungiella* spp.

1. Whereas the *Arabidopsis* cycle can be completed in 6–10 weeks, *T. salsuginea* requires from 16 to 20 weeks from sowing to harvest, in comparison to 12–16 weeks for *T. parvula* (Fig. 1).
2. Growing conditions that heavily influence flowering time and life cycle include light (short/long day), temperature, watering and nutrition, plant density, containers, presence of pests,



**Fig. 1** (a) Adult plants of *Arabidopsis thaliana* and *Thellungiella parvula*. (b) An adult flowering plant of *Thellungiella parvula*



and the type of facilities used, e.g., growth chambers vs. greenhouse.

3. When optimal conditions are maintained as uniformly and consistently as possible, shorter harvesting times and higher seed quality result. Any prolonged stress will result in weak, unhealthy plants, delayed and poorer harvest, or outright plant losses.
4. It is crucial to understand that general plant health has a much larger effect on *Agrobacterium*-based transformation of *Thellungiella* compared to *Arabidopsis*. The activation of the innate immune response controls significantly the ability of *Agrobacterium* to successfully mediate gene transfer in *Arabidopsis*. Because *Thellungiella* species are perennial-like (they continue growth after flowering), it is tempting for convenience to use old plants that continue to flower, but because of previous stress and pathogen episodes (root aphids are a common example), transformation frequency will be very low.
5. Both *T. salsuginea* and *T. parvula* show a greater degree of seed germination variability than *A. thaliana*. This is related to the higher percentage of dormant seeds generally present in *Thellungiella* spp. It is good practice therefore to stratify seeds for several days (1 week) at 4 °C in the dark and to work with seeds of uniform age and good quality.
6. Cold treatment of dry seeds is not effective, whereas seeds maintained in a constant moist environment (either water suspension or moist soil) for 7 days will germinate promptly and more uniformly.

### 3.1.1 Seed Storage and Preservation

1. Seeds should be dried to a moisture content of 5–6 %, by air-drying for about 4 weeks or in desiccators with Drierite or silica gel for 3 or 4 days.
2. Seeds are then commonly stored at room temperature (24–27 °C) in scintillation vials or paper envelopes kept in desiccators. In these conditions, seeds will be viable for at least 3 years and we have experienced *Thellungiella* seeds to preserve viability for 10–12 years. For longer storage time, seeds should be sealed in moisture-proof containers and kept at 4 °C.

### 3.1.2 Seed Germination

1. For sowing, seeds are mixed with sand and distributed on the soil using a salt/pepper shaker to facilitate uniform dispersion. Alternatively, seeds kept at 4 °C in water in the dark for 7 days are further diluted in abundant water and uniformly distributed on soil using a squeeze bottle.
2. Final density of sowing depends on experimental purposes and dictates the ratio of seeds and sand/water to be used.

3. After making sure the mixture of seeds and sand (or the dispersion of seeds in water) is homogeneous, proceed with sowing on well-watered soil.
4. For low density in small pots or limited surfaces (10–20 plants in 4–5 in. pots or in the case of celled trays), dry seeds placed on a piece of paper can be effectively dispersed by tapping.
5. Seeds should not be covered with soil. Pots or containers sowed with dry seeds on moist soil can then be placed at 4 °C in the dark for 7–14 days.
6. Containers should be checked periodically and moved out of the cold room as soon as germination is achieved. This will avoid etiolation of the plantlets.

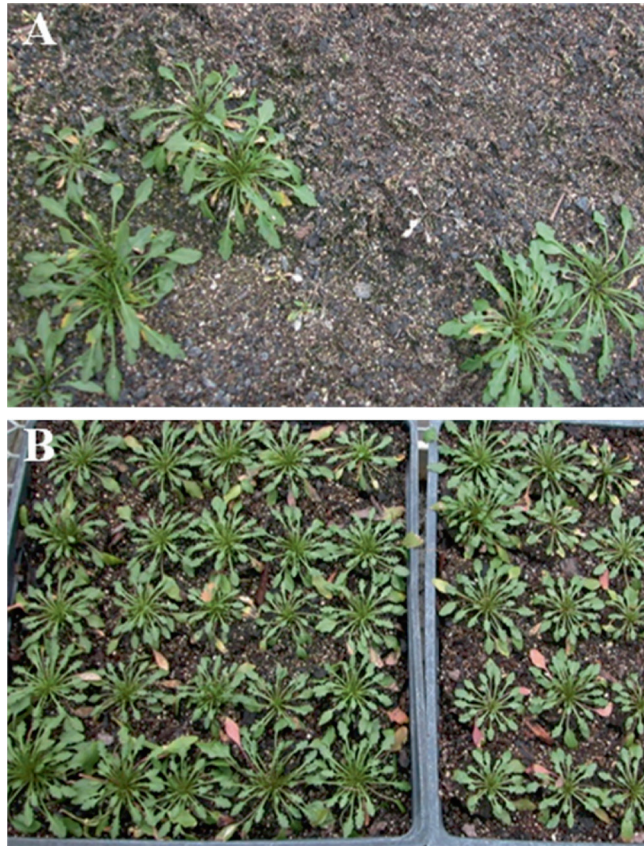
### 3.1.3 Growth Conditions

1. Temperature requirements do not differ greatly for *T. salsuginea* and *T. parvula* and a range between 24 °C at daytime and 18 °C at nighttime with a 16 h photoperiod and a light intensity of 130–150  $\mu\text{mol}/\text{m}^2 \text{ s}$  is adequate. Both species grow well even at wider temperature and light ranges (typically encountered in a greenhouse compared to a growth room or growth chamber) since plants in nature undergo wider day/night fluctuations than those experienced in controlled environments. *Arabidopsis*, as well as *Thellungiella* spp., adapt well to more uniform regimens.
2. *T. parvula* benefits from intense photosynthetic active radiation. Additional lighting might be required in the greenhouse mainly to control photoperiod, depending also on the season and the location.
3. Plants should be watered regularly and thoroughly from above, with a gentle shower, or by infiltration from the bottom. To reduce fungal and algal growth and infestation of fungus gnats (*Mycetophilidae* and *Sciaridae*), containers/soil should drain well the extra water and the soil surface should be allowed to dry between watering.
4. Young plantlets will require thinning at about 3 weeks from germination. This will allow the remaining plants to grow stronger.

### 3.1.4 Vernalization

1. *T. salsuginea* requires vernalization. In order to promote uniform flowering, plants of 4–5 cm in diameter (Fig. 2b) should be well watered and then placed at 4 °C for 21–28 days, with a 16-h photoperiod. Plants should not require much care at this stage. The use of plastic domes (generally utilized for propagation) can reduce dehydration and watering intervention.
2. Vernalization can be initiated any time after germination, but in general older plants require longer vernalization times. After vernalization, plants are placed at normal growing temperature and watered regularly (*see Note 2*).





**Fig. 2** *Thellungiella salsuginea* plants resistant to glufosinate. Panel (a) shows glufosinate-resistant seedlings of *T. salsuginea* transformed with vector pSK115 15 days after treatment with 5 mg/L. (b) An aerial view of flats of young *T. salsuginea* seedlings resistant to glufosinate after transfer from the selection tray (source: <http://thellungiella.org/>)

#### 3.1.5 Post-flowering Maintenance

1. *T. salsuginea* bolts and grows upright, while *T. parvula* has a recumbent habit (Fig. 1). It is helpful to train older plants of both species which can form numerous branches by tying them to wooden skewers. Tied bundles also facilitate final harvest. When plants begin to dry, whole stalks can be cut for seed collection.
2. It is important to recognize that *Thellungiella* seeds will mature much more asynchronously than *Arabidopsis*. It is advisable to repeat harvest as needed (see **Note 3**).
3. Regular and frequent watering is required during flowering and until siliques are well formed (see **Note 4**).
4. Watering should be gradually decreased in proximity of plant senescence.

### 3.1.6 Seed Harvest

1. Harvest generally occurs 2–4 weeks after termination of watering. When appropriate, plants can be harvested in bulk by cutting the stalks at the base and letting them undisturbed to dry completely on large sheets of paper (brown packing type).
2. Seeds are threshed by hand rolling and cleaned through sieves and strainers. Common tea strainers are very useful. Several passages through different size sieves might be necessary to clean seeds from all residues and debris.

### 3.1.7 Pests

1. Scouting for pests should be done regularly, since high density of plants of the same species in a controlled environment, at optimal growing conditions, in the absence of natural antagonists, increases the chance of pest attacks. Early detection and prompt intervention are critical to avoid major pest explosions.
2. Good watering and fertilization regimens, as well as adequate ventilation, are essential to ensure healthy plants. Vigorous plants are less susceptible to pests and diseases and will also respond better to treatments.
3. A short list of most common problems we encountered and effective measures to solve them follows, with indications on specifically biological antagonists (*see Note 5*).
  - (a) Powdery mildew (*Erysiphe* spp.)—sulfur is effective.
  - (b) Fungus gnats (*Mycetophilidae* and *Sciaridae*)—controlled by the nematode *Steinernema feltiae* and by *Bacillus thuringiensis israelensis*.
  - (c) Thrips (*Thysanoptera*)—generally not as lethal as on *Arabidopsis*, still require care and spraying with insecticides approved for thrips, in case of heavy infestation. The predatory mites *Neoseiulus cucumeris* and *Hypoaspis miles* have been proven effective.
  - (d) Aphids—a prompt intervention is key, as well as all measures aimed at limiting insect presence in the greenhouse (screens on all openings, reduced traffic, use of coats when entering each greenhouse contained area). Insecticidal soaps are helpful, though repeated or too extensive treatments can damage tender parts of the plants (inflorescences).
  - (e) Root aphids—since they affect roots, they are not as easily detected as other aphids. Symptoms are pronounced leaf yellowing and slow growth. Plants stop developing with consequent dramatic reduced seed maturation and yield (*see Note 6*).

## 3.2 Other Candidate Halophytic/ Extremophyle Model Species

Whereas *Thellungiella* spp. have been so far the most studied *Arabidopsis* relatives, other related crucifers are subject to an increasing interest due to specific characteristics, such as high tolerance to heavy metals in *Thlaspi* spp. [40–43]. A short description

of some of the most promising candidates is provided, describing main features of their life cycle and plant development. Table 1 summarizes light, photoperiod, temperature, and watering requirements for the described species.

### 3.2.1 *Barbarea verna*

*B. verna* is a biennial herb native from Eastern Europe and southwestern Asia, usually found in damp soils, roadsides, or waste places.

1. Seeds should be planted shallowly, at about 1 cm depth. Germination occurs in 1–2 weeks from sowing, while in 4 weeks about two to four true leaves are found.
2. Flowering starts about 6–7 weeks from sowing, when the plant has about ten leaves.
3. At full maturity, the plant may reach size of 0.3 m width and 0.3 m height.
4. Flowers are hermaphrodite and the plant is self-fertile.

### 3.2.2 *Capsella bursa-pastoris*

*C. bursa-pastoris*, also known as shepherd purse, is an annual plant, native from Eastern Europe, usually found in arable lands, waste areas, and road margins.

1. Seeds will germinate in 1–2 weeks from sowing and will present 2–6 leaves in 4 weeks and about 15–20 leaves in 6 weeks, when first flowers may appear.
2. At full maturity, the plant may reach size of 0.3 m width and 0.2–0.5 m height.
3. Flowers are hermaphrodite and the plant is self-fertile.

### 3.2.3 *Descurainia pinnata*

*D. pinnata* is an annual or biennial plant, native from desert regions from Nevada southward into north central and northwestern Mexico. It is also native to deserts of North Africa and the Middle East. It is usually found in sandy fields, gravel, white saline areas, dunes, open desert, waste ground, disturbed sites, open woods, prairies, glades, roadsides, and railroads. It may grow in sterile soils, such as sandy or gravelly, although in fertile soil, the plant will be larger in size.

1. Germination occurs in about 2 weeks from sowing, and the plant will develop a rosette in about 4–6 weeks, with stems in which flowers will thereafter appear.
2. At this stage, blooming will start and last about 2 months. At full maturity, the plant may reach size of 0.3 m width and 0.6 m height.
3. Flowers are hermaphrodite and the plant is self-fertile.
4. Plants exhibit extreme soil desiccation tolerance, based almost entirely on root growth characteristics.

**Table 1**  
**Other candidate halophytic/extremophile model species**

	<b>Common name</b>	<b>Ploidy</b>	<b>Radiation (<math>\mu\text{mol}/\text{m}^2 \text{ s}</math>)</b>	<b>Photoperiod (h light/dark)</b>	<b>Temperature (°C, min–max)</b>	<b>Watering</b>	<b>References</b>
<i>Barbarea verna</i>	Yellow flower, winter cress	$n=8$ $2n=16$	250–1,000	16/8	14–24	Frequent, avoid flooding	[16, 44]
<i>Capsella bursa-pastoris</i>	Shepherd's purse	$n=8, 16$ $2n=16, 32$	250–1,000	16/8	14–24	Can tolerate mild drought stress	[16, 40, 44–47]
<i>Descurainia pinnata</i>	Western tansy mustard	$n=7$ $2n=28$	500–1,000	16/8	18–24	Can tolerate drought stress	[16, 44, 48]
<i>Hirschfeldia incana</i>	Conil yellow, Mediterranean hoary-mustard	$n=7$ $2n=14$	500–1,000	16/8	16–24	Can tolerate mild drought stress, avoid flooding	[16, 40–42, 44]
<i>Lepidium</i> spp.	Common pepperweed, prairie peppergrass Virginia pepperweed	$n=16$ $2n=32$	100–1,000	16/8	15–24	Can tolerate mild to severe drought stress	[16, 44, 47, 49, 50]
<i>Malcolmia triloba</i>	Conil blue	$n=7, 14$ $2n=28$	500–1,000	16/8	12–20	Frequent, but avoid flooding, may tolerate mild drought	[16, 44]
<i>Sisymbrium officinale</i>	Hedge mustard	$n=7$ $2n=14$	500–1,000	16/8	14–24	Frequent, but avoid flooding	[16, 44, 47]
<i>Thlaspi arvense</i>	Pennycress	$n=7$ $2n=14$	250–1,000	16/8	12–20	Frequent	[16, 43, 44, 47]
<i>Arabisopsis lyrata</i>	Northern rock cress	$2n=16$	250–400	16/8	8–16	Frequent	[33, 61–64]
<i>Cakile maritima</i>	Sea rocket	$n=9$ $2n=18$	500–800	16/8	12–25	Not frequent/scarcely	[65–69]

- 3.2.4 *Hirschfeldia incana* *H. incana* is a perennial plant, native to the Mediterranean basin, usually found in waste places, roadsides, and canyons.
1. Germination occurs in 1–2 weeks from sowing, with development of a rosette of lobed leaves within 4–5 weeks, from which stems will develop, covered by dense, soft, and white hairs.
  2. Flowers will set between 6 and 12 weeks from sowing and blooming may last a few months.
  3. At full maturity, the plant may reach size of 0.5 m width and 1 m height.
  4. Flowers are hermaphrodite and the plant is self-fertile.
- 3.2.5 *Lepidium spp.* *Lepidium* spp. are perennial plants native to Eurasia, but spread in all continents, except Antarctica. They are usually found in sandy soil, waste places, coastal regions, sea cliffs, dry creek beds, and dry plains.
1. Germination occurs in 1 week from sowing.
  2. The plant reaches full size (0.10–0.50 cm tall) within 8–10 weeks, in the shape of a rosette of lobed leaves from which the flowering stems will develop.
  3. Flowers are hermaphrodite and the plant is self-fertile.
  4. Plants present considerable salt tolerance close to *Thellungiella* [16].
- 3.2.6 *Malcolmia triloba* *M. triloba* is an annual plant native to Asia and the Mediterranean region and usually found in waste and disturbed areas, gravel pits.
1. Seeds germinate in 1–2 weeks.
  2. Flowers will appear in 6–8 weeks, when plant will reach their full size (0.15–0.50 m height).
  3. Flowers are hermaphrodite and the plant is self-fertile.
- 3.2.7 *Sisymbrium officinale* *S. officinale* is an annual or biennial plant native to the Mediterranean region and usually found in disturbed sites.
1. Germination occurs in 1–2 weeks.
  2. Plants reach full size (up to 1 m height) in 5–8 weeks, when flowering starts. Blooming lasts 2 months.
  3. Flowers are hermaphrodite and the plant is self-fertile.
- 3.2.8 *Thlaspi arvense* *T. arvense* is an annual plant native to central and western Asia, usually found in roadsides and waste places.
1. Germination will occur in 2 weeks, and the plant will develop the basal rosette of glabrous leaves within 4–6 weeks.
  2. Flowering will start 8–12 weeks after sowing, when plants have reached their full size (about 0.75 m height).

3. Flowers are hermaphrodite and the plant is self-fertile.
4. *Thlaspi* species are notably tolerant of heavy metals.

### 3.2.9 *Arabidopsis lyrata*

*A. lyrata* (also known as northern rock cress) is the closest well-studied relative of *A. thaliana*. It may complete its cycle within a single season, but is normally a perennial. Native to cool temperate areas around the Arctic, it is usually found in disturbed habitats, with scarce vegetative competition, such as humid rocky places, coastal cliffs, pine forests, or sandbars.

1. Germination time is within 2–3 weeks from sowing and the plant presents a simple rosette within 4–6 additional weeks.
2. It may reproduce vegetatively via stolons or gamically via insect pollination, producing a high number of seeds.
3. Flowering starts 8–12 weeks after sowing.
4. Differently from *A. thaliana*, *A. lyrata* plants are outcrossing diploids.
5. The *A. lyrata* genome has been sequenced.

### 3.2.10 *Cakile maritima*

*C. maritima* (also known as sea rocket) is an annual plant sometimes behaving as perennial. Native to Europe, is an invasive species in North America that grows easily along the coast often in sand dunes.

1. Germination occurs in 2–3 weeks.
2. Flowers will set after 6–8 weeks, when plants reach their full size of 0.3 m height.
3. The plant is easily grown on a well-drained sandy soil at high solar radiation and can tolerate salt exposure.

## 3.3 Genetic Transformation Using *Agrobacterium*

### 3.3.1 *Agrobacterium* Transformation

1. *Agrobacterium tumefaciens* transformation of *T. parvula* and *T. salsuginea* can be carried out following the floral-dip method widely used for *Arabidopsis* [27].
2. *Agrobacterium*-mediated transformation has been successfully obtained for both species, with similar degrees of efficiency (0.1–2 %), either by flower-dip or spraying techniques, using the bacterial strain GV3101.
3. Since the aim of both techniques is to infect the maximum number of unopened floral buds, and considering that flowering is not synchronous, several *Agrobacterium* treatments are required, generally at 3–5 day intervals.
4. For random activation tagging mutagenesis, the vector pSKI15 [51] is used.
5. Start from a frozen glycerol stock of *A. tumefaciens* GV3101 (pMP90RK) (C58 derivative) stored at –80 °C.



6. The *Agrobacterium* strain GV3101 was transformed with a binary vector (pSKI15) for activation T-DNA insertional mutagenesis. The pSKI15 plasmid contains four transcriptional enhancers derived from the cauliflower mosaic virus (CaMV) 35S RNA promoter cloned in tandem near the right border sequence and an expression cassette for herbicide resistance (*bar* gene, encoding phosphinothricin acetyltransferase).
7. *Agrobacterium* selectable markers are:
  - (a) Resistance to ampicillin/Ticar/carbenicillin (pSKI15).
  - (b) Resistance to gentamicin (Ti plasmid).
  - (c) Resistance to kanamycin (Ti plasmid).
  - (d) Resistance to rifampicin (GV3101).
8. The T-DNA also contains a bacterial origin of replication (*oriC*) for plasmid rescue in *Escherichia coli*.
9. To culture *Agrobacterium* for transforming plants, chip off pieces of frozen culture with a 200  $\mu$ L pipette tip from the  $-80^{\circ}\text{C}$  *Agrobacterium* stock and inoculate 5 mL YEP or LB medium plus appropriate antibiotics in culture tubes ( $25 \times 150$  mm).
10. Incubate on a shaker in the dark at  $28^{\circ}\text{C}$  for 24 h at 230 rpm. The medium should look saturated (cloudy,  $\text{OD}_{600} = 1.5\text{--}2.0$ ).
11. Add 3 mL of culture to a larger amount of medium (YEP or LB+antibiotics) in a flask, according to the amount of plants to transform. 500 mL of grown culture are sufficient for floral dipping of three pots of 5 in. in diameter containing ten plants each. The volume of medium should be no more than 1/5 of the volume of the flask, to assure proper ventilation during shaking.
12. Incubate on a shaker at  $28^{\circ}\text{C}$  and 230 rpm to an  $\text{OD}_{600}$  of  $= 1.5\text{--}2.0$  (16–18 h).
13. Centrifuge the culture to form a pellet ( $4,500 \times g$  for 20 min). Decant the supernatant and add about half of the original culture volume of infiltration medium (*see* Subheading 2) into the bottle.
14. Resuspend the pellet completely by vigorous shaking and dilute the suspension with infiltration medium to a final  $\text{OD}_{600}$  of 0.8–1.
15. Proceed with floral dip or plant spraying.
16. In order to avoid rapid dehydration of the *Agrobacterium* infiltration solution from the flowers, plants should be protected from air and covered for a period of 24–36 h (flowers should stay wet for at least 24 h). This can be obtained in situ, covering the plants with plastic sheets, or moving plants to closed cabinets or similar structures (*see* **Note 7**).

17. Post-transformation care is identical to the standard plant growth conditions described above. As indicated early, the health of the plants has a dramatic effect on transformation efficiency and may even cause total failure.

### 3.3.2 Identification of *T<sub>1</sub>* Plants Based on Herbicide Resistance Gene Expression

For a large amount of seeds, it is convenient to screen transformants directly in the soil; hence the use of a herbicide-tolerance selective marker is advantageous. The following protocol has been extensively utilized for *T. salsuginea* (and *A. thaliana*), for the production of large tag-insertional mutagenesis line collections:

1. Typically, 1 g of seeds harvested from *Agrobacterium*-treated plants is uniformly sowed in greenhouse flats (53 × 27 × 5 cm) with loose soil previously well watered. Use a sand/seeds mixture (1 part of seed to 2–4 parts of sand) and distribute the seeds using the salt-pepper shaker method. Proceed as previously described for stratification and germination.
2. Begin spraying the seedlings with the herbicide immediately after plantlets form the first pair of true leaves. For herbicide application, use a final concentration of 5 mg/L of glufosinate ammonium (active ingredient). One liter of diluted solution is sufficient for about ten flats (*see* **Note 8**).
3. Spray for 3–5 consecutive days each week until clear distinction between dead vs. surviving plants is visible. Repeated herbicide treatments may be required depending on plant density and uneven germination.
4. Transformed plants should continue to grow undisturbed (Fig. 2a). Mark survivor plants from earlier germination with toothpicks, while secondary germination is starting. This is important to help avoid later generating non-transformed plants that escape herbicidal effects.
5. Transplant putative transformants to 25 × 25 × 5 cm trays, 25 plants/tray (Fig. 2b).
6. Protect plants from transplant shock by immediately watering them and covering them with propagation domes and/or by shading for 1–2 days.
7. Once plants are established (4–5 cm diameter rosette, 4–6 weeks), vernalize at 4 °C, following the vernalization procedure above described.
8. For large-scale mutagenesis, seeds collected from each tray are pooled for further study. Alternatively, *T<sub>1</sub>* plants can be isolated in vitro (*see* **Note 9**).
9. Frequency of transformed plants recovered can be determined by PCR of the insertion plasmid and or herbicide gene sequences.

**Table 2**  
**Genomic tools developed for *Thellungiella* spp.**

Tool	Species	Tissue of origin/stress treatment	Notes	Database accession nos.	Reference
EST collection	<i>T. salsuginea</i>	Seedlings/salt	About 1,700 ESTs sequenced	GenBank, see paper for Acc. Nos.	[52]
EST collection	<i>T. salsuginea</i> Yukon	Adult plants, aboveground tissue/chilling, freezing, salt acclimation, salt shock, drought stress	6,578 ESTs were sequenced from cDNA libraries obtained from different treatments	GenBank, Acc. Nos. DN772677–DN779205	[31]
cDNA library	<i>T. salsuginea</i> Shandong	Several tissues/chilling, freezing, salt, ABA	20,000 full-length enriched <i>Thellungiella</i> cDNAs (RTFL) were generated	DDBJ, Acc. Nos. BY800476–BY835646; Clones available at: <a href="http://www.brc.riken.go.jp/lab/epd/Eng/">http://www.brc.riken.go.jp/lab/epd/Eng/</a>	[53]
EST collection	<i>T. salsuginea</i> Shandong	Whole plants/salt	946 EST sequences generated	GenBank, Acc. Nos. EC598928–EC599965	[54]
Microarray chip	<i>T. salsuginea</i> Yukon	N.A. <sup>a</sup>	ESTs spotted on the chip. Specifically developed for <i>Thellungiella</i> , it can analyze “novel” genes	N.A. <sup>a</sup>	[32]
BIBAC Library	<i>T. salsuginea</i>	Partially digested <i>T. salsuginea</i> genomic DNA	BIBAC (binary bacterial artificial chromosome) library expected to cover 4× genome of <i>salsuginea</i> was generated	N.A. <sup>a</sup>	[55]

<sup>a</sup>Not applicable

### 3.4 Tools for Comparative Genomics Analyses of *Arabidopsis* Relatives: An Example with *T. parvula*

In addition to the genome sequences [17, 34], several genomic tools have been developed for *T. parvula* and *T. salsuginea*, including EST and cDNA libraries, BiBAC libraries, and microarray chips ([11], Table 2). Below, we describe methods to compare, align, and assemble genomic contigs and scaffolds of a newly sequenced crucifer species.

The chromosome structures of plant species within the *Brassicaceae* family have been studied with comparative chromosome painting (CCP) techniques using pools of *A. thaliana* BACs as probe [56, 57]. The ancestral *Brassicaceae* genome was inferred to contain 24 ancestral karyotype (AK) blocks, named A to X, which constitute eight chromosomes [56]. Genomes of most crucifer species consist of combinations of these AK blocks in different numbers of chromosomes [57]. For example, crucifers in the Lineage II with  $2n=14$  karyotypes, including *Thellungiella*, evolved from eight ancestral *Brassicaceae* chromosomes to genomes with seven chromosomes after multiple translocation and inversion events [58]. As an example, here we describe current tools to compare the genomes of a newly sequenced ARMS (*T. parvula*, *Tp*) to the genome of the model plant, *A. thaliana* (*At*). Comparison of the larger *Tp* contigs with the AK blocks in the *At* genome helped the assembly of the seven *Tp* chromosome pseudomolecules [34].

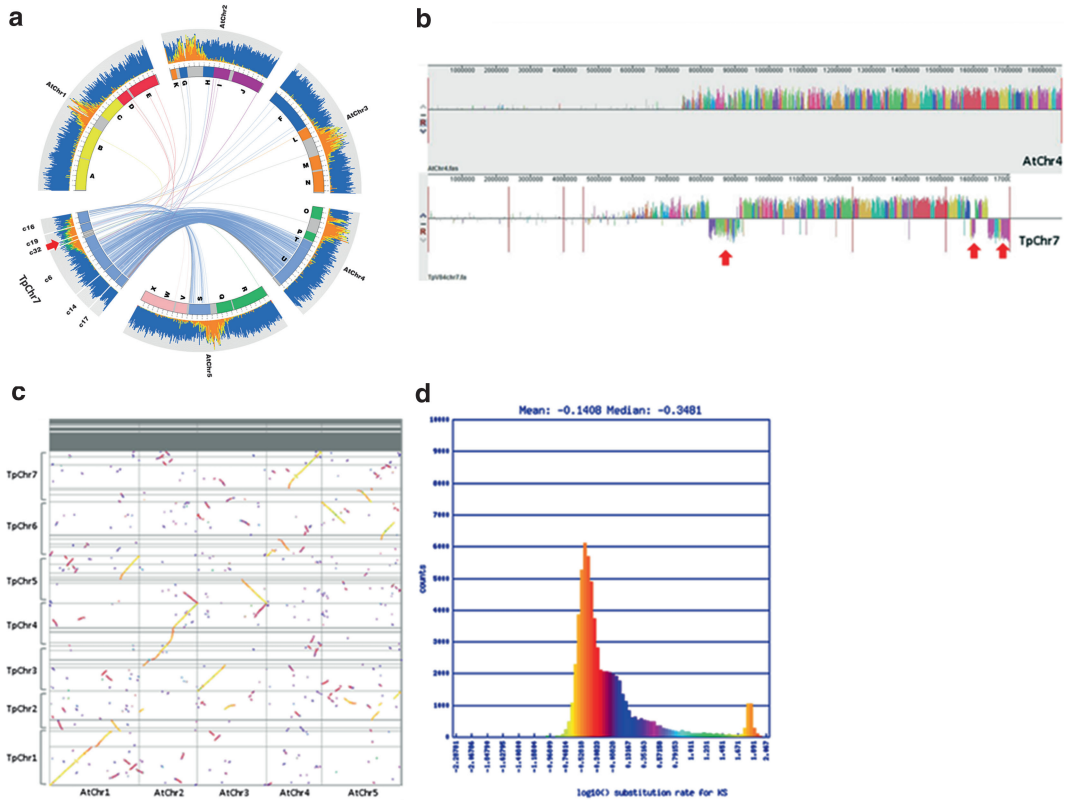
### 3.4.1 Identification and Visualization of Global Synteny Using Nucmer and Circos

#### Alignment of Genomes Using Nucmer

1. The genome contigs and scaffolds were aligned as FASTA files with the *At* genome sequence using Nucmer. An example of command line and parameter is “\$ nucmer --maxmatch --maxgap 1000 --prefix <project\_name> <input\_genome\_sequence\_file\_name.fasta> <At\_genome\_file\_name.fasta>”
2. Run delta-filter. An example of command line and parameter is “\$ delta-filter -r -q -l 500 project\_name.delta> project\_name.filter”
3. Run show-coords. An example of command line and parameter is “\$ show-coords -c -d -l -r -T project\_name.filter> project\_name.txt”
4. The resulted file should contain the coordinates of genomic regions that show sequence similarity with genomic regions from *At* in text delimited file format.

#### Visualization of Nucmer Results with Circos

1. Circos is a visualization tool for comparative genomics that runs with a configuration file. The alignment results obtained using Nucmer can be fed into Circos as <links> in the configuration file. An example of visualization comparing *Tp* chromosome 7 with five *At* chromosomes is shown in Fig. 3a.
2. When *Arabidopsis*-relative crucifer genome sequences are compared with those of *At*, extensive colinearity is usually found. If a karyotype model with AK blocks is available [9] for the *Arabidopsis*-relative species, the contig/scaffolds can be mapped to the model by comparing them with the *At* genome. Using the Nucmer alignment result obtained as described earlier in this section (*Alignment of Genomes Using Nucmer*), identify which *At* AK blocks the contig/scaffolds show colinearity. The coordinates of AK blocks in the *At* genome are available in Schranz et al. [56]. For example, *Tp* contigs c16 and c19 are colinear with the AK block S in *At* genome (Fig. 3a). Similarly,



**Fig. 3** Tools for identification and visualization of synteny between the genome sequences of *Thellungiella parvula* (*Tp*) and *Arabidopsis thaliana* (*At*). (a) Synteny between *Tp* chromosome 7 and *At* genome. *Tp* genome contigs that are colinear to the ancient karyotype (AK) blocks S, T, and U are assembled to *Tp* chromosome 7 according to the model developed by comparative chromosome painting (CCP) results [56–58]. Synteny regions were identified using Nucmer [37] and visualized with Circos [38]. The outer histogram shows the distribution of genes, retrotransposons, DNA transposons, and unidentified repetitive sequences in blue, orange, yellow, and green, respectively. The red arrow indicates the centromeric region of the *Tp* chromosome 7. (b) Synteny blocks between *At* chromosome 4 and *Tp* chromosome 7 were identified by MAUVE [39]. Genomic regions with sequence similarity were indicated with the same color between the two chromosomes. Red arrows identify inversions. (c) Identification of genome-wide synteny between *At* and *Tp* using SynMap (<http://genomevolution.org/r/r4gyq>) included in the CoGe tools [59, 60]. The protein-coding sequences (CDSs) of *At* and *Tp* were compared and dots were plotted where the coding sequences from the two species show similarity. Colors of the dots indicate the synonymous nucleotide substitution ratio ( $K_s$ ) as indexed in (d)

c32, c6, c14, and c17 showed synteny with AK blocks T and U in *At*. Since chromosome 7 of *Thellungiella* species consists of AK blocks S, T, and U [57, 58], the contigs c16, c19, c32, c6, c14, and c17 can be mapped to *Tp* chromosome 7 (Fig. 3a).

3. If genome annotation is available, the distribution of coding sequence (CDS) and transposable element (TE) can be plotted in the Circos diagram as a histogram. The correct chromosome assembly will reveal a TE-rich centromeric region, as indicated with a red arrow in Fig. 3a.

### 3.4.2 Alignment of Genomes Using MAUVE

MAUVE [39] is a sequence alignment tool suitable for identifying synteny as well as chromosome scale inversions.

1. MAUVE runs with a graphical user interface and takes two or more genome sequences in FASTA format as input. Figure 3b displays an example of MAUVE results comparing the *At* chromosome 4 and *Tp* chromosome 7, as assembled above using Nucmer.
2. When mapping the genome contig/scaffolds to the chromosome model with AK blocks, MAUVE is particularly useful support tool in deciding the direction of each contig/scaffold.

### 3.4.3 Comparison of *Tp* and *At* Genomes Using CoGe

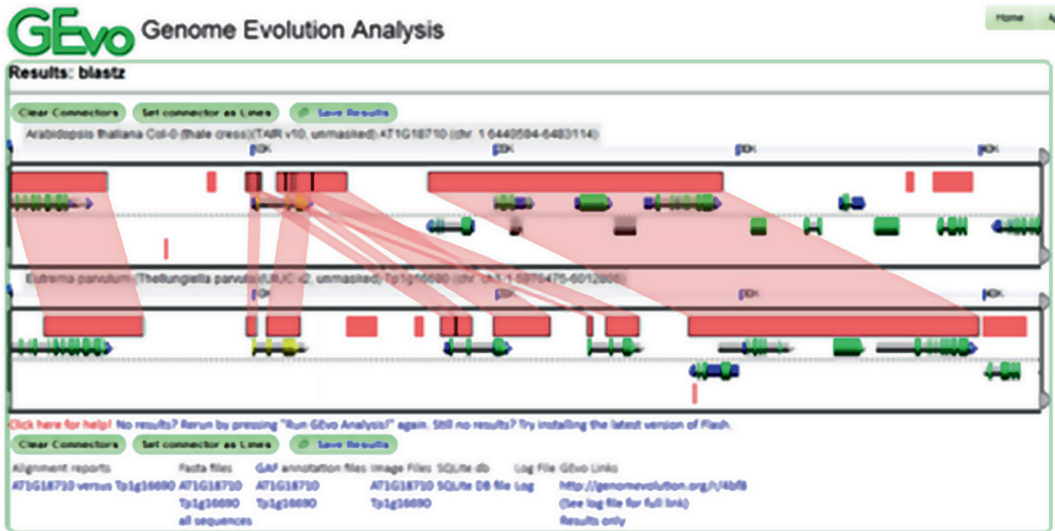
#### Genome-Wide Comparison Using SynMap

1. The version 2 of *Tp* genome and annotation is available for comparative studies in CoGe database (<http://genomeevolution.org/CoGe/>) [59, 60]. Using SynMap, a tool included in CoGe, the *Tp* genomes and coding sequences can be compared to any genome deposited in CoGe. Comparison of the entire *Tp* CDSs with those of *At* is available in <http://genomeevolution.org/r/4gyq>. The comparison is visualized in a dot plot (Fig. 3c).
2. When the annotation of CDS available for both species is being compared, SynMap calculates synonymous substitution rate ( $K_s$ ) for all homologous CDS pairs between the two species in comparison and generates a histogram of  $K_s$  values (Fig. 3d). The dot plot will be colored according to the  $K_s$  value of the CDS pairs. In Fig. 3c, the lines with yellow dots consist of CDS pairs with  $K_s$  values around or less than 0.3, while red dots indicate  $K_s$  values around or larger than 0.5. CDS pairs with  $K_s$  values higher than 0.8 are shown with blue dots (Fig. 3d). SynMap generates links to the sequences of all homologous CDS pairs, as well as the list of tandem duplicated CDSs.
3. Part of the SynMap can be magnified by clicking and dragging with the mouse cursor. The magnification will be shown in a separate window. Clicking any dots in this magnified window will open another tool, GEvo, for comparison in higher resolution.

#### Localized Comparison of Genomic Features Using GEvo

1. There are two different ways to start GEvo. Firstly, clicking a dot in the magnified SynMap window will open the GEvo analysis around the selected dot. Secondly, the name of the CDS can be directly entered from the GEvo window (<http://genomeevolution.org/CoGe/GEvo.pl>). Entering the CDS name in the "Name:" window will automatically bring up the genomic sequences around the CDS. For example, entering "AT1G18710" as the name for Sequence 1 and "Tp1g16690" for Sequence 2 and pressing "Run GEvo Analysis!" button will bring up Fig. 4. More than two sequences can be compared by clicking "Add sequence."
2. The pink ribbons in the example presented in Fig. 4 indicate genomic regions showing homology or high-scoring segment





**Fig. 4** Comparison of homologous genomic regions of *At* and *Tp* using GEvo. *T. parvula* genome sequence and annotation is available for comparative genomic studies in CoGe database (<http://genomeevolution.org/CoGe/index.pl>). Shown is an example snapshot of GEvo results (<http://genomeevolution.org/CoGe/GEvo.pl>), part of the CoGe toolbox, comparing the genomic regions near the *AtMYB47* (*At1g18710*) and the three putative *TpMYB47* homologs (*Tp1g16690*, *Tp1g16700*, and *Tp1g16710*). Pink ribbons indicate blocks with sequence similarity between the two species. Gene models are shown as cylinders with exons, introns, and noncoding conserved sequences in green, gray, and blue colors

pairs (HSPs). By clicking on the ribbon, the link between homologous regions will be toggled on and the alignment between the two regions will appear in a separate window. The link can be toggled off by clicking one of the ribbons connected by it again. The gene models are shown as cylinders. Clicking the cylinder will bring up a separate window containing the annotation and information of the CDS, as well as the link to the CDS sequence.

3. The example in Fig. 4 shows a local tandem duplication event specific to *T. parvula*, where putative *Tp* homologs of *AtMYB47* were amplified to three copies. GEvo is suitable for browsing and analyzing local tandem duplication in detail.

## 4 Notes

1. If using pesticides, it is imperative to alternate product types to reduce occurrence of resistance in the pest population. Furthermore, not all products are licensed to be used in controlled environments and regulations and product availability differ in different countries. Restricted entry intervals (REI), i.e., the period of time after plants and/or soil is treated with a pesticide during which restrictions on entry are in effect to protect

persons from potential exposure to hazardous levels of pesticide residues and protective measures should be adopted.

2. The EMS mutant162 of *T. halophila* does not require vernalization. This mutant flowers very early and has a smaller size allowing it to be manipulated much like *Arabidopsis* (Bressan, personal communication).
3. Waiting for dehydration of the whole plant may lead to non-uniform silique dehiscence and consequent seeds loss, especially transformed seeds if the maturation of the flowers that were sprayed with *Agrobacterium* is not followed closely.
4. At this stage, watering should be done carefully at the base of the plants or by bottom infiltration. Above-canopy watering will cause seed loss.
5. In general, with the possible exception of powdery mildew, *Thellungiella* spp. seem to be less prone to pest and disease infestations than *Arabidopsis*. To date, we have not observed impatiens necrotic spot virus (INSV) on *Thellungiella* species.
6. Root aphid infestation is a good example of a condition that will demonstrate to affect the transformation efficiency without alarmingly affecting the appearance of the plants.
7. It is very important to avoid overheating conditions and provide adequate shading.
8. The herbicide diluted solution can be kept for several days, in the dark, since light promotes the herbicide degradation.
9. In case of in vitro isolation of T<sub>1</sub> plants, using glufosinate ammonium (Crescent Chemical Company, Islandia, NY), we have noticed that whereas the response of *A. thaliana* is optimal at 5 ppm (5 mg/L), the response of *T. parvula* appears more variable, displaying some plants with higher tolerance to the herbicide. This species seems to respond more slowly to the action of the herbicide, resulting in a suggested optimal concentration of 10 mg/L. The response of *T. salsuginea* to in vitro herbicide screening is also slower than *A. thaliana*. Resistant plants can be hardened by moving them onto regular medium without herbicide before being transplanted into soil.

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