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*Full Length Research Paper*

# Effects of gibberellin mutations on *in vitro* shoot bud regeneration of *Arabidopsis*

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**Tissue culture provides a useful system to investigate how plant hormones are involved in this process. Auxin and cytokinin are widely used in plant regeneration. Gibberellin is also an important plant hormone in regulating plant growth and development. It is interesting to know the effects of gibberellin and its signalling pathway on plant regeneration. In this report *Arabidopsis thaliana landsberg* (wild type), *ga1-3* (gibberellin biosynthesis deficiency mutant), *gai* (gibberellin insensitive mutant), *penta* mutant (lacking GA1, GAI, RGA, RGL1, RGL2) and *tetra* mutant (lacking GAI, RGA, RGL1, RGL2) were used as materials to investigate how plant regeneration progress was affected in these mutants under different conditions. The results showed that more shoot buds were regenerated in *ga1-3* and *gai* than in *wild type, penta* and *tetra* mutant in the normal shoot induction medium. The frequency of shoot bud regeneration in different mutants also varied remarkably when auxin : cytokinin ratio in the medium changed. Only *penta* mutant had shoot bud regeneration without auxin in the medium. When the ratio increased *wild type*, *ga1-3* and *gai* had higher performance in shoot bud regeneration than *penta* mutant. When cytokinin level increased from 0.1 to 0.5 mg $l^{-1}$, shoot bud regeneration frequency of *ga1-3*, *wild type* and *tetra* increased remarkably except for that of *penta* and *gai*. Only the shoot bud regeneration frequency of *gai* did not change significantly when cytokinin level increased from 0.5 to 5 mg $l^{-1}$. These gibberellin-related mutants also responded differently to NPA (an auxin polar transport inhibitor) in plant regeneration. Our results indicate gibberellin and its related pathway are also involved in plant organogenesis: gibberellin inhibitor and auxin polar transport inhibitor can promote plant organogenesis. This might provide a new way for the regeneration of recalcitrant species.**

**Key words:** Shoot bud regeneration, *Arabidopsis thaliana,* gibberellin, DELLA protein mutant.

## INTRODUCTION

The ability of higher plants to produce new shoots or roots via tissue culture provides a useful system to investigate how plant hormones function in this progress. According to Christianson and Warrick's (1983), plant regeneration can be divided into 3 phases: 1, the acquisition of competent cell 2, the induction phase: the developmental fate of regenerating tissue is specified by hormonal composition of the medium. 3, the newly determined tissue forms a functional meristem and develops into a complete shoot or root independent of exogenous hormones.

Auxin and cytokinin are widely used in tissue culture. Many researches have shown how these two plant hormones affect plant tissue culture. Gibberellin is also very important for plant to regulate growth and development. However, up to date just a few reports show that gibberellin is also involved in plant embryogenesis at very earlier stage. Tokuji and Kuriyama (2003) reported gibberellin inhibited the early stage of embryogenic cell differentiation/ development to the globular stage. Uniconazole, another inhibitor of gibberellin synthesis, promoted the secondary embryogenesis during somatic embryogenesis of carrots. Hiroshi Ezura had also shown *in vitro* shoot regeneration of A*rabidopsis thaliana* was influenced by endogenous gibberellin (Ezura and Harberd,

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**Abbreviations**: **IP,** isopentenyladenin; **IAA**, 3-indoleacetic; **PAC**, paclobutrazol; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **NPA**, N-(1-naphthyl) phthalamic acid; **CIM**, callus induction medium; **SIM**, shoot induction medium.

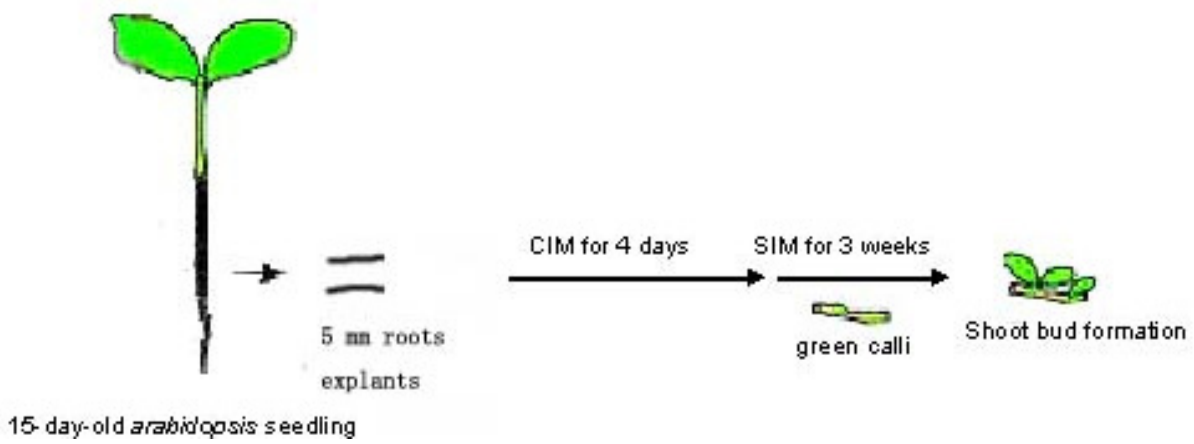


**Figure 1.** Shoot or root regeneration from root explants of *Arabidopsis thaliana* 5 mm root segments were cut from 15-day-old seedling, cultured on CIM for 4 days, and then transferred to SIM.

1995). DELLA protein played an important role in gibberellin signalling pathway, it negatively regulated gibberellin signalling (Peng et al., 1997; Silverstone et al., 1998; Wen and Chang, 2002; Cheng et al., 2004). DELLA protein is involved in many aspects of plant growth and development. Recent researches have discovered DELLA protein also was interacted with auxin and ethylene (Fu and Harberd, 2003; Achard et al., 2003). It is worthy to know whether DELLA protein is also involved in *in vitro* organogenesis. It is well known that *Arabidopsis* have been the model plant for scientific researches. There are many papers published about gibberellin biosynthesis and signalling pathway in *Arabidopsis*. A plethora of gibberellin biosynthesis and response mutants of *A. thaliana* are available. These provide a useful system to investigate how gibberellin affecting plants regeneration or other biological activity. Banta and Pigliucci (2005) have already examined the effects of gibberellin in tolerance to apical meristem damage with different gibberellin-related mutants. In order to examine the role of gibberellin and its responding genes in plant organogenesis, shoot bud regeneration ability of different gibberellin related mutants was characterized. In this paper we confirmed that the shoot bud regeneration frequency and efficiency in *A. thaliana* were deeply affected in different gibberellin-related mutants.

## MATERIALS AND METHODS

### Plant materials and growth condition

All plants including mutants are of the *A. thaliana landsberg* ecotype. Seeds of *wild type*, *ga1-3*, *gai*, *penta* mutant (lacking GA1, GAI, RGA, RGL1, RGL2), *tetra* mutant (lacking GAI, RGA, RGL1, RGL2) were freshly harvested in our laboratory.

Seeds were sterilized in 70% ethanol for 5 min, and then rinsed in sterilized distilled water for 3 times, 5 min for each rinse. Twenty five seeds were sown on each plates containing GM medium. All the plates were chilled at 4°C for 4 days, then placed vertically and incubated in a growth room (25°C, 16 h light).

### Preparation of explants and shoot induction

Twenty days after incubation in the growth room, five-millimetre long root segments were cut from the young seedlings and incubated on CIM (callus induction medium, B5 + 2,4-D 0.5 mg $l^{-1}$ + kinetin 0.1 mg $l^{-1}$) for 4 days in the growth room. Then the pre-cultured roots were transferred from CIM to SIM (shoot induction medium), with 25 root segments in each plate. Each experiment was done with 4 repeats. The process was shown in Figure 1.

### Evaluation of shoot bud regeneration

Shoot buds regeneration from root explants were evaluated using a microscope at 3 weeks after the incubation of root explants in SIM in the growth room. The frequency of shoot buds regeneration was measured by determining the root explants from which the shoot buds were regenerated in total explants per dish. The shoot bud number was determined by evaluating the regenerated shoot buds from per root explant of different GA-related mutants.

## RESULTS AND DISCUSSION

### Shoot bud number per root explant

The number of differentiated shoot buds per root explant of different gibberellin-related mutants was shown in Figure 2. *Ga1-3* and *gai* had much better performance in shoot bud regeneration. The shoot bud number of *ga1-3* is significantly different from that of *penta* (T-test, 1% level). The shoot bud number of *wild type* is significantly different from that of *tetra* (T-test, 5% level). The shoot bud number of *gai* is also significantly different from *wild type* (T-test, 5% level). *Ga1-3* is a recessive gibberellin biosynthesis deficiency mutant; it is seriously dwarf with dark-green leaves and decreased apical dominance. The phenotype of *ga1-3* can be recovered by spraying the plants with gibberellin. *Gai* is a semi-dominant gibberellin insensitive mutant; it is also seriously dwarf with dark-green leaves but its phenotype cannot be recovered

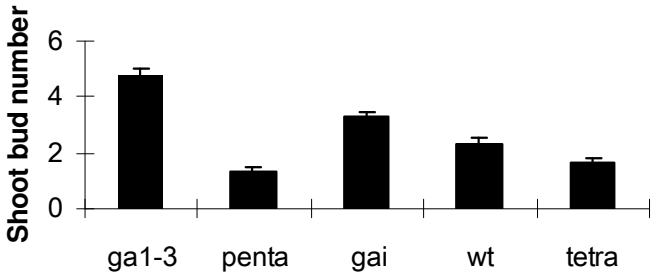


**Figure 2.** Shoot bud number regeneration from per root explant of different GA-related mutants. Each value was expressed as the shoot bud number per root explant, taken from a data set consisting of a total 100 explants. Shoot bud regeneration was evaluated after 3 weeks incubation in SIM.

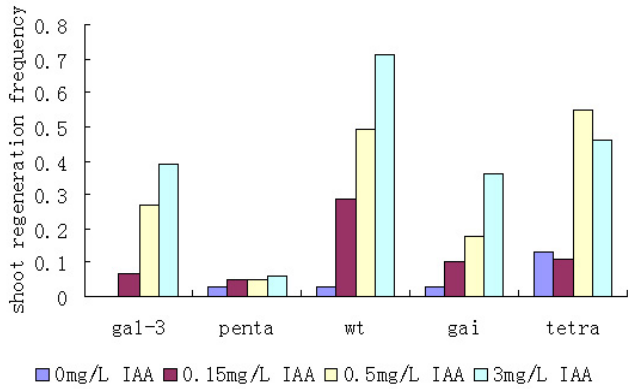


**Figure 3.** Shoot bud regeneration frequency at different level of IAA in SIM. Each value was expressed as the shoot bud regeneration frequency, taken from a data set consisting of a total 25 explants with 4 repeats.

by spraying the plants with gibberellin. The phenotype of *penta* mutant and *tetra* mutant are much similar to *wild type*. The phenotype of *ga1-3* and *gai* having decreased apical dominance and more branches, might suggest that *ga1-3* and *gai* have more potential to produce more meristem. This is consistent with shoot bud regeneration results. This result indicates gibberellin and its signalling pathway inhibit shoot bud regeneration of *Arabidopsis*.

## Shoot bud regeneration responses to different IAA level

In order to detect how gibberellin-related mutants respond to different auxin level in the medium during shoot bud regeneration, four different IAA levels 0, 0.15, 0.5 and 3 mg $l^{-1}$ were applied in SIM independently while cytokinin level was stable at o.5 mg $l^{-1}$. Interestingly there are dramatic differences in shoot bud regeneration frequency between the different lines when IAA level increased. The results are shown in Figure 3.

*Tetra* mutant has the best performance in shoot bud regeneration when there is no IAA in the SIM, while all the other lines have very poor shoot bud regeneration. The shoot bud regeneration frequency of *wild type*, *ga1-3* and *gai* mutant increases remarkably when IAA level in SIM increases. When IAA level increases from 0.15 to 0.5 mg $l^{-1}$, significant difference exists between *ga1-3* and *penta* (T-test, 1% level). Significant difference also exists between *wild type* and *tetra* (T-test, 5% level), *wild type* and *gai* (T-test, 5% level). Among all the lines the shoot bud regeneration frequency of *penta* mutant does not change remarkably, it is always at very low level. The shoot bud regeneration frequency of *tetra* mutant increases when IAA level changed from 0.15 to 0.5 mg $l^{-1}$ in SIM. However, when high IAA level (3 mg $l^{-1}$) was applied in SIM, the shoot bud regeneration frequency of *tetra* mutant began to fall down. It is very interesting to point out that the behaviour of *penta* and *tetra* mutant responding to varying auxin level was different from the *wild type*, *ga1-3* and *gai*.

It is also interesting to point out that without IAA in SIM, the shoot bud regeneration frequency of *tetra* mutant is significantly better than *wild type* and *gai* (T-test, 1% level). There are no shoot buds differentiated from root explants of *ga1-3* mutant in SIM without IAA in the medium, while the shoot bud regeneration of *penta* mutant is still fine. These suggest that gibberellin and its signalling pathway can promote shoot bud regeneration in the absence of auxin.

## Shoot bud regeneration responses to NPA during shoot induction

Auxin polar transport is an essential factor in the morphogenesis of zygotic and somatic embryos in monocots and dicots (Fischer et al., 1997). During somatic embryogenesis of carrots, the development of somatic embryos from globular stage to heart-shaped stage was seriously inhibited by the presence of 10 µM TIBA (2, 3, 5-triiodobenzoic acid), an inhibitor of auxin polar transport. However they did not have critical effects on the frequency of somatic embryogenesis (Tokuji and Kuriyama, 2003). NPA is also an inhibitor of auxin polar transport, it can stabilize DELLA protein in gibberellin signalling pathway (Fu, 2003). In order to examine how shoot bud regeneration of gibberellin-related mutants was affected by NPA, two concentrations of NPA 1, 10 µM were applied in SIM independently during root explants incubation in the medium in growth room. The results are shown in Figure 4.

The shoot bud regeneration frequency of all the lines increases remarkably compared with that in normal SIM without NPA. It is more interesting to point out that the shoot bud regeneration frequency of *ga1-3* and *gai* did not change much when NPA level changed from 1 to 10 µM, while the shoot bud regeneration frequency of *wild type*, *penta* and *tetra* mutant increased significantly (T-test, 5%

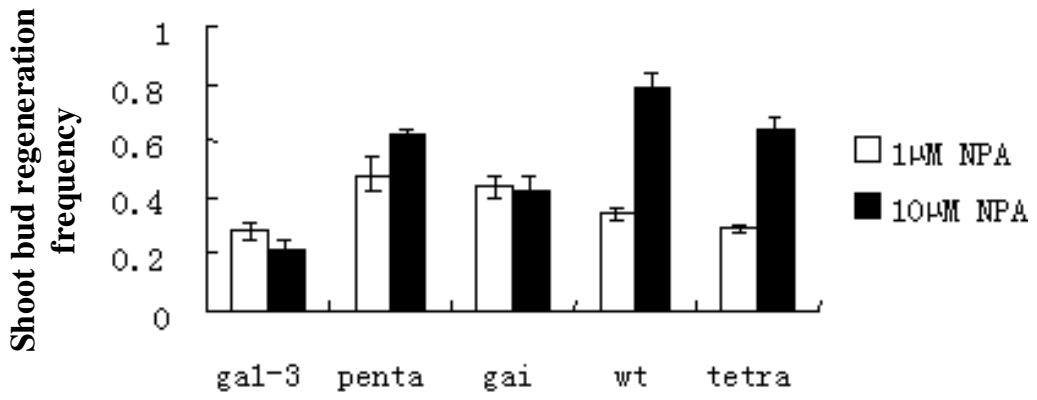


**Figure 4.** Shoot bud regeneration frequency at different level of NPA in SIM. Each value was expressed as the shoot bud regeneration frequency, taken from a data set consisting of a total 25 explants with 4 repeats.

level). The responses of *wild type* and *tetra mutant* to increased NPA level are more similar and stronger than *penta* mutant. This seems not consistent with Tokuji's results. It might be due to the differences between somatic embryogenesis and shoot bud regeneration. During Tokuji's experiments, somatic embryogenesis still happened, but the development of somatic embryo was seriously inhibited with 10 uM TIBA. During shoot buds regeneration process, little shoot buds would come out if organogenesis and development is seriously inhibited.

When NPA is applied in the medium, DELLA protein is stabilized and gibberellin signalling pathway is seriously blocked, shoot bud regeneration ability is promoted. This is consistent with the results from Figure 2.

## Shoot bud regeneration responses to different cytokinin levels

In order to check how shoot bud regeneration of gibberellin-related mutants were affected by different cytokinin levels, three concentrations of 2-ip at 0.1 m, 0.5 and 5 mg $l^{-1}$ were applied to SIM independently while IAA level was kept stable at 0.15 mg $l^{-1}$. The shoot bud regeneration frequency was evaluated 3 weeks after incubation in the SIM. The results are shown in Figure 5.

When 2-ip changed from 0.1 to 0.5 mg $l^{-1}$, significant difference of shoot bud regeneration frequency exists between *wild type* and *gai* (T-test, 5% level), *tetra* and *gai* (T-test, 5% level), *ga1-3* and *penta* (T-test, 5% level). When 5 mg $l^{-1}$ 2-ip was added into SIM, the shoot bud regeneration frequency of *wild type*, *tetra, ga1-3* and *penta* mutant decreased dramatically except that of *gai* mutant. It seems that too high concentration of 2-ip inhibits shoot bud regeneration of *wild type*, *ga1-3*, *tetra* and *penta* mutant except for that of *gai* mutant. The shoot bud regeneration of *gai* was more stable than all the others. This might be due to the special character of *gai* mutant. It is well known that *gai* mutant is a semi-dominant gibberellin related mutant.

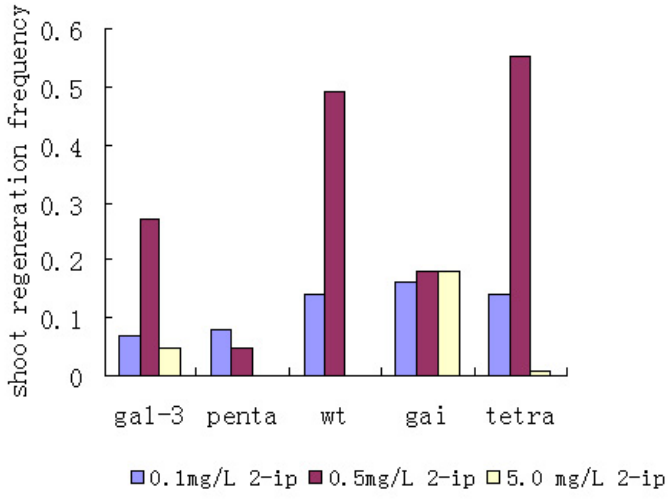


**Figure 5.** Shoot bud regeneration frequency with different level of NPA in SIM Each value was expressed as the shoot bud regeneration frequency, taken from a data set consisting of a total 25 explants with 4 repeats.

It cannot be recovered by spaying gibberellin to the plants. GAI protein is also very stable and difficult for degradation in the presence of gibberellin.

During shoot induction process in *in vitro* tissue culture of *A. thaliana*, auxin and cytokinin are deeply involved. Since gibberellin is also interacted with auxin and cytokinin, it is also very important to examine the role of gibberellin in plant regeneration. In this paper, tissue culture has provided a useful system to investigate how gibberellin biosynthetic and response genes affect shoot regeneration in conjunction with varying concentrations of IAA, NPA and 2-IP. In our other experiments (data and pictures not shown), callus induction of all the lines was seriously inhibited when 10 μM purine riboside (an inhibitor of cytokinin biosynthesis during callus induction)

was applied. However, callus induction was not affected with the 10 µM PAC (inhibitor of gibberellin biosynthesis) or 10 µM NPA (an inhibitor of auxin polar transport) being applied. This suggests cytokinin is necessary for competence acquisition. It might be possible that callus induction by cytokinin was independent of auxin and gibberellin. Only in shoot induction process, gibberellin was involved.

There are just a few reports described that gibberellin was involved in shoot bud regeneration. Shoot bud regeneration of *wild type A. thaliana* was very poor when 10 µM $GA_3$ was applied to SIM (data not shown). This is consistent with the shoot bud regeneration frequency of *penta* mutant. It is also interesting to note that without IAA in SIM, the shoot bud regeneration from the root explants of *ga1-3* mutant was totally inhibited while the root explants of *penta* mutant still formed shoot buds. Similarly, the shoot bud regeneration from the root explants of *tetra* mutant was much better than *wild type*. There might be two possibilities: 1. there is higher concentration of endogenous auxin level in *tetra* meant and *penta* mutant than in *wild type* and *ga1-3*; 2. it is also possible that auxin regulates embryogenesis or organogenesis via DELLA protein. Since auxin polar transport was reported to be interacted with DELLA protein (Fu, 2003), it might be possible that auxin regulates organogenesis or embryogenesis via modification of gibberellin signalling pathway.

Cytokinin is also very important in plant tissue culture, it is known that cytokinin can stabilize ACC biosynthesis pathway. Its effects on the inhibition of root and hypocotyls elongation were coupled to Ethylene (Chae, 2003). It has been shown that DELLA protein was involved in ethylene signalling pathway (Achard et al., 2003). It might be possible that gibberellin can be interacted with cytokinin directly or indirectly via ethylene signalling pathway. The results in our experiments had shown that the shoot bud regeneration of *penta* and *gai* mutant did not change too much compared with *wild type* and *tetra* mutant when cytokinin level increased in SIM. It is also very interesting to point out *gai* still had better shoot bud regeneration in the high concentration of cytokinin level in SIM. These suggest that *gai* is less sensitive to cytokinin in shoot bud regeneration. *Gai* has more branches and decreased apical dominance, suggesting decreased auxin level or increased cytokinin level exist in the mutant.

Arabidopsis may be an inroad to other systems where regeneration is poor, if not impossible. From our results, perhaps, the use of gibberellin inhibitors or auxin polar transport inhibitor could allow the regeneration of recalcitrant species successfully.

## ACKNOWLEDGEMENT

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