A Simple, Rapid, and Quantifiable System for Studying Adventitious Root Formation in Grapevine

XinYu Chang
Hunan Agricultural University
Kai Zhang
Hunan Agricultural University
Yunzhang Yuan
Hunan Agricultural University
Peiyi Ni
Hunan Agricultural University
Jing Ma
Hunan Agricultural University
Hui Liu
Hunan Agricultural University
Shiyu Gong
Hunan Agricultural University
Guoshun Yang
Hunan Agricultural University
Miao Bai (baimiao@hunau.edu.cn)
Hunan Agricultural University https://orcid.org/0000-0002-8842-0506

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Abstract

Woody cutting is customarily utilized as material in research of grape adventitious root formation (ARF). However, phenotypic heterogeneity caused by the complex background influenced its use for molecular mechanism research of ARF of grape. The present study tested various types of explants from grape tissue culture plantlets and found the whole leaf: blade with petiole (LP) was the simplest unit that can easily form adventitious root (AR). LP explants which can be easily obtained, directly generate ARs via *de novo* organogenesis from the base of the petiole. The plantlet age, node position, blade size, the health condition of leaves, and light intensity have been demonstrated to affect the homogeneity of ARF phenotype in LP. By controlling these parameters, selected LPs cultured on medium with 6 $g{\cdot}L^{-1}$ agar and 10 $g{\cdot}L^{-1}$ sucrose under dark conditions started rooting at 6-7 day after culture (DAC) and reached 100% rooting rate within 13-14 DAC. Using this system, the core role of auxin on ARF was verified by exogenous application of indole butyric acid (IBA) and N-1-naphthylphthalamic acid (NPA). Strikingly, we found light promoted ARF in the absence of sucrose, but inhibited ARF in the presence of sucrose (10 $g{\cdot}L^{-1}$), while a low concentration of 0.1 $mg{\cdot}L^{-1}$ NPA partially relieved the inhibition. Finally, this study confirmed exogenous plant growth regulators (PGRs), including 6-benzyl aminopurine (6-BA), gibberellic acid 3 (GA_3), and 2,4-Epibrassinolide (EBR) inhibited ARF significantly. This simple, rapid, quantifiable ARF research system provides a new approach to study the factors influencing the formation and development of grape adventitious roots and establishes a framework for investigating the mechanism of grape adventitious root induction and initiation.

Introduction

Grape (*Vitis* sp.) is one of the most economically important crops which occupies a pivotal position in the global fruit market. As perennial vines, the propagation of grapes conventionally using by dormant hardwood cuttings that taken from current season's canes (Creasy and Creasy 2009). Cutting propagation guarantees characteristics of species or new varieties. In asexual propagation, the adventitious roots formation (ARF) is the premise of successful propagation (Pijut et al. 2011; Steffens and Rasmussen 2016). However, adventitious roots are difficult to be induced in many grape species or varieties with important breeding utilization, such as *V. davidii*, *V. quinquangularis* (syn. *V. kiusiana*), and *V. amurensis* which are wild grapes native to East Asia (Shiozaki et al. 2013). The *V. rotundifolia* (Muscadine) (Castro et al. 1994), *V. champinii* (Saleh 2019), *V. aestivalis* (Keeley et al. 2004), *V. berlandieri* (Kracke et al. 1981; Smart et al. 2003), and some cultivars of *V. vinifera* are also known to be difficult to root from their woody cuttings.

Combined with studies on lateral root formation, a mode of ARF has been formed (de Klerk et al. 1999; da Costa et al. 2013; Joshi and Ginzberg 2021). The formation of AR can be divided into three stages: induction, initiation, and expression. In the induction stage, some competent cells with regenerative ability, such as procambium and some vascular parenchyma cells, are induced and activated their fate, and then transformed into AR founder cells. Subsequently, AR founder cells divide to form root primordia.

Finally, newly formed adventitious roots appear through the epidermis, and AR protrusion can be observed by eyes. Some studies divide ARF into two or four stages (An et al. 2020; da Costa et al. 2013).

ARF is essentially a regenerative process that involves reprogramming cells and changes in cell fate, and the primary induction signal is wounding (Xu 2018). Environmental conditions, exogenous application, and endogenous biochemical substances all have important influence on AR formation. In terms of endogenous biochemical substances, auxin plays a central role, and numerous inducible factors that respond to ARF will eventually be reflected in the concentration, transport, or homeostasis changes of auxin (Gonin et al. 2019; Busov et al. 2006). In plants prone to rooting, auxin produced in shoots or leaves and transported to the cutting site is often sufficient to meet the needs of ARF. For plants that are difficult to root, exogenous auxin treatment or other stimuli may induce endogenous auxin accumulation and restore AR induction. It is important to note, nonetheless, that not all plants can overcome the difficult rooting phenotype by increasing growth factors. ARF depends to a large extent on the nutritional status of the mother plant, or the explants being cut, including minerals and carbohydrates, and sink establishment at rooting zone (Chen et al. 2014; Ahkami et al. 2013). Moreover, the presence of some inhibitors may also make ARF difficult to achieve in some species. Rooting is a complex trait controlled by multiple factors and genes. The key genes related to ARs are auxin signal response genes, and some genes related to cell wall regulation are also related to ARs regulation. In addition, crosstalk and interaction among many molecular processes produce complex networks that regulate AR production. Ethylene, cytokinin, jasmonic acid, gibberellin, abscisic acid, brassinolide, strigolactone, and other biochemical substances may regulate the production of ARs through molecular processes and crosstalk. These hormones may both promote and inhibit ARF, which appears to depend on hormone type, dose, or species (Bannoud and Bellini 2021; Sharma et al. 2021)

It is time-consuming and labor-intensive to investigate the causes of difficulty in rooting of grape by conventional genetic methods. In reference to AR formation molecular regulatory network obtained from model plant studies, the researcher could identify the characteristic of ARF in grapes and clarify the influence of various factors on ARF and its mechanism. This would be of great value to solve the problem of grape rooting difficulty. In grapes, basic biology of ARF progresses slowly, and recent important advances are mainly based on the study of model plants. Model plant *Arabidopsis thaliana* could regenerate ARs from exfoliated leaves, intact hypocotyls of etiolated seedlings, hypocotyls of root-removed seedlings, and stem segments removed from inflorescence stems (Correa et al. 2012; Gutierrez et al. 2012; Della Rovere et al. 2013). Due to the different habits and lifestyles of model plants such as *Arabidopsis*, it remains to be seen whether the knowledge derived from them can be directly translated into more complex vine or woody plants.

In the studies on grape adventitious roots, the hardwood cuttings with dormant bud collected from current seasons canes were usually utilized (Jaleta 2019; Thomas et al. 2003), and greenwood leafy cuttings were also used in some research (Thomas and Schiefelbein 2004). However, woody cuttings are a highly complex system in which endogenous hormones levels, transport, dormancy, storage, and inhibitory compounds affect adventitious root formation and growth, all of which depend on pretreatment

(Smart et al. 2003). Our previous study found that the rooting of woody cuttings is slow, generally requires more than 20 days, and varies with varieties, the node site and sampling time. In addition, the ARF phenotype of woody cuttings differed greatly, that is, there was obvious heterogeneity among samples. Moreover, the sprout of buds on cuttings maybe affects rooting (Zhou et al. 2020; Smart et al. 2003). In addition, the acquisition of cuttings has seasonality. These make it difficult to investigate the function and molecular regulation mechanism of the factors affecting ARF of grape by using cuttings.

Blade or whole leaves of *Arabidopsis* were utilized for adventitious root regeneration studies (Chen et al. 2014; Bustillo-Avendano et al. 2018), which is probably the smallest and simplest unit that can form ARs, and without the influence of sprouting. Nevertheless, the system has not been applied to grapes. Referring to this method, young grape leaves from field could not induce root, while the replacement of leaves from tissue culture plantlets can generate root, but the speed and efficiency are low, the sample is not homogeneity, so it is still difficult to apply on grapes. Hence, we found that ARs were regenerated when whole grape leaves (leaf with petiole, LP) were used without any added nutrients and hormones. We described some internal factors affecting ARF of LP in grapes. Using this system, the role of carbohydrate, auxin, light and exogenous plant growth regulators has been tested on grape ARF.

Materials And Methods

Plant materials and culture conditions

Nine-year-old *Vitis labruscana* × *Vitis vinifera* 'Shine Muscat' (SM) and 'Summer Black' (SB) which planted in a research field (E 113°19', N 28°13') at Hunan Agricultural University (Changsha, China) were used in this experiment. Woody current canes with ca 3-5 nodes (N3, N4 and N5) were obtained from these vines on October 19, 2020. Canes then were cut into single bud stem segments and put into glass bottles with clear water and cultured in plant tissue culture chamber (GZ-400-GII, Guangzhou, China), at 25℃, with a 16-h light (4000 Lux, cool white fluorescent lamp) and 8-h dark photoperiod. Water was refreshed every 2 days. Rooting data were recorded daily. The cutting with root (>1 mm) was classified as a rooted cutting.

The explants of single bud stem section with leaf (SBS-L), single bud stem section (SBS), whole leaf (called as a leaf with petiole, LP), blade (B), petiole (P) and stem section (SS) were used to observe ARF phenotype. They were obtained from field or tissue culture plants. AR induction medium and culture conditions were changed according to the experiment. Without special instructions, the basic medium was 6 $g{\cdot}L^{-1}$ plant agar (P1001, Duchefa Biochemie) without (A6) or with 10 $g{\cdot}L^{-1}$ sucrose (A6+S10), and the pH value was adjusted to 5.8 and the culture conditions were at 25℃, with 16h photoperiod irradiance of 4000 Lux provided by cool white fluorescent lamps (expressed by Light) or 24h-dark (expressed by Dark) and air humidity of 90%.

In the field, leaves on a 9-year-old grapevine or 2-year-old cutting propagation plant of 'Summer Black' were also used in this experiment. From the top to the base, leaves at different nodes were taken as

explants and cultured on the A6 medium under culture conditions mentioned above. Grape tissue culture explants from *Vitis* interspecific crossing varieties 'Vidal blanc' were preserved in our laboratory. The medium for propagation was the base of Woody Plant Medium (WPM) with 10g·L^{-1} sucrose and 3 g·L^{-1} activated carbon (WPM+S10+AC3). With no special description, the LP was taken from the 45-60-day-old tissue culture plantlets with 6 leaves/nodes as explants for the experiment.

Hormonal and Inhibition Treatments

Exogenous plant growth regulators (PGRs) were used to investigate the effects of different hormones on grape ARF. Exogenous auxin (indole butyric acid, IBA, I5386, Sigma-Aldrich) and N-1-naphthylphthalamic acid (NPA, PESTANAL®, 33371, Sigma-Aldrich), a polar auxin transport inhibitor, were utilized to investigate the key functions of auxin in ARF. Cytokinin (6-benzaminopurine, 6-BA, A8170, Solarbio), ethephon (ET, E8021, Solarbio), abscisic acid (ABA, A8060, Solarbio), gibberellin acid 3 (GA_3, G7645, Sigma-Aldrich), jasmonic acid methyl ester (MeJA, M8640, Solarbio), 2,4-epibrassinolide (EBR, IE0110, Solarbio), were first prepared into 10 mg·mL^{-1} stock solutions using DMSO or NaOH. At the time of exogenous hormone treatment, the stock solution was added to a warm growth medium before pouring into plates to provide a final concentration.

ARF phenotype observation and analysis

In this study, the phenotype of ARF was determined by the appearance of AR protrusions (at the expression stage) observed under stereomicroscope (SZX7, Olympus, Tokyo, Japan). The proportion of explants with ARF phenotype was defined as rooting rate, and AR data curve was made by the dynamic change of rooting rate by daily (day after culture, DAC) (Fig. 1). The curve was served to judge the homogeneity or heterogeneity of samples by characterizing the speed, uniformity, synchronism, and the error of ARF phenotype. Where, the speed is defined as the time F (d) from the beginning of processing to the formation of the first AR. Uniformity was defined as U (%) of the rooting rate. It is expresses as U_{max} when stability eventually ceases to take root. S (d), defined as synchronism, represents the number of days from the first AR formation to the U_{max}. The smaller S is, the higher the synchrony of rooting is. The standard deviation (SD) of rooting rate represents the statistical error E (%) between three or four biological replicates (N>30). The smaller E is, the smaller individual differences are. In this study, the number and length of ARs and other growth states of explants were further investigated.

Statistical analysis

Data values were statistically analyzed by ANOVA with a Duncan's multiple range test, using SPSS Statistics 20 software (IBM). Significant differences were collected with 5% level of significance (P-value,0.05).

Results

Phenotypic heterogeneity of ARF in hardwood cuttings

The rooting of hardwood cuttings taken from node 3 to node 5 (N3, N4 and N5) of current canes of Shine Muscat (SM) and Summer Black (SB) was investigated, respectively (Fig. 2(a) and (c)). Cuttings of two cultivars were rooted after 22 DAC (F = 22 DAC). The ARF phenotype of SB was more consistent than SM, U_{SB} = 90~98% and U_{SM} = 53~75% at 38 DAC. The rooting rate of SM was different in nodes. The N5 was higher than N3 and N4. From the first root induced to the stable rooting rate, S_{SB} and S_{SM} were taken 16 days (S=16 days). In addition, the standard deviation (E) of rooting rate among samples was large. Some of them were more than 22%. At the same time, there were also inconsistent rooting and sprout in cuttings from the same node (Fig. 2(b) and (d)).

Differences in ARF phenotype among various types of explants

Field and tissue culture materials were used to obtain different types of explants to study the phenotypic heterogeneity of ARF. Young and mature leaves (Blade or LP) were taken from top to base of current canes of SB and cultured on medium A6 or A6 with 0.1 $mg{\cdot}L^{-1}$ IBA (A6+IBA0.1) under light condition (Light: 16h-photoperiod, 4000lux). After 60 days till the leaves were scorched, no ARs appearance. AR induction was also attempted on leaves (N8, N6, N4 and N2) from infantile two-years-old SB vines but was also unsuccessful (Fig. 3(a)). Field leaves cultured on medium A6 with $10g{\cdot}L^{-1}$ sucrose (A6+S10) were easily contaminated with bacteria or fungi, and statistical results could not be obtained.

By distinguishing the different nodes and culturing under the light condition in WPM+S10+AC3, the results showed that rooting rate of the first and second nodes (N1~N2) was low (U=34.44~46.67%), while the rooting rate of N3~N4 and terminal bud (NT) was 80.00%~88.89% at 45 DAC (Fig. 3(b)). Heterogeneity of N1~N2 samples was larger than that of N3~N4, with a partial error (E) was more than 15% (Fig. 3(b)). These results indicated that explants from different nodes exhibit different rooting abilities.

Subsequently, six types of explants were selected from N3~N6 (the basal two nodes and NT were not taken) of plantlet with relatively consistent growth potential and these ARF phenotype were compared (Fig. 3(c)). The explants were cultured on A6 medium at 25℃ under light condition (Light: 16h-photoperiod, 4000lux) and results showed that petiole (P) and stem segment (SS) did not regenerate root even after 60 days of continuous culture. The SBS-L, LP and SBS began to root at 7-10 DAC, faster than the blade (F=11 DAC). The highest rooting rate of explants was LP (67.78%±14.99% at 20 DAC). The rooting rate of SBS-L was 44.4% (44.44%±10.30% at 20 DAC), SBS was 10.0% (10.00%±2.70% at 20 DAC), respectively (Fig. 3(d)). However, only 1-2 explants of the blade (B) rooting occasionally after 20 DAC culture (N>90). Different types of explants were further cultured on A6 medium under dark condition.

After 20 DAC, only LP rooting was observed (17.80%±15.00% at 20 DAC) (Fig. 3(e)), and no rooting was observed till 30 DAC in other explants (Fig. 3(e)).

Then, different explants were cultured on medium A6+S10 under dark condition. The results showed that P, B and SS did not root after continuous observation of 30 DAC. However, after culture of 45 DAC, 37.78% (37.78%±22.19%) of SS developed roots (Fig. 3(c)). These indicating that neither leaves nor buds were necessary for ARF in this condition, while sugar and darkness were important factors promoting rooting. The rooting efficiency of SBS-L and LP on A6+S10 medium under dark was significantly greater than that of A6 medium under light, and almost all the explants of SBS-L and LP could root in 13 DAC (U=100%). The rooting rate of SBS was also increased significantly and reached 73.33%±5.40% at 20 DAC (Fig. 3(g)).

Interestingly, when different explants were cultured on A6+S10 medium under light condition, the rooting of SBS-L, SBS and LP were significantly slower than that under dark culture (Fig. 3(f) and 3(g)). Moreover, the rooting rate of these explants was significantly reduced at 20 DAC, only 43.3%±8.20% for SBS-L and 10.00%±5.40% for SBS, and only 4.40%±4.20% for LP (Fig. 3(f)), which was easy to root in dark culture (Fig. 3(g)). Subsequently, rooting rates of SBS increased gradually with the extension of culture time but were ultimately not as high as that under dark culture. Rooting rates of SBS-L and LP did not change with observation time. Meanwhile, P and SS did not generate root after continuous culture at 60 DAC, while B occasionally took root. These results suggest that light significantly inhibited ARF of explants with a leaf in the presence of additional sucrose (10 $g \cdot L^{-1}$), which was confirmed by subsequent experiments.

Combined with the above results, it is indicated that the ARF phenotypes of SBS-L and LP were fast, synchronous, and consistent between samples. Leaves accelerate rooting and it may act as a supplier or distributor of carbohydrates and hormones needed for ARF. The comparison of ARF results from field woody cuttings and leaves suggested that the tissue culture materials with continuous subculture was easier to root that might be because of its juvenile state, which is difficult for field young leaf samples to be simulated.

ARF phenotypic characteristics of grape LP

LP was an ideal research system for grape ARF, which excluded the influence of sprouting, and was the simplest and most readily rooted unit of the explants investigated. Wounding was the primary factor to induce AR formation in LP explants. When the petiole was picked with tweezers, the middle part of the petiole was occasionally clipped, and these wounds can form AR (Fig. 4(a)). With attention to the clamping force, LP-induced AR appears more frequently about 2mm above the cut site, rather than at the base of the cut. Observing under the stereomicroscope, the ARs formation did not undergo callus, but was direct *de novo* organogenesis from petiole (Fig. 4(b)).

High light intensity inhibited ARF of grape LP

In an accidental culture process, we noticed that when the light intensity was ≥6000lux, the leaves cultured on A6 medium were damaged to varying degrees within 12h under 16h-photoperiod condition, and part of the leaf margin was burnt, which seriously affected rooting (from 67.78% reduced to 12.22% at 20 DAC), although injured LP can take root occasionally (Fig. 4(c)). Therefore, the light intensity was determined as 4000lux in the subsequent experiments.

The selection and control of LP explants influenced the consistency of ARF phenotype

In tissue culture, the growth potential of the SBS propagated plantlets were not completely consistent at different ages, and the leaf size and growth state of the same plant at different nodes were also different. Usually, leaves at the basal nodes were small and some of them may become brown and senile, while the middle and upper nodes are large and healthy, and the apical leaves are young and tender (Fig. 5(a)). These are different from the leaves of 12-day-old seedlings that are most suitable for ARF study in *Arabidopsis thaliana* by precisely controlling seedling age (Chen et al. 2014).

Leaf size was reflected in length×width (L×W cm^2) to analyze the correlation between leaf size and rooting (rooting rate and speed). In this study, the size (L×W) of healthy leaves of tissue culture plantlets ranged from 1 to 6. Cultured 20 days under dark (D) condition on A6 medium, 17.80% LP could form ARs. Forty LPs that rooted (D-R) and unrooted (D-NR) were randomly selected for statistics, and it is shown that when the average L×W was less than 1.8 (median), most of the leaves had no spontaneous rooting ability. While, in LP that could spontaneously root, the L×W was above 3.8 (Fig. 5(b)). Then LP was placed on A6 medium under light condition (L), most healthy leaves could generate root (67.8%) at 20 DAC. There was also a positive correlation between the rooting capacity and the size of leaves, which was mainly reflected in the small LP of unrooted (L-NR), with the size shrinking at about 1.6, however, the rooted LP (L-R) are widespread in a wide range of sizes (Fig. 5(b)). In addition, leaves with senescence at the base of the plant or withered patches at the leaf margin were found difficult to root under various conditions. These results suggest that the amount of nutrients stored in healthy leaves (under dark condition) and the ability to synthesize photosynthates and auxin-related to leaf photosynthetic area (under light condition) determines the ability of ARF. Therefore, leaf size and health status are important factors affecting the homogeneity of ARF.

Carbohydrate provision is prerequisite in the formation of AR from LP

In order to further prove the importance of carbohydrate in ARF, N3-N6 nodes were selected according to the previous results, and the leaf size was controlled at (2<L×W<6) to compare the difference of ARF in A6 and A6+S10 culture under dark and light, respectively. After controlled blade size and node position (Controlled), the rooting rate of LP in A6 medium under dark condition (16.7%±7.80% at 20 DAC) was not

significantly different from that without control (Uncontrolled) (Fig. 5(c)). The rooting rate of LP with strict selection was increased (82.8%±6.80% at 20 DAC) under light culture on A6 medium compared with that without control, and the errors were significantly smaller during the rooting process (Fig. 5(d)). When sucrose (10 $g \cdot L^{-1}$) was supplemented by the A6 culture medium, all healthy LP could root under dark condition, and the rooting rate finally reached 100% after 13 DAC (Fig. 5(e)). The leaves specially selected for senescence or withered spots at the leaf margin at the base of plantlets could hardly generate root on A6+S10 medium under dark. These results suggest that the amount of nutrients and hormones stored in healthy leaves (under dark condition) and the ability to synthesize photosynthates and hormones as determined by leaf photosynthetic area (under light condition) determines the ability of ARF. Therefore, leaf size and health status are the factors that need to be considered to affect the uniformity of ARF.

We used different mineral-containing medium without sucrose (B5, WPM and C2D) and cultured for 30 days under dark and found that only about 20% LPs could root (data not shown). However, it was difficult for LP to root on A6 medium under dark condition. In the absence of added sucrose, we did not find a culture regimen that increased LP under dark culture conditions.

The importance of auxin supply in ARF of grape LP

In this study, grape LP could generate root without adding exogenous auxin, suggesting that LP explants could provide endogenous auxin to meet the need of AR formation. In A6+S10 medium, different concentrations of NPA (0.1, 1 and 3 $mg \cdot L^{-1}$) were added and cultured under dark conditions. The results showed that the inhibition of NPA on ARF was related to concentration. With the increase of concentration, the inhibition effect of ARF became more obvious, and 3 $mg \cdot L^{-1}$ NPA could completely inhibit the rooting (Fig. 6(a)). In A6 medium, light culture showed that 0.1 $mg \cdot L^{-1}$ NPA had no noticeable inhibition on ARF, while 1 $mg \cdot L^{-1}$ and 3 $mg \cdot L^{-1}$ NPA could completely inhibit rooting (Fig. 6(b)). IBA was added to A6 medium with 0.1 and 1 $mg \cdot L^{-1}$, respectively, and cultured under darkness. The results showed that 0.1 $mg \cdot L^{-1}$ IBA significantly promoted ARF, and the rooting rate stabilized to 78.90%±10.30% after 11 DAC. However, 1 $mg \cdot L^{-1}$ IBA (36.70%±19.60%) had a less promoting effect on ARF than 0.1 $mg \cdot L^{-1}$ IBA (Fig. 6(c)). In addition, the adventitious roots produced after IBA treatment continuously was different from the control, showing an increase in the number of roots, rooting sites were not limited to the base of petiole, and AR elongation slowed down (Fig. 6(d) and 6(e)).

Light inhibited rooting in the presence of exogenous sucrose

Higher plants can integrate multiple signal transduction pathways such as light, auxin and reactive oxygen to fine-regulate adventitious root formation, to adapt to changing environmental conditions (Bai et al. 2020). Light exerts a strong influence on multiple aspects of the auxin system, controlling auxin

level, transport and responsiveness (Halliday et al. 2009). Sugar and light signal transduction is associated with auxin biosynthesis and root distribution changes (Garcia-Gonzalez et al. 2021).

In A6 medium and under light culture, leaves could produce photosynthetic products through photosynthesis, partially satisfying ARF (67.78-82.22%, 20 DAC) (Fig. 5(d)). However, as mentioned above, damage to leaves caused by high light intensity will affect ARF (Fig. 4(c)). In the medium supplemented with sucrose (A6+S10), the light was controlled at 4000lux to prevent the damage of strong light. After 20 DAC, only a few LP explants (4.40-5.60%) rooted, and the initiation of rooting was significantly delayed (F=11 DAC) (Fig. 6(f)). From the appearance of the leaves, there were no obvious injury symptoms. Since the addition of sugar may promote auxin synthesis, it is not clear whether the difficulty of rooting is due to the influence of auxin homeostasis (possibly including high concentration or polar transport). Under light condition, in the mediums A6+S10 with 1 or 3 $mg{\cdot}L^{-1}$ NPA (A6+S10+NPA1 and A6+S10+NPA3), LP did not root. However, LP in A6+S10+NPA0.1 can root (66.67%±2.72% at 20 DAC) (Fig. 6(f)), which is significantly higher than the control A6+S10. Since NPA inhibits the polar auxin transport, it is speculated that the difficulty of rooting A6+S10 under light culture may be related to the excessive polar auxin transport. In conclusion, light-induced inhibition of ARF of LP explants in grapes after sugar addition, while low concentration of NPA partially relieved this inhibition, suggesting that light and sugar signal may interact, but molecular biological evidence is needed.

Investigation of PGRs affecting ARF phenotype of LP

The effect of exogenous hormone on rooting may be either promotive or inhibitory and may be dose or species dependent (Bannoud and Bellini 2021). Small changes may have the opposite effect on ARF. Due to the heterogeneity of woody cuttings, it is difficult to accurately show the phenotypic changes of ARF after exogenous PGRs treatment. Based on the established LP study system for rapid and stable ARF phenotype identification, this study preliminarily investigated the effect of exogenous PGRs on grape ARF.

Under dark condition, LP explants could not root when the A6+S10 medium added $1mg{\cdot}L^{-1}$ 6-BA and GA_3, respectively. Meanwhile, 24-epibrassinolide (EBR) ($1\ mg{\cdot}L^{-1}$) also showed significant inhibition of ARF. ABA had no significant affection for ARF (Fig. 7(a)). The base of LP petiole treated by 6-BA expanded significantly (Fig. 7(b)). Lower concentrations of GA_3 (0.01 and 0.1 $mg{\cdot}L^{-1}$) were then examined, and it was found that they also inhibited rooting completely. SBS, SBS-L and woody cuttings were treated with GA_3 ($0.1\ mg{\cdot}L^{-1}$), and the rooting inhibition effect was also obvious, no root could be observed till 60 DAC. The results showed that GA_3 had a significant inhibitory effect on grape ARF (Fig. 7(d)). Studies have shown that the inhibition of GA on ARF may be due to the inhibition of auxin polar transport by GA (Mauriat et al. 2014). Based on this, LP was cultured on A6+S10 with GA_3 ($0.1\ mg{\cdot}L^{-1}$) and IBA ($1\ mg{\cdot}L^{-1}$) under dark conditions, and partial rooting of LP was found (Fig. 7(c)). It indicates that IBA ($1\ mg{\cdot}L^{-1}$) could partially reduce the inhibition effect of GA_3 ($0.1\ mg{\cdot}L^{-1}$). The characteristics of rooting explants in this treatment were similar to those in IBA treatment: the number of adventitious roots increased, and the

elongation slowed down. Therefore, it is speculated that GA_3 may inhibit rooting by inhibiting endogenous growth factors in grapes.

Endogenous ethylene and jasmonic acid (JA) can be induced by wounding, and both ET and JA may promote rooting in the early stage and inhibit rooting·Later (da Costa et al. 2013). After culture on A6+S10 with 0.01, 0.1 or 1 $mg·L^{-1}$ ethephon (ET) under dark condition, there were no significant effect on ARF of grapes (Fig. 7(e)). In this study, the rooting rate was reduced after culture with MeJA (0.1 and 1 $mg·L^{-1}$) on A6+S10 medium under darkness (Fig. 7(f) and 7(g)). This suggests that MeJA has some inhibitory effect on ARF, at least at these two tested concentrations. After culture on A6 medium with MeJA (0.1 and 1 $mg·L^{-1}$) medium under darkness, rooting rate increased, but there was no significant difference. In addition, leaves treated with MeJA in this study were uniformly yellow (Fig. 7(h)), which may be related to the initiation of systemic defense by MeJA.

Discussion

Some wild grapes show genotypic differences in rooting characteristics, which could be reflected in differences in the levels of endogenous hormones or nutrients or other endogenous molecules in the explants. Phenotypic heterogeneity is a concept in cell biology, and the heterogeneity of cells in plants is the prerequisite for the formation of different organs in plants (Hong et al. 2018). We introduce this concept to indicate the phenomenon that the target phenotypes are inconsistent due to the differences of endogenous factors in the samples at the physiological level. Phenotypic heterogeneity makes it difficult to analyze the function and mechanism of influencing factors of phenotypes. In present study, we hope to introduce a simple, rapid, phenotypic consistent and quantifiable research system. This system could reduce the confusion caused by the heterogeneity of materials in the analysis of ARF influencing factors.

In grape, the comparison of factors related to the differential phenotype of ARF by different treatments of hardwood or greenwood cuttings with leaves has been reported in many studies (Kose et al. 2011; Patil et al. 2020; Saleh 2019; Kose 2007; Amiri et al. 2019; Thomas and Schiefelbein 2004). These studies confirmed that endogenous auxin (indole-3-acetic acid, IAA), vitamins, carbohydrates, organic nitrogen and/or rooting cofactors, as well as some genes (Thomas et al. 2003) or miRNA(Chen et al. 2020) were involved in cuttings rooting. The effect of sprouting process on cuttings is also mentioned (Zhou et al. 2020). However, these studies have not clearly explained the process of AR formation in grapes, particularly the regulatory mechanism of AR induction and initiation. Here, we need a consistent research system of ARF phenotype, but the phenotypic heterogeneity of ARF in grape woody cuttings is severe (Fig. 2), and its acquisition is seasonal. The physiological and biochemical quality of the mother plants affects the rooting ability of cuttings (Bannoud and Bellini 2021). In vitro propagation plants have many advantages, such as ease of preparation at any time of the year, rapid growth and relative homogeneity, good hygiene and space saving (Valat et al. 2018). We found that the ARF of tissue culture explants was more consistent than woody cuttings (Fig. 3). However, different explants from diverse sources, different types of explants, and the physiological conditions, age, leaf size and node location of the same type of explants also affected the homogeneity of ARF phenotype (Fig. 5). Studies using Blade and LP showed

that the difference of ARF phenotype in *Arabidopsis thaliana* was significantly correlated with leaf age, and suitable explants were limited to 12day-old plants. The contents of sugar, starch and auxin in leaves of different growth time were different, which had great influence on ARF (Chen et al. 2014; Della Rovere et al. 2013; Correa et al. 2012). The ability to produce adventitious roots in young leaves of *Arabidopsis thaliana* is stronger than that in mature and senile tissues, which may be related to the content of endogenous auxin, because the addition of auxin can partially save the AR regeneration defect of mature leaves (Diaz-Riquelme et al. 2014; Chen et al. 2016). Tissue culture can restore plant vitality and rooting ability, especially in some species that are difficult to root, which may be due to the high content of endogenous auxin in juvenile tissue culture plantlets (Haapala et al. 2004). In grapes, this study showed that, even with the addition of auxin, leaves of all leaf ages sampled in the field were difficult to root, while LP from tissue culture plantlets was easy to root in (Fig. 2). It is proposed that the increase of rooting potential in tissue culture may not only be due to the increase of endogenous auxin, but also to fewer rooting inhibitors or fewer physicochemical characteristics of root retarding in explants.

We hypothesized that the distinction in ARF caused by leaf age (node position) could be reflected in the difference in the supply of endogenous carbohydrate and auxin in leaves. Leaf age alone could not completely explain the difference in grape explants. We concluded that leaf size, node location, and health status affected our judgment of ARF phenotype. In this study, it was found that under the condition of strictly controlling the source of explants and culture conditions, without the need to add other mineral nutrients or auxin, under the condition of dark culture in A6+S10 medium, grape LP could form AR within 6-7 days, and the rooting rate reached 100% in 13-14 DAC (Fig. 5(e)). LP is the smallest and simplest ARF unit in grapes. Owing to the absence of exogenous mineral nutrients and hormones, and the absence of sprout, the system eliminated other factors affecting ARF to the maximum extent and could screen key genes for AR induction and initiation more conveniently. We determined the conditions under which LP uniformly produces ARF phenotype and verified the influence of related factors on ARF. This system offers a better approach to study adventitious root induction and initiation. The system can be used to study the factors affecting adventitious root formation and growth of the grapevine.

Recent research on ARF and its influencing factors was described in the following reviews (Li 2021; Sharma et al. 2021; Gonin et al. 2019; Ikeuchi et al. 2019). ARF is controlled by many endogenous and environmental factors, including endogenous nutrient levels, auxin and other hormones, and some secondary metabolites. Exogenous factors include wounding, light, temperature, biological or abiotic stress and other factors. At the molecular level, complex networks are associated with the regulation of AR production in plants. Key molecular events of ARF occur during root induction and initiation. When an organ or tissue is detached from the mother plant, the injury cuts off the basal auxin transport channel, and auxin accumulates in the cells of the wounded tissue by polar transport (Yu et al. 2017; Gonin et al. 2019). Wounding also induces some biochemical reactions immediately (da Costa et al. 2013; Druege et al. 2014; Rasmussen et al. 2015), and triggers endogenous auxin changes that help target cells reprogram into regeneration. Endogenous auxin (IAA) plays a key role in ARF, and other hormones mainly regulate ARF through interaction with auxin. Many genes involved in auxin perception, transport, and homeostasis, as well as in cell division, cell wall regulation, and root meristem formation and

maintenance, have been identified to regulate ARF (Brinker et al. 2004; Sorin et al. 2006; Holmes et al. 2010; Gutierrez et al. 2012; Mashiguchi et al. 2011; Li et al. 2012; Peret et al. 2012). In this process, some miRNAs also regulate the expression of auxin response factors (Xu et al. 2017)In addition to auxin, other signal pathways of endogenous hormones such as cytokinin, ethylene, gibberellic acid, jasmonic acid, and its crosstalk modulate ARs regeneration. Crosstalk and interaction among various molecular processes produce complex networks that regulate ARF. The role of hormones in ARF can be quickly identified by mutations, transgenes, or exogenous PGRs, as well as inhibitors of endogenous hormones. In conclusion, ethylene and brassinosteroids may promote adventitious root initiation, while cytokinin, jasmonic acid, gibberellic acid, strigolactone and ABA may inhibit adventitious root formation. However, the effects of hormones on ARF may vary by dose or species (Busov et al. 2006; Chen et al. 2006; Ramirez-Carvajal et al. 2009; Bergonci et al. 2014; Negi et al. 2010).

This study confirmed that LP was difficult to root on medium without sucrose in dark culture, while it was easy to root on medium with 10g·L^{-1} sucrose under dark condition or without sucrose but under light condition (Fig. 5). This is consistent with the results in *Arabidopsis thaliana*, suggesting that carbohydrates are necessary for ARF (Chen et al. 2014). Studies have shown that sucrose can affect auxin homeostasis in the form of signaling molecules (Rolland et al. 2006). Sucrose has also turned out to be an initial regulatory signal of apex dominance affecting budbreak (Mason et al. 2014). However, it has yet to be studied whether sugar only serves as a carbon supply or whether it acts as a signaling molecule on ARF (Ahkami et al. 2013).

Although the effect of exogenous PRGs on ARF cannot fully reflect the function of endogenous hormones, the mechanism of the effect of exogenous PRGs on ARF can be studied through the treatment of endogenous hormone analogs and inhibitors. Under the premise of satisfying the carbohydrate supply, the utilization of NPA confirmed the key role of auxin in grape ARF. NPA is an inhibitor of auxin polar transport, and its concentration on grapes was determined to be greater than or equal to 3 mg·L^{-1}, which can guarantee complete inhibition of LP rooting (Fig. 6(a) and 6(b)). Studies have shown that high concentration of auxin is required to induce AR at the induction stage after cutting, but it has an inhibitory effect on subsequent AR formation (da Costa et al. 2013). The rooting rate of LP was increased when IBA was added into A6 medium for continuous dark culture in this study (Fig. 6(c)). Moreover, the relationship between the effect of auxin on ARF and its concentration was confirmed by quantitative data. The quantifiable rooting data of this system can give assistance to more detailed research in the future.

The relationship between sugar, light and auxin and its function in ARF were embodied in the experiment of this system. Sugar may affect the homeostasis of endogenous auxin together with light through affecting PIF (Sairanen et al. 2012), which may be the justification why light inhibits rooting in the presence of exogenous sugar in this study. These hypotheses will be tested in future studies.

In application, AR can be induced from asexually propagated cuttings. However, when exogenous auxin is added and/or other culture conditions are combined, some species or varieties may fail to achieve the goal of rooting, suggesting that there may be other physiological factors or compounds that inhibit

rooting in these plants (Wei et al. 2019). In this study, the application of 6-BA, GA_3 and EBR severely inhibited the rooting of grapes (Fig. 7).

In this study, it was confirmed that 6-BA completely inhibited the rooting of grape LP (Fig. 7(a)). According to studies, some decreased ability of ARF is related to endogenous cytokinin. In *Arabidopsis*, cytokinin in the *WUS* expression region can regulate *YUC1* and *YUC4* through *ARR1*, *ARR10* and *ARR12*, and inhibit the biosynthesis and accumulation of auxin, thus inhibiting the formation of AR (Meng et al. 2017). In poplar, cytokinin act as negative regulator of ARF (Saito et al. 2019), and this process is caused by cytokinin affecting the polar transport of auxin (Ramírez-Carvajal et al. 2009). In conclusion, cytokinin may negatively regulate auxin synthesis, transport and homeostasis by affecting multiple genes, thus inhibiting ARF (Li 2021).

GA_3 has been proved to significantly inhibit the ARF of grape in this study (Fig. 7(a) and 7(c)). Reports from different species suggest that gibberellins (GAs) have an inhibitory effect on AR formation and development (Niu et al. 2013; Mauriat et al. 2014). External application of GA_3 has been shown to inhibit rooting in poplar (Busov et al. 2006), possibly by inhibiting auxin polar transport by GA (Mauriat et al. 2014), which could be eliminated by miR476 (Bannoud and Bellini 2021). However, some studies have shown that endogenous GA is necessary for AR formation, which seems to be related to the functional differences of different types of GAs. Studies have shown that endogenous GA_1 may be a promoter of ARF (Ibanez et al. 2019; Xu et al. 1995; Michaels and Amasino 1999). In all these cases, the relationship between GA biosynthesis and GA signaling appears to be both complex and context-specific and warrants further investigation. In grapes, Kracke et al. (1981) mentioned that the species that are difficult to root have high endogenous GA content (Kracke et al. 1981), which indicated whether the high endogenous GA content is a limiting factor for these species or varieties is worth investigating.

Application of MeJA can promote the formation of adventitious rooting in tobacco (Fattorini et al. 2009). AR Induction may be related to early induction of endogenous JA by wounding, which in turn promotes the accumulation of IAA (Druege et al. 2019). In *Arabidopsis*, JA can activate the expression of *ASA1* and *YUCs* genes through *ERF109* and enhance auxin synthesis, thus promoting the occurrence of lateral roots (Cai et al. 2014). However, MeJA long-term treatment inhibits adventitious root initiation (Gutierrez et al. 2012). Endogenous auxin and JA may interact with each other. COI1 is the receptor of JA pathway and is regulated by miRNA, ARF and GH3 proteins. The binding of ARF and GA_3 to auxin and JA leads to activation, inactivation, or degradation, and regulates homeostasis of auxin and JA. Long-term exogenous JA treatment may lead to feedback regulation of endogenous IAA, thus affecting adventitious root occurrence (Westfall et al. 2010; Li 2021). In this study, the application of MeJA on A6+S10 had an inhibitory effect on grape ARF, which was related to concentration. In the absence of sucrose, MeJA feebly promoted rooting on A6 medium (Fig. 7(f) and 7(g)). This complex result suggests that the function of MeJA in grape ARF may be related to the crosstalk between JA, auxin and sugar, which needs to be further explored in more detailed experiments.

Studies in model plants show that the various hormones and their crosstalk are extremely intricate, but this is not illustrative of the real situation in grapes. This study developed a simple and rapid LP system suitable for the study of AR in grapes, which can assist with explaining the particularity of ARF in grapes. LP materials from tissue culture are readily available. The ARF phenotype of the constructed system was easy to observe, and the data of rooting rate was easy to quantify. The design of various processing can facilitate the data analysis, which is conducive to the detailed investigation of ARF influencing factors. Based on these characteristics, we revealed some interesting effects of sucrose and hormone treatment on grape ARF. Much remains to be done, and the system will show its advantages.

Declarations

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Author Contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by XinYu Chang, Kai Zhang, Yunzhang Yuan, Peiyi Ni, Jing Ma, Hui Liu and Shiyu Gong. Data analysis was performed by XinYu Chang and Miao Bai. The first draft of the manuscript was written by Guoshun Yang and Miao Bai and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

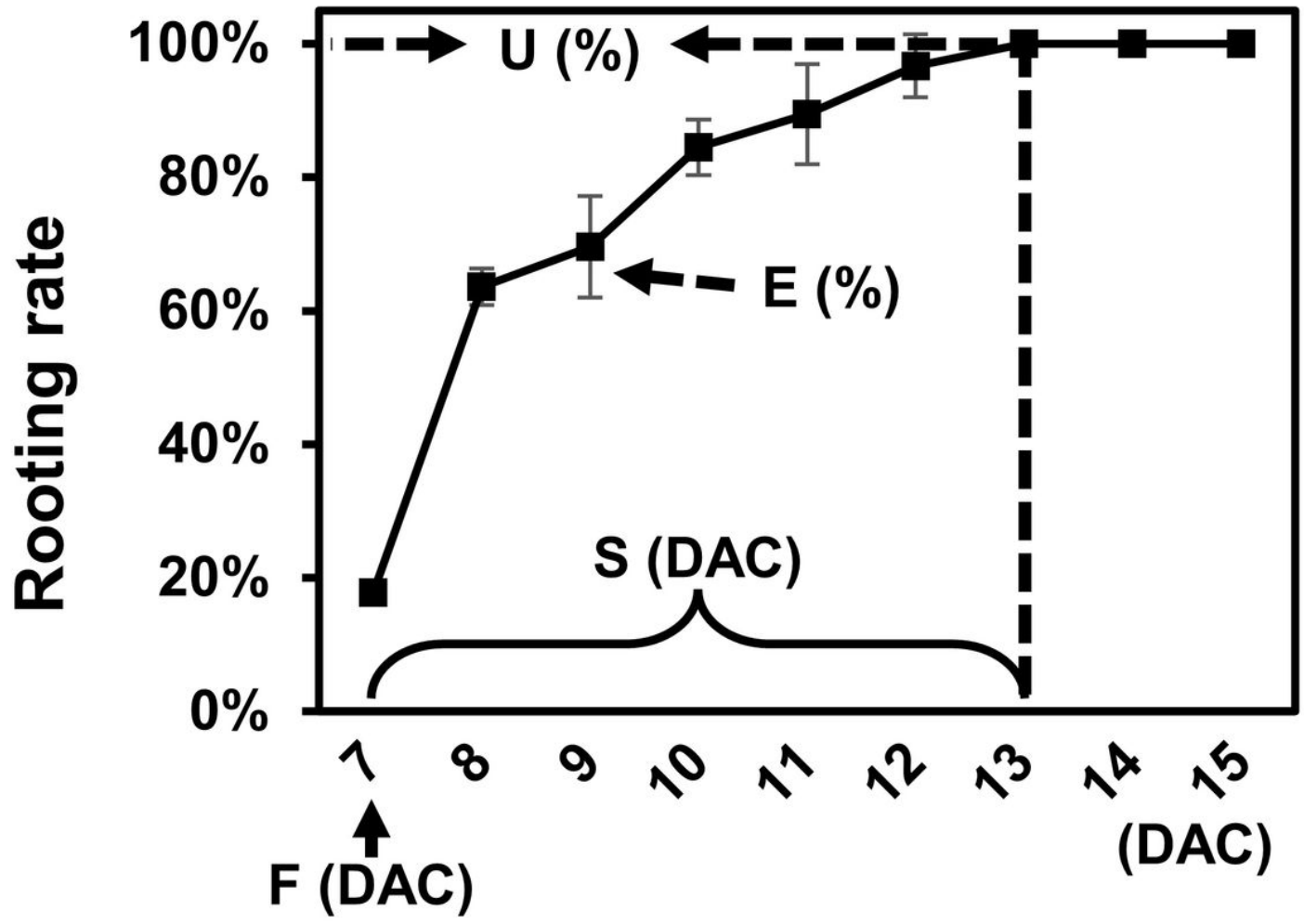

Figure 1

The parameters of rooting rate curve indicate the homogeneity of ARF

The proportion of explants with ARF phenotype was defined as rooting rate, and AR data curve was made by the dynamic change of rooting rate by daily (day after culture, DAC). The curve was served to judge the homogeneity or heterogeneity of samples by characterizing the speed, uniformity, synchronism, and the error of ARF phenotype. Where, the speed is defined as the time F (d) from the beginning of processing to the formation of the first AR. Uniformity was defined as U (%) of the rooting rate. It is expresses as U_{max} when stability eventually ceases to take root. S (d), defined as synchronism, represents the number of days from the first AR formation to the U_{max}. The smaller S is, the higher the synchrony of rooting is. The standard deviation (SD) of rooting rate represents the statistical error E (%) between three or four biological replicates (N≥30). The smaller E is, the smaller individual differences are.

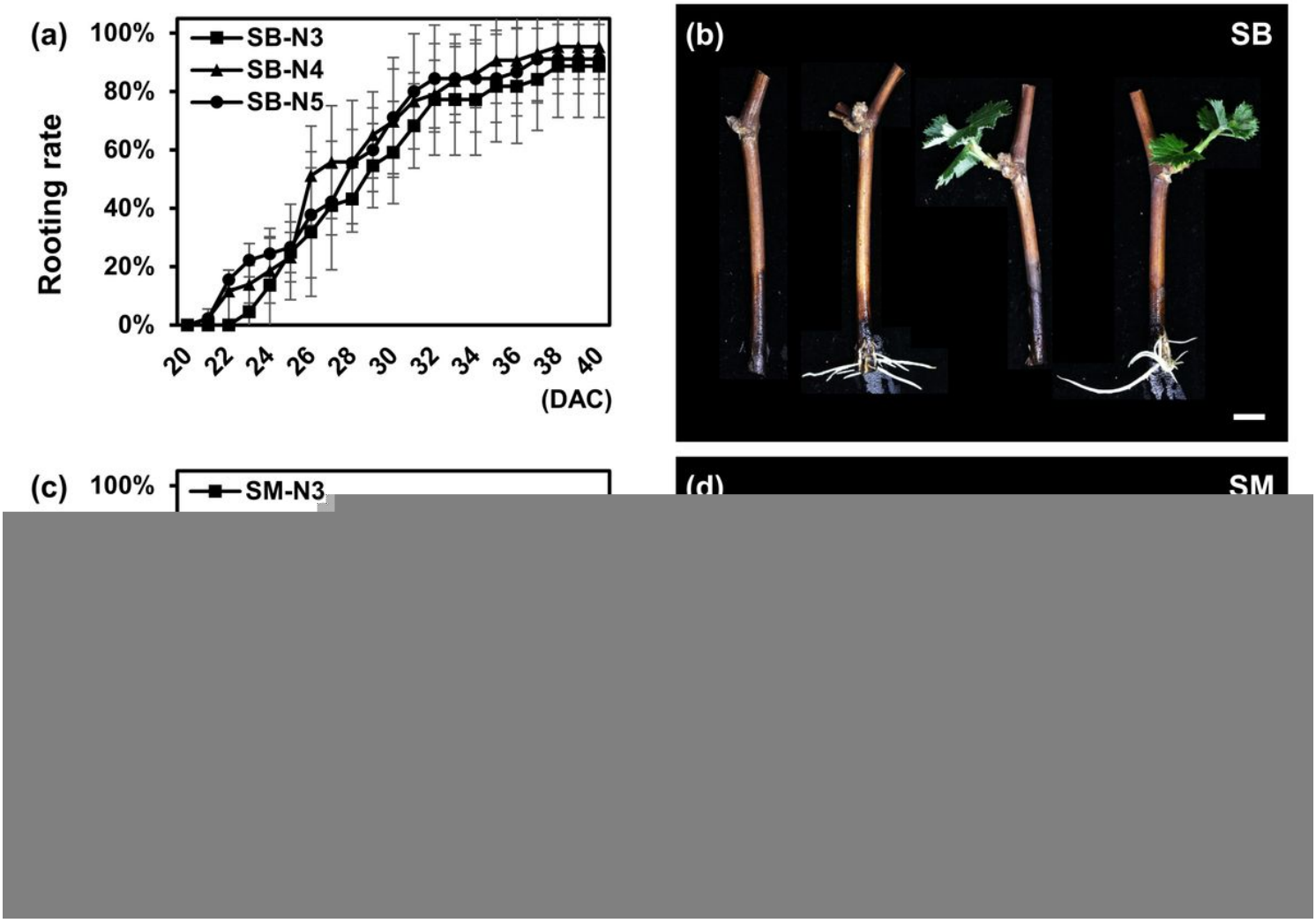

Figure 2

Phenotypic heterogeneity of hardwood cuttings

Rooting rates of hardwood cuttings of ‘Summer Black’ (SB) and ‘Shine Muscat’ (SM) were recorded every day after culture (a and c). N3-N5 represented the cuttings with a single bud from the 3rd to 5th nodes of

current canes, respectively. The phenotypic heterogeneity between rooting and sprouting of cuttings at the same node (b and d). Scale bars: 1cm.

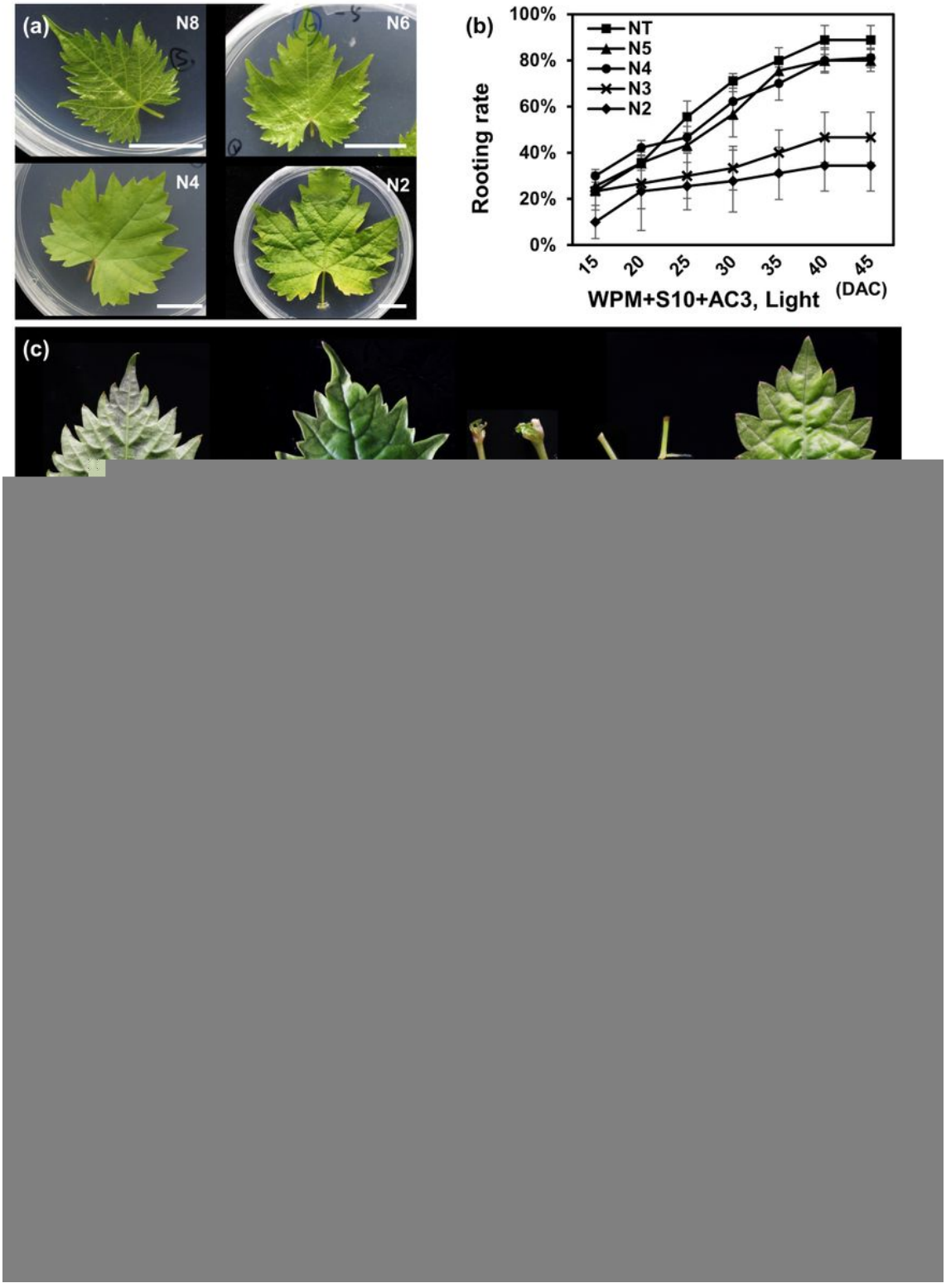

Figure 3

ARF phenotype of different types of explants

The leaves of 2-years-old vines in the field at different nodes (N8, N6, N4 and N2, respectively) could not take root in A6 or A6+IBA0.1 medium. The state of leaves after culture was presented in (a). Scale bars: 1cm. SBS taken from nodes (N2-N5 and NT) were cultured on WPM+S10+AC3 medium under light, and the differences of rooting rate were shown in (b). Rooting of different types of explants was displayed in (c). Arrow shows the growth of explants 7days after root regenerated. (d-g) showed rooting rates of different types of explants, which were cultured in A6 medium under light (d) and dark (e) condition, in A6+S10 medium under light (f) and dark (g) condition, respectively.

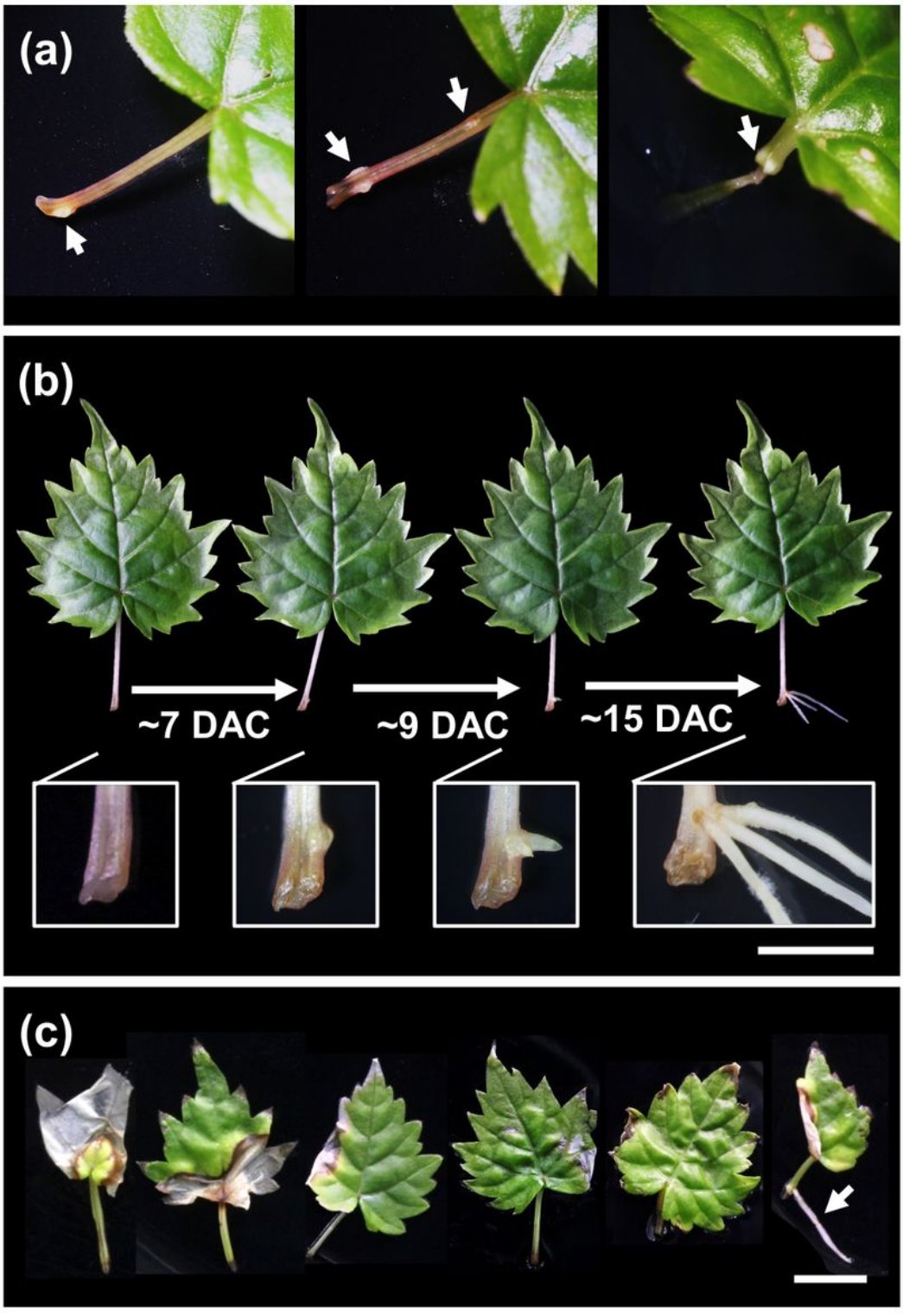

Figure 4

Characteristics of AR formed on LP and damage of LP by high intensity light

The presence of ARs at wounded site of the petiole was shown in (a); ARF process of LP was shown in (b); (c) shows the damage of leaves under high intensity for 24h, which further affects ARF. The arrow shows where AR or AR occurs. Scale bars: 1cm.

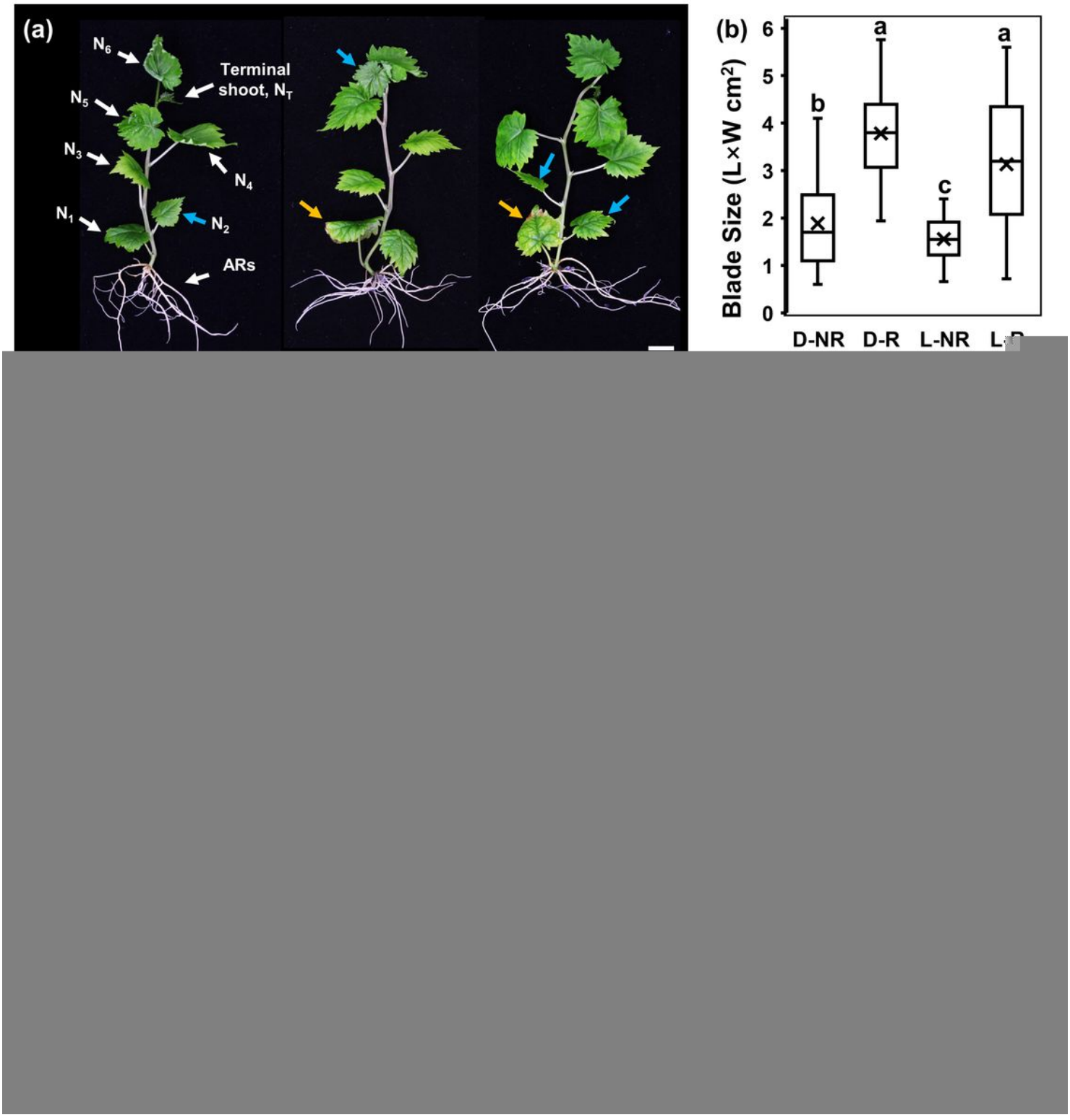

Figure 5

Control of LP explants and their effect on phenotypic heterogeneity of ARF

The growth status of tissue culture plantlets was presented in (a). Arrows indicate nodes from the base up. The blue arrows indicate small leaves (2<L×W), yellow arrows indicate aging or unhealthy leaves. ARF characteristics of LP with different leaf sizes in A6 medium under dark or light condition were shown in (b). D-NR and D-R represent the size distribution of unrooted and rooted LP under dark condition respectively, while L-NR and L-R represent the distribution under light culture. After controlling the leaf size (2<L×W<6) and node position (N3-N6), the rooting rate of LP were recorded in A6 medium under dark (c) and light (d) condition, and in A6+S10 medium under dark (e) and light (f) condition, respectively. Scale bars: 1cm. Different lowercase letters in each graph indicate significant differences ($p \leq 0.05$).

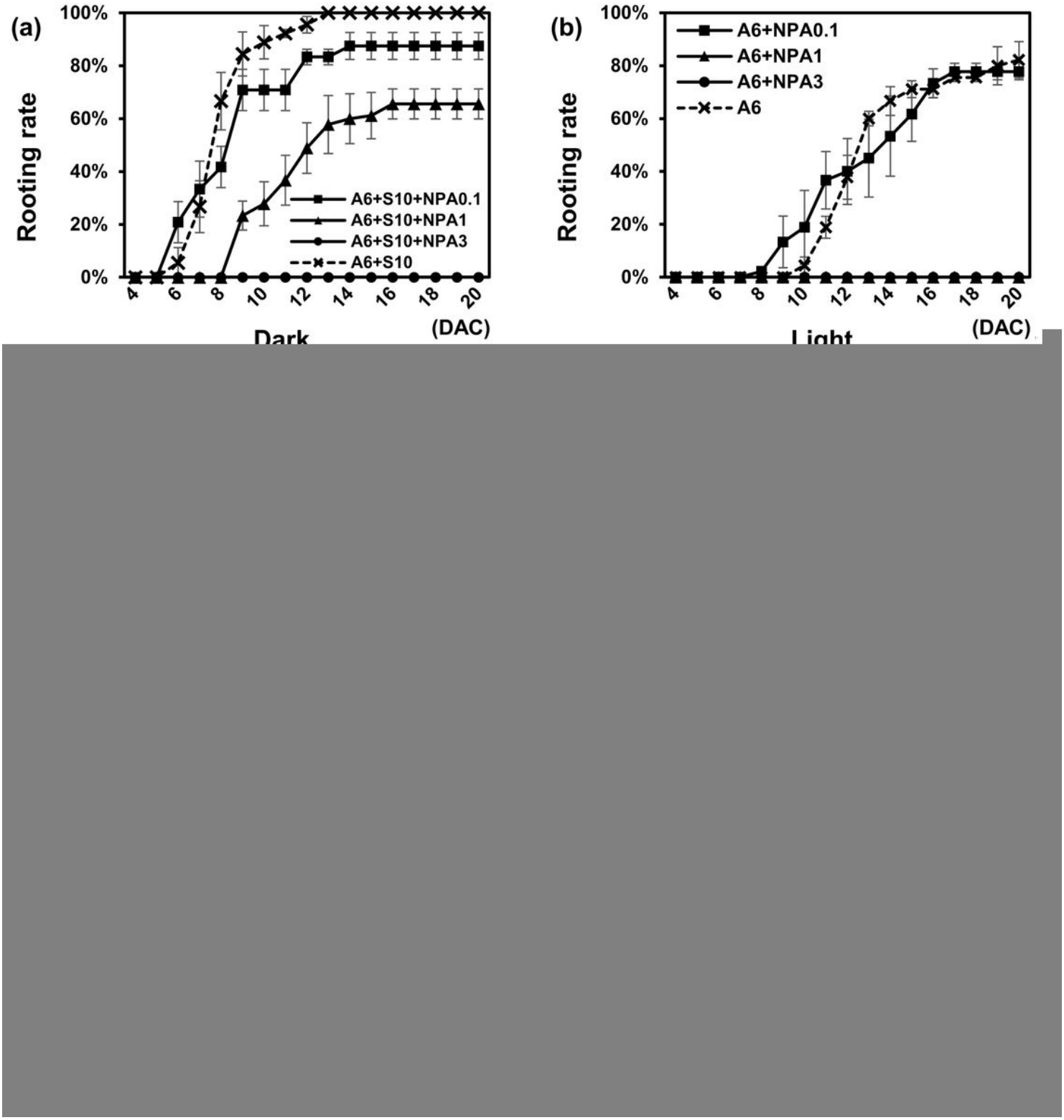

Figure 6

The importance of auxin supply in ARF of grape LP

Rooting rate of LP cultured in A6+S10 with 0.1, 1 or 3 mg·L^{-1} NPA under dark or light condition were shown in (a) and (b), respectively. Rooting rate of LP cultured in A6 with 0.1 or 1 mg·L^{-1} IBA under dark condition was shown in (c), and the state of LP after rooting was presented in (d). The arrow shows

where AR occurs after 30 DAC. Scale bars: 1cm. The number and length distribution of ARs of LP on A6 (N=10) and A6 with 0.1 or 1 mg·L-1 IBA medium cultured under dark at 20 DAC were shown in (e). Different lowercase letters in each graph indicate significant differences ($p \leq 0.05$). Rooting rate of LP cultured on A6+S10 with 0.1, 1 or 3 mg·L^{-1} NPA under light was shown in (f).

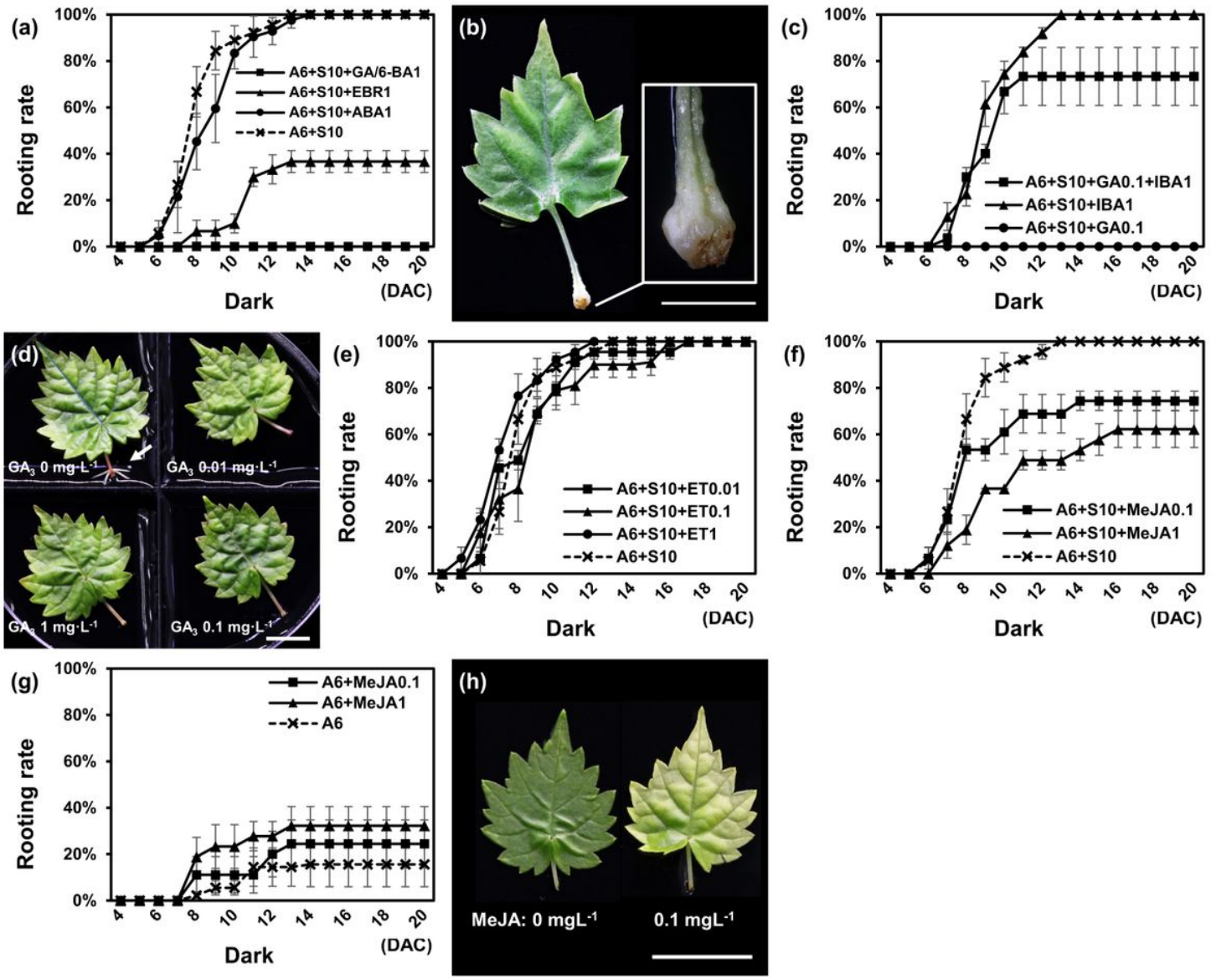

Figure 7

Investigation of PGRs affecting ARF phenotype of LP

Rooting rate of LP under dark condition on A6+S10 with 1 mg·L^{-1} 6-BA, GA_3, EBR or ABA medium was shown in (a). The growth state of LP explants cultured on A6+S10 with 6-BA 1 mg·L^{-1} medium under dark condition was presented in (b). Rooting rate of LP under dark condition on the medium of A6+S10 with GA_3 and/or IBA was shown in (c). The growth state of LP explants cultured on A6+S10 with GA_3 0.01, 0.1 or 1 mg·L^{-1} medium under dark condition was presented in (d). White arrow indicates ARs. Rooting rate of

LP under dark condition on A6+S10 with ET 0.01, 0.1 or 1 $mg{\cdot}L^{-1}$ medium was shown in (e). Rooting rate of LP under dark condition on A6+S10 with MeJA 0.1 or 1 $mg{\cdot}L^{-1}$ medium was shown in (f). (g) showed rooting rates on A6+MeJA 0.1 or 1 $mg{\cdot}L^{-1}$ medium under dark condition. Leaf chlorosis caused by MeJA was shown in (h). Scale bars: 1cm.