Suppressor Screening with an Auxin-resistant Mutant *axr1-12* of *Arabidopsis thaliana*

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Abstract *axr1-12* was used as the original mutant to screen secondary mutants those were more sensitive to auxin and could develop siliques apparently. 38 mutants were isolated and two un-allelic recessive suppressor, *sa1* and *sa2*, were characterized. Results showed that mutations in *sa1* and *sa2* altered the leaf shape and the plant posture, and suppressed *axr1-12* in root response to 2,4-D. *sa1* and *sa2* also recovered the defect of *axr1-12* in silique. However, abnormal seed development was observed in these two mutants. Siliques of *sa1* developed both seeds and seed-like structures with no embryos, while siliques of *sa2* had seed setting partially and some ovules arrested in the full-grown siliques. Results suggest that proteins coded by *sa1* and *sa2* might change the response of siliques to auxin, and *sa1* may play roles in controlling initiation of seed development before fertilization.

Key words *Arabidopsis*; auxin response; suppressor; silique development; *axr1-12*

In *Arabidopsis*, several auxin response loci were identified by screening mutants that are resistant to auxin. Most of these genes have been characterized at the molecular and biological level. They are involved in the physiological and morphological events associated with auxin action. Axr1 is a gene identified and characterized in such way. It codes a protein (AXR1) required for rapid auxin responses [1–3]. AXR1 is a subunit in the RUB-activating enzyme [4]. It heterodimerizes with ECR1 to form the ubiquitin-activating enzyme E1 and conjugates the ubiquitin-related protein RUB to members of the cullin family, and thus it is required for the normal function of the E3 SCF^{TIR1} and has a role in protein degradation [4–6]. The degradation of the Aux/IAA family, like AXR2 (IAA7) [7], AXR3 (IAA17) [8] and AXR5 (IAA1) [9], by the ubiquitin-proteasome pathway through interacting with SCF ubiquitin E3 ligase complex results in derepression of auxin-regulated genes [9–11]. Thus the AXR1 could regulate diverse aspects related to auxin response through the protein degradation pathway.

Mutations in the AXR1 gene result in reduction in auxin response and diverse defects in auxin-regulated growth and development [1]. The mutant *axr1-12*, carrying a mutation in AXR1, was originally isolated in a screen for auxin-resistant seedlings. It displays reduced hypocotyl elongation in the dark, lack of apical hook formation, a reduction in apical dominance, and defects in leaf, inflorescence, and flower morphology. It produces significantly less pollen, and fails to elongate the filaments, and as a result, it exhibits greatly reduced fertility and does not produce siliques normally [1,11].

Although a number of studies have been performed focusing on the AXR1 and auxin signaling, the molecular mechanism of AXR1 in regulating development, especially the seed and fruit development of arabidopsis is unclear. As suppressor isolation is one of the best ways to further investigate gene function and to define additional loci (or genes) that affect the same developmental process as the original mutation, some studies were performed successfully in identifying the components involved in auxin response [13,14], ABA biosynthesis [15], gibberellin signal transduction [16,17], hypocotyl growth [18] and even the RNA slicing [19]. To further understand the function of the AXR1 protein, a screen for suppressors which are able to recover the phenotype of *axr1-12* in silique development was performed in this research.

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The main purpose is to define additional AXR1-interacting genes that control the silique development processes predicted to be highly dependent on auxin signaling.

1 Materials and Methods

1.1 Plant materials and growth conditions

axr1-12, the original mutant used for suppressors screen, is an auxin insensitive mutant, derived from Columbia ecotype [1,12].

Plants were grown in a growth chamber with a 14-h day length, a light intensity of 120 $\mu mol \cdot s^{-1} \cdot m^{-2}$, at 22 ℃, 65% relative humidity (day) / 18 ℃, 75% relative humidity (night).

For screen, 0.75 g, estimated 40 000 *axr1-12* seeds, were soaked for 16 h in 100 ml 0.3% (*V/V*) EMS (methanesulfonic acid ethyl ester), and then washed in water over a period of four hours. These M1 seeds were sown at a density of approximately one seed per cm^2 on nutrient soil. At maturity, putative mutants were harvested separately.

1.2 Light Microscope

6 to 7 days old siliques and seeds or seed-like structures were photographed with a Axioskop microscope (Carl Zeiss) coped with a Nikon Coolpix 990 camera.

1.3 Determination of auxin sensitivity

The surface sterilized seeds were distributed on the surface of MS agar (10 g/L) medium plates. Plates were placed in a culture room in a vertical position so that the roots could grow along the agar surface. After four days, seedlings were transferred to new plates supplemented with 0, 0.03, 0.3 μmol/L of 2,4-D and allowed to grow for additional three days. Root elongation was measured and the percent root growth inhibition was calculated relative to the root elongation on the MS medium without hormone. At least fifteen seedlings per treatment were analyzed.

1.4 Genetic analysis

The genetic analysis was done to determine if the new mutation was recessive. Mutants (♀) were crossed to the original mutant *axr1-12*, and the resulted F1 plants as well as their parents were assayed for the auxin-response of root growth.

To test if mutants were allelic, crosses between mutants were performed and the auxin-response of roots was assayed in the F1 generation.

2 Results

2.1 Suppressors screen from *axr1-12*

The auxin resistant mutant *axr1-12* mutant has got defects in reproductive organ, and produces abnormal siliques [1,12]. In the screen, we isolated the plants being able to recover the defect in fruit development and could develop siliques obviously at the later flowering stage [about forty days after sowing (DAS)].

In about 30 000 M1 plants, forty-six putants (putative mutants) were isolated and ten of them maintained their phenotypes in the M2 generation as those in M1. The rest M1 plants were harvested to generate the mixed M2 seed pools. 3 000 seeds from each twenty-one independent M2 pools were grown up for screening the plants which developed siliques to a significant extent at maturity. Twenty-eight putants were isolated again. These Twenty-eight lines together with the ten lines from M1 were backcrossed with *axr1-12* to clean up the background, and then self-crossed for two generations to generate the stable-phenotype homozygous ones. The specific CAPS (cleaved amplified polymorphic sequences) analysis of a 590 bp- genomic DNA fragment in *AXR1* demonstrated that all these lines carry the original mutation *axr1-12* and had the CAPS maker as the *axr1-12* mutant did, proving that their special phenotype was due to a new mutation.

In this paper, two typical mutant lines named *sa1* (suppressor of *axr1-12*) and *sa2*, which were conferred to be two distinct mutants later, are described.

2.2 Morphological phenotype of the mutants

All screened lines suppressed the defect of *axr1-12* in silique development. In addition, they exhibited other specific morphological phenotype in both seedlings and adult plants.

Fig.1 shows the phenotypes of 3-week seedlings of *sa1*, *sa2*, and wild type Col and the original mutant *axr1-12*. *sa1* was larger than *sa2* in seedling size, and its phenotype was similar to the wild type's. Leaves of these new mutant plants curled down much slightly as

compared to those of *axr1-12*, but did not expand as plainly as those of Col. Margin teeth of leaves of them were shallower than those of *axr1-12*. And petioles of them were shorter than Col's but longer than *axr1-12*'s. Thus, seedlings of *sa1* and *sa2* presented intermediate phenotype between wild type and the original mutant *axr1-12*. The phenomenon showed that new mutations in *sa1* and *sa2* partially recovered the defective phenotype of *axr1-12* in the shape of rosette leaves.

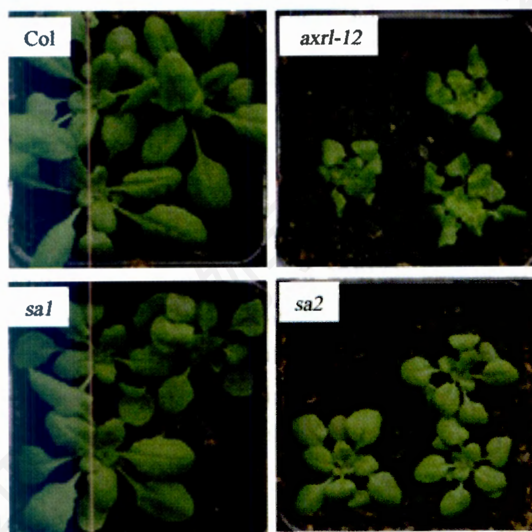

Fig.1 3-week old seedlings of *sa1*, *sa2*, Col (control) and *axr1-12* grown on soil in 10 cm×10 cm pots

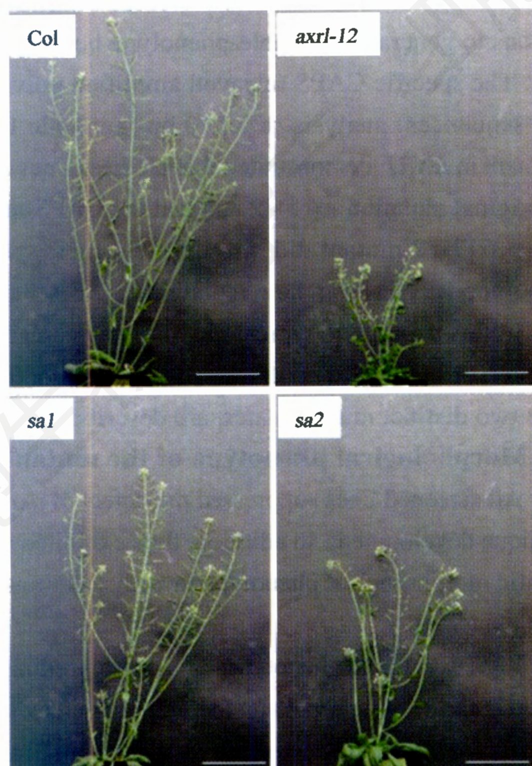

Fig.2 Morphology of adult plants of *sa1, sa2*, wild type Col (control) and *axr1-12* (45 DAS) (Bar=10 cm)

The adult plants of *sa1* and *sa2* also presented similar phenotype as wild type. They had long branches and were taller than *axr1-12*, the *sa1* had even evident display (Fig.2). They developed siliques very well. However, unlike wild type plants which developed siliques with seed fully setting when fertilized in nature (Fig. 3A), *sa1* and *sa2* plants showed abnormal seed development. Siliques of *sa1* developed normal seeds and seed-like structures with abnormal embryos (Fig. 3C, Fig.3G, Fig.3H). While siliques of *sa2* had partial seeds setting and partial ovules arrested (Fig.3D). What different from *axr1-12* (Fig.3B) was that the silique development of *sa2* was not restrained by low seed-

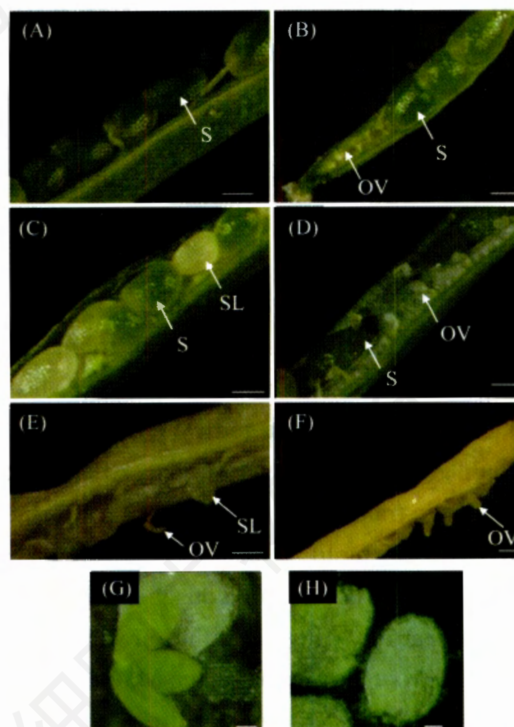

Fig.3 Seeds and siliques developed in wild-type Col (control) original mutant *axr1-12*, and suppressor *sa1, sa2 Arabidopsis* plants

A: silique of wild-type; B: silique of *axr1-12*; C: silique of sa1; D: silique of *sa2*; E: silique of emasculated *sa1*; F: silique of emasculated *sa2*; G: seed of *sa1* slightly broken under coverslip. The robust embryo is clearly shown in it; H: seed-like structure of sa1 slightly broken under coverslip. No embryo is found in it. Seed (S), seed-like structure (SL) and non-developed ovule (OV) are pointed by arrows. Bar: 0.5 mm (A–F) and 0.1 mm (G–H).

setting rate. When emasculation was performed to avoid fertilization, some seed-like structures could be still produced in *sa1* autonomously but not in *sa2* (Fig.3E, Fig.3F), Results showed that new mutations altered the fruit and seed developmental manner.

2.3 Root growth of mutants in response to 2,4-D

Mutant *axr1-12* is an auxin resistance mutant. Seedlings of *axr1-12* are resistant to 2,4-D at approximate 50-fold higher concentration than that for wild type [12]. To determine the response of the suppressors to auxin, root growth of seedlings was determined at treatments of different concentrations of 2,4-D.

In auxin-free medium, *sa1*, *sa2*, as well as *axr1-12* had less root elongation when compared with the wild type (Fig.4), suggesting that the new mutations in *sa1* and *sa2* exerted no effect upon root growth of the original *axr1-12* mutation. When seedlings were transferred to fresh MS solid plus 2,4-D (0, 0.03, 0.3 μmol/L), root elongation of the two suppressors presented different phenotypes (Fig.5). At 0.3 μmol/L of 2,4-D, the roots of Col elongated hardly, while the root elongation of *axr1-12* was only inhibited about 30%. And roots of *sa1* and *sa2* were inhibited around 60% and 78% respectively, just fall in-between the Col and *axr1-12*. At 0.03 μmol/L, the inhibition of root growth of Col, *axr1-12* and *sa1* was around 50%, 18%, and 22%, respectively, while no inhibition was showed on *sa2*. Results indicated that these mutants were more sensitive than *axr1-12* and less sensitive than Col in response to auxin, and *sa2* response to auxin in a narrow threshold of sensitivity.

2.4 Genetic segregation

To analyze the genetic basis for suppressors of the *axr1-12*, *sa1* and *sa2* was backcrossed to original mutant *axr1-12* respectively. The auxin-response of the root elongation in F1 and F2 generation from their crosses was determined.

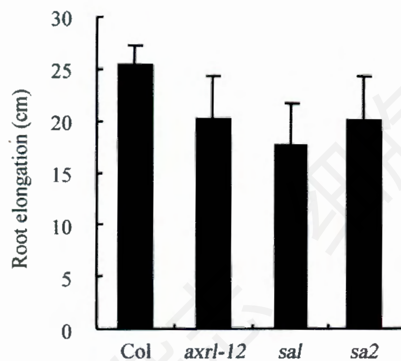

Fig.4 Root elongation of *sa1*, *sa2*, the control seedlings (Col) and *axr1-12* (*n*=30, ±SD)

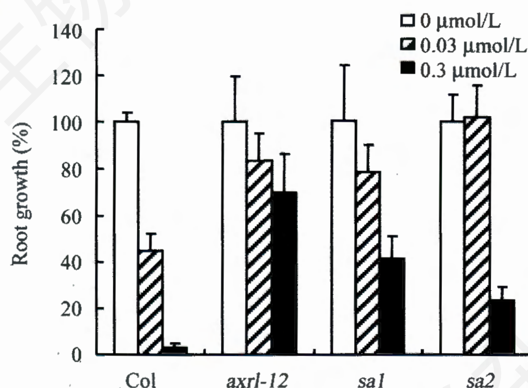

Fig.5 Root growth of seedlings of *sa1*, *sa2*, the control (Col) and *axr1-12* treated with 2,4-D (*n*>15, ±SD)

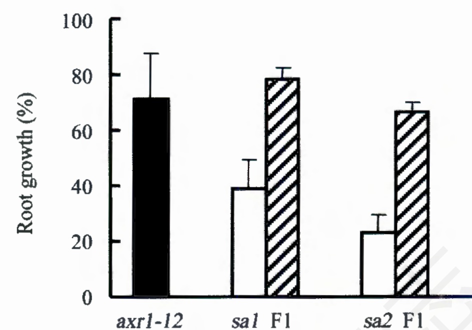

Fig.6 Root growth of seedlings of *axr1-12*, suppressors (*sa1*, *sa2*) and their F1 seedlings

Root growth of *axr1-12*, suppressor, and F1 of *axr1-12* crossed to each suppressor are indicated by solid, open and hatch columns, respectively (*n*>15, ±SD).

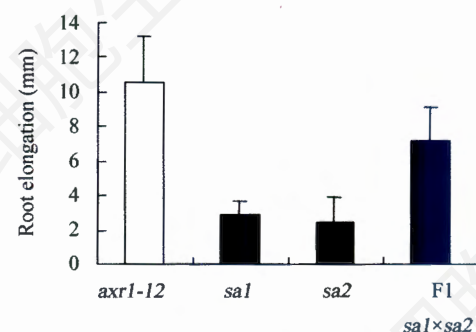

Fig.7 Root elongation of *sa1*, *sa2* and their F1 seedlings (*n*>15, ±SD)

F1 seedlings from crosses of *axr1-12* (♀) with each suppressors (♂) were cultivated on the media containing 0.3 μmol/L 2,4-D. Result showed that F1 seedlings were insensitive to 2,4-D as their maternal *axr1-12* seedlings (Fig. 6), suggesting that these suppressors carried recessive mutations.

2.5 Allelic test

To determine if mutants carry mutations at the same locus, crosses between *sa1* and *sa2* were performed. 2,4-D response of root elongation showed that the F1 seedlings of *sa1* × *sa2* were insensitive to 2,4-D resembling the original mutant *axr1-12* (Fig.7). Results implied that *sa1* carried a mutation at a distinct locus as that of *sa2*.

3 Discussion

To gain insight into fruit development, *axr1-12* was used as the original mutant to screen secondary mutants that could develop siliques apparently. Ten mutants from M1 and twenty-eight mutants from M2 were isolated. All these thirty-eight mutants were dramatically different from the original mutant *axr1-12* in the phenotype. Results revealed that the new mutations suppressed, at least partially, the defects of the *axr1-12* phenotype. Among the suppressors, two un-allelic recessive suppressor *sa1* and *sa2* were characterized in terms of root response to 2,4-D. Our studies showed that the *sa1* and *sa2* mutations altered the leaf shape and the plant posture of *axr1-12*, suppressed auxin resistance in *axr1-12* and increased auxin inhibition of root growth in *axr1-12* background. In addition, *sa1* and *sa2* recovered the defect of *axr1-12* in silique. However, abnormal seed development was observed in their siliques. Siliques of *sa1* develop both seeds and seed-like structures with no embryos, while siliques of *sa2* had seed setting partially and some ovules arrested in the full-grown siliques.

Previous study showed that the mutation (*axr1-12*) in AXR1 reduced the sensitivity of silique to 2,4-D and application of 2,4-D stimulated silique and ovule growth of emasculated flower [20]. It was proposed that action of auxin stimulated the auxin transduction related to AXR1 via the ubiquitin pathway and broke the function of negative regulators (e.g. ARF8, ARF2, ARF6) in carpel or even integument growth [21–23]. *sa1* and *sa2* developed full-grown siliques rather than abnormal silique as those in *axr1-12*, and got even better appearance than *axr1-12* in the case of unfertilization. It is possible that mutation in *sa1* and *sa2* boost apperception of siliques to auxin through elevation of auxin levels or auxin sensitivity. This should lead to efficient function disruption of negative regulator of silique development. Another possibility is that *sa1* and *sa2* carry mutations in genes coding for the negative regulators of silque development. And the mutation results in alleviation of the inhibition of the negative regulators to fruit development, therefore the silque could grow.

In *sa1*, seed-like structures were produced both occasionally in siliques in nature and in unfertilized siliques. The preliminary observation showed that no normal embryo formed in these structures. The possible reason for seed-like structures development would be that the unfertilized ovules are able to get the female gametes developed. Their ovule integuments and central cells develop into seed coat and endosperm respectively. Thus protein coded by *sa1* gene may play roles in controlling initiation of seed development, which should be confirmed further.

Phenotype of *sa1* was similar to maternal gametophytic mutants *mea* [24], *fis* [25,26] and *fie* [27]. These mutated genes were involved in imprinting in endosperm materally [28–31]. Considering that *sa1* altered auxin response, it is inferred that auxin signal might be involved in controlling imprinting in *Arabidopsis*.

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拟南芥抗生长素突变体*axr1-12*的抑制型突变体筛选

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摘要 利用抗生长素突变体*axr1-12*筛选获得了对生长素敏感并且果荚发育良好的突变体38个, 对其中的两个非等位隐性突变体*sa1*和*sa2*进行了分析。新突变体*sa1*和*sa2*在叶形、株型、以及根系生长对生长素的反应等方面都在一定程度上表现出与原突变体*axr1-12*相反的特征。*sa1*和*sa2*还使*axr1-12*的果荚发育缺陷得到了修复, 但其种子发育表现异常。*sa1*的果荚中可见种子和无胚的种子状结构, 在*sa2*的果荚中则同时存在部分健康种子和发育不良的胚珠。结果显示, *sa1*和*sa2*编码的蛋白质可能参与果荚对生长素的反应, *sa1*还可能在受精前种子发育初级阶段起调控作用。

关键词 拟南芥; 生长素反应; 抑制型突变体; 荚果发育; *axr1-12*

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