J Plant Growth Regul
DOI 10.1007/s00344-016-9651-9

Pyramid Screening: Combining Three Genetic Screens into One Efficient Screen for Shoot Regeneration Mutants in *Arabidopsis thaliana*

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Received: 5 August 2016 / Accepted: 5 October 2016

Abstract Gain-of-function genetic mutants are typically found by creating a non-permissive condition and screening for plants that overcome the stress. Separate genetic screens are conducted for each condition, a potentially time-consuming effort. In severed *Arabidopsis thaliana* leaves, high light, suboptimal hormone exposure and old age were each independently found to reduce frequency of shoot regeneration. Rather than conducting three separate mutant screens to dissect these three pathways, a laborious process, we hypothesized that we could undertake a single economical screen to retrieve mutations specific for each trait as well as cross-talk alleles between pathways. Instead of creating non-permissive conditions for each of our three traits of interest, we combined the three suboptimal stress conditions such that only when combined was shoot regeneration abolished. No one stress was primarily responsible for loss of our trait, thus ensuring that we could recover mutant alleles in any of the three pathways of interest. Screening of 18,000 mutagenized plants resulted in 12 *SHOOTING UP* (*stu*) mutants. Secondary screening revealed that we had recovered alleles that were both specific for a pathway (light, hormones or age) and which acted through multiple pathways. Our approach, which we refer to as pyramid screening, represents an economical method for mutant screening of multiple pathways in parallel (three screens in one) and has the potential to recover alleles that cross-talk between multiple pathways that underlie a complex trait such as organ regeneration. Pyramid screening should be widely applicable across species.

Keywords *Arabidopsis* · Mutant screen · Shoot regeneration · Hormone · Abiotic stress

Electronic supplementary material The online version of this article (doi:10.1007/s00344-016-9651-9) contains supplementary material, which is available to authorized users.

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Introduction

Mutant screens typically proceed by screening for a mutation that targets one specific trait under a specific environmental or experimental condition. There are two limitations to this approach. First, when a mutant screen is time and labor-consuming or requires extensive growth space, then the one-condition, one-pathway approach is not economical. Second, because mutant screens are typically conducted with a narrow phenotype focus (for example, sugar tolerance), cross-talk with other biochemical or signaling pathways may not be revealed until years later when an independent research group conducts a mutant screen

Published online: 21 January 2017

for their trait of interest only to discover that the underlying gene had been studied for years in a different pathway. For example, in the model plant, *Arabidopsis thaliana* (L.), alleles of abscisic acid hormone biosynthetic genes and ethylene hormone signaling genes were later found in screens for sugar insensitivity (Arenas-Huertero and others 2000; Laby and others 2000; Gibson and others 2001; Ljung and others 2015). If cross-talk can be revealed early, then the suite of phenotypes associated with a novel gene might be characterized faster.

We are interested in isolating genes that regulate shoot organ regeneration from detached *Arabidopsis thaliana* tissues (Zhao and others 2002; Nameth and others 2013; Xu and Huang 2014). Post-injury organ formation requires regeneration of a stem cell population (shoot apical meristem, SAM) responsible for elaborating all shoot organs (Perianez-Rodriguez and others 2014; Gaillochet and Lohmann 2015). Plant regeneration studies in *Arabidopsis* have demonstrated that increased light intensity (Chaudhury and Signer 1989; Nameth and others 2013), reduced auxin and cytokinin hormone duration (Cary and others 2002; Gordon and others 2007; Motte and others 2014) and old tissue age (Zhao and others 2002) can inhibit this developmental process. Microarray cluster analysis (Kilian and others 2007) suggest that these and other pathways may be coordinately upregulated or downregulated during shoot regeneration (Cary and others 2002; Che and others 2006). Some progress is being made in elucidating the underlying molecular mechanisms (Cheng and others 2013; Xu and Huang 2014). We have confirmed and tried to understand how these three conditions regulate shoot regeneration using detached embryonic leaves (cotyledons) of *Arabidopsis* (ecotype Landsberg *erecta*, Ler-0) as a model system (Nameth and others 2013). To further dissect each trait at the genetic level, we had planned to create three non-permissive regeneration conditions (for example, very old age, minimal hormones, high light), and then undertake three separate gain-of-function mutant screens. However, we realized that each screen would be laborious: we calculated that the in vitro screening of 20,000 ethylmethane sulfonate (EMS)-mutagenized lines would require 80,000 manual tissue transfers, as each tissue would have to be transferred individually between four types of tissue culture media (germination, induction, regeneration and basal). Furthermore, we were not only interested in finding mutants in each pathway, but also mutations in hypothetical master cross-talk proteins controlling or responsive to two or more of these pathways.

In this paper, we describe a novel mutant screen method that achieved our objectives in a single experiment rather than three separate screens. This has resulted in considerable labor and cost savings and the uncovering of alleles that are both specific to a "pathway" as if we had conducted three separate mutant screens, as well as other alleles that affect two or three pathways. Pyramid screening is a method that should be widely applicable across species to help elucidate the genetic mechanisms underlying somatic regeneration.

Materials and Methods

Light, Hormone and Age Regeneration Assays

The *Arabidopsis* ecotype used was Ler-0 (Lehle Seeds, Round Rock, Texas, Catalog WT-4). Growth and cotyledon-based regeneration conditions and media were as described previously (Nameth and others 2013). Seedlings were germinated in Germination Media under 50–65 μmol m^{-2} s^{-1} continuous cool white fluorescent light at 25 °C. The age treatments consisted of cotyledons detached from 4-, 6-, 8-, 10-, 12- or 14-day post-germination seedlings, then transferred onto callus induction media (CIM, containing 0.5 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin) for 5 days, and then shoot induction media (containing 0.5 μM NAA and 4.4 μM 2-iP) for 28 days. Continuous low light was used (20–25 μmol m^{-2} s^{-1}). The post-detachment light treatments consisted of either 30 days of 24 h continuous cool white fluorescent light (low, 20–25 μmol m^{-2} s^{-1}; medium, 44–55 μmol m^{-2} s^{-1}; high, 80–94 μmol m^{-2} s^{-1}), or a shift treatment (5 days of continuous high light, followed by 10 days of darkness, then 18 days of high light). 7-day-old cotyledons were used, transferred onto CIM for 5 days, then SIM for 28 days. The post-detachment hormone treatment consisted of 5 days of callus induction media followed by 0, 1, 2, 3, 4 or 5 days of SIM, and then SIM lacking hormones (Basal Media). 7-day-old cotyledons were used, and exposed to continuous low light (20–25 μmol m^{-2} s^{-1}). Regeneration was scored 4–5 weeks after detachment.

Defining a Non-permissive Regeneration Condition by Pyramiding Three Suboptimal Growth Conditions

Cotyledons from 4-, 7-, 14- and 18-day-old seedlings were detached, treated for 5 days on CIM, transferred onto SIM for 1, 2, 5 and 10 days, then transferred onto basal media (no hormones) for the duration. Cotyledons were exposed to low, medium or high light (20–25, 50–60, 90–120 μmol m^{-2} s^{-1}, respectively, using cool white fluorescent bulbs) for continuous 24 h periods. The light-shift treatment consisted of 5 days of high light, 10 days of darkness, followed by high light, at 23 °C in a large Conviron growth chamber. There were 64 treatments of which 36 are shown (Fig. 1). For all experiments, seeds and cotyledons were evenly plated at a density of 26 cotyledons per plate.

Mutant Screen

A total of 18,000 M2 EMS-treated Landsberg *erecta* (Ler-0) lines (Lehle Seeds, Round Rock, Texas, Catalog M2E-04-06) were pyramid screened for enhancement of shoot regeneration under non-permissive conditions. Shown is the isolation of an enhancer mutant (arrow), on a plate of non-regenerating wild-type siblings (Fig. 2). Growth and cotyledon-based regeneration conditions and media were as described previously (Nameth and others 2013). For the final screen, seedlings were germinated in Germination Media under 50–65 µmol m^{-2} s^{-1} continuous cool white fluorescent light at 25 °C; 7- or 8-day-old detached cotyledons were treated for 5 days on CIM Media, then transferred onto SIM Media for 24 h, then transferred onto basal media for 30 days; regeneration occurred at 110–130 µmol m^{-2} s^{-1} continuous (24 h) cool white fluorescent light at 23 °C. Mutants were scored that regenerated a green shoot, defined as having at least two distinct leaves, within 4 weeks following detachment. Heritability was confirmed in the M3 and M4 generations.

Determining the Pathway Affected by Each Mutant

To test for enhancement of old age inhibition, cotyledons from 4-day-old versus 12-day-old seedlings were detached, then placed onto CIM media for 5 days, then SIM media for 30 days (optimal) at 20–30 µmol m^{-2} s^{-1} continuous light (optimal). To test for enhancement of high light inhibition, 7-day-old cotyledons (standard) were detached,

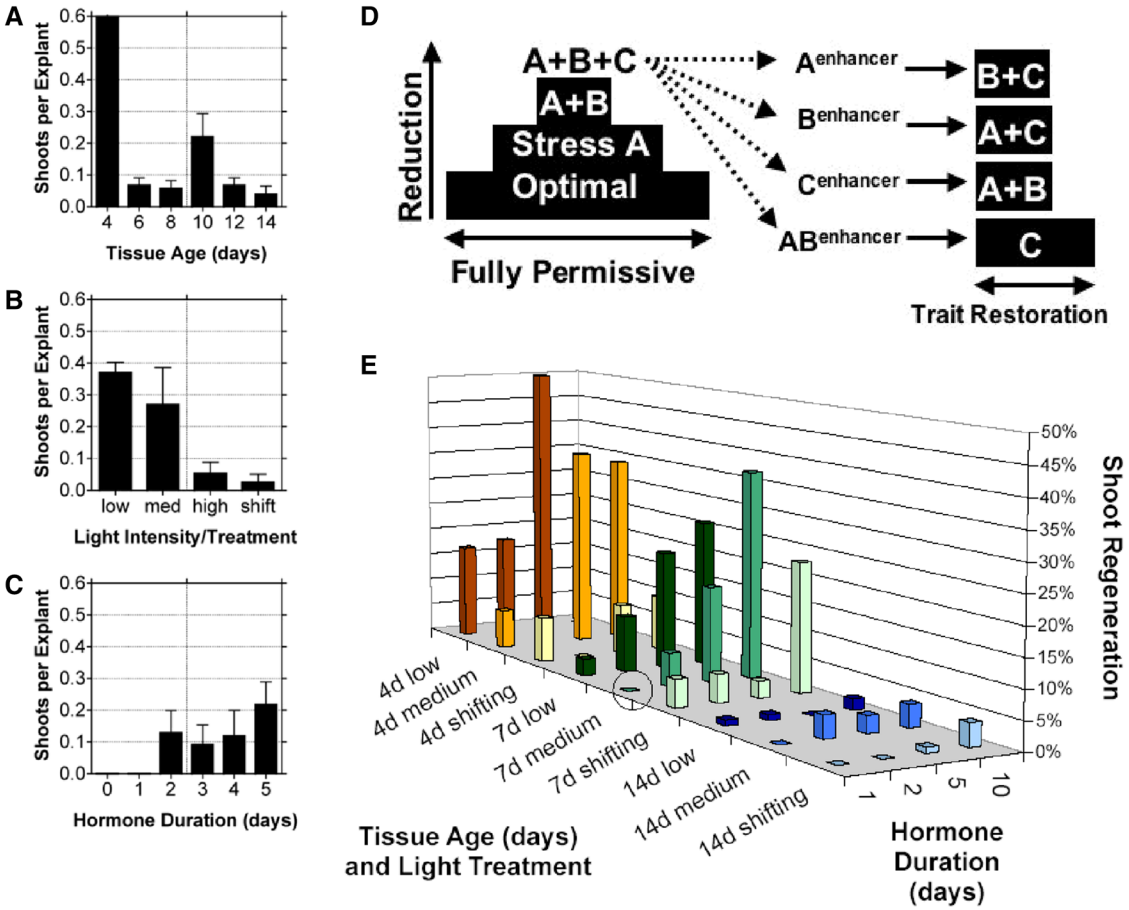

Fig. 1 Establishing the conditions for a pyramid enhancer screen for shoot organ regeneration from severed leaves of *Arabidopsis* seedlings. **a–c** The phenotype of interest (regeneration) was first tested under three different stress conditions: **a** the age of the tissue at the time of detachment; **b** the intensity and duration of post-detachment light quantity; **c** the duration of exposure to shoot regeneration media (SIM) hormones following detachment. Old age, high light and minimal hormone exposure were all stress conditions that caused a reduced phenotype. **d** *Left* to find enhancer mutants in each pathway and across multiple pathways in a single screen, we conceptualized a pyramid screen. In this screen, a non-permissive condition was created by stacking multiple suboptimal stress conditions (that is, ABC), with each stress having an equal inhibitory effect. *Right* in theory, a gain-of-function mutation (enhancer) should then be able to partially restore regeneration. **e** Based on these criteria, the optimal non-permissive pyramid screening condition was investigated by combining all three stress conditions; the final screening condition selected is *circled*. Each histogram shown is the mean of 3 replicates of 26 (n = 78) per treatment. The *error bar* represents the standard deviation (SD)

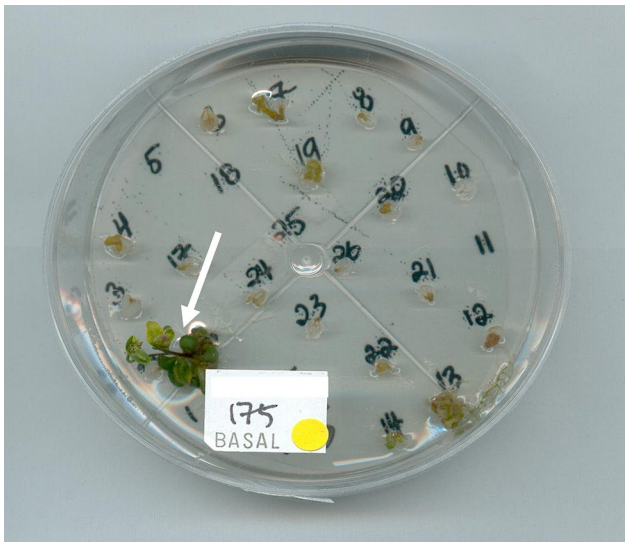

Fig. 2 Isolation and analysis of cotyledon-derived shoot regeneration enhancer mutants in *Arabidopsis* could overcome up to three inhibitory "stress" conditions

placed onto CIM media for 5 days, then SIM media for 30 days, under low (20–30 µmol m^{-2} s^{-1}) or high light (90–120 µmol m^{-2} s^{-1}). To test for enhancement of limited regeneration hormone (SIM) exposure, 7-day-old cotyledons were detached, then placed onto CIM media for 5 days, then SIM media for 1, 2 or 28 days, followed by basal media (SIM media without hormones) under low light. 28 days of hormone treatment represents the duration of hormones experienced by the age and light treatments and hence is included for comparison. Regeneration was scored 5 weeks after being detached.

Results and Discussion

Defining Three Separate Non-permissive Conditions for Shoot Regeneration

We were interested in finding alleles that regulate how light intensity, hormone duration and/or tissue age regulate shoot regeneration in *Arabidopsis*. We set out to create a non-permissive regeneration condition for each trait and then undertake three separate gain-of-function mutant screens. To define each non-permissive condition, we generated graphs for shoot regeneration ability under altered age, light or hormone conditions (Fig. 1a–c). We found that old age (6–14-day-old cotyledons) was less permissive for shoot regeneration than young age (4 days old) (Fig. 1a). Similarly, we observed that high light intensity (60–100 µmol m^{-2} s^{-1}) was less permissive than low light (~20 µmol m^{-2} s^{-1}) (Fig. 1b) (Nameth and others 2013). Finally, we found that 1 day of post-detachment hormone exposure to auxin and cytokinin hormones was much less permissive than more than 2 days of hormone exposure (Fig. 1c). However, a prerequisite for a gain-of-function mutant screen is that the treatment must abolish the trait of interest in the wild-type, but this requirement was only met in one of three treatments (Fig. 1c).

Pyramiding Three Equal Suboptimal Growth Conditions Strategically to Undertake a 3-in-1 Mutant Screen

We realized that we would have to experiment with more extreme non-permissive treatments (for example, very old tissue age) that abolished shoot regeneration before we could undertake mutant screening. Rather than defining such a condition for each pathway and then having to undertake three separate and laborious mutant screens, we wondered (1) whether we could create a non-permissive condition by stacking the three suboptimal treatments, and (2, more novel), whether by strategically selecting which suboptimal treatments to stack, we could undertake a single mutant screen that would retain the power of identifying mutations in each of the three pathways and possibly even identify signal cross-talk mutants that might not otherwise be uncovered. To achieve these goals, we conceptualized progressively creating a non-permissive condition for shoot regeneration, resulting in a pyramid shape (Fig. 1d).

We had to define a non-permissive condition for shoot regeneration by combining three suboptimal "stress" conditions such that each stress was equal, and no two stress conditions completely abolished regeneration. To accomplish this, we tested a matrix of 64 different combinations of light quantity, hormone duration and donor-tissue age, a screen requiring 4992 donor leaves. A subset of the treatments is shown (Fig. 1e). For each non-permissive condition uncovered, defined as causing 0% shoot regeneration, we searched for evidence that the above criteria were met and that a moderate improvement in any of the three pathways would permit some regeneration; this would suggest that genetic mutants specific for each pathway could be found. We identified at least one such non-permissive condition, which consisted of 7-day-old cotyledons, exposed to 1 day of hormones and continuous higher light intensity (>60 µmol m^{-2} s^{-1}) (Fig. 1e, circled). We chose 7-day-old cotyledons, because the next more permissive age, 4 days old, could regenerate when light levels and hormone exposure were kept constant. Similarly, we chose 1 day of hormone exposure, because 2 days of exposure permitted regeneration. Finally, we chose continuous high light intensity (60–100 µmol m^{-2} s^{-1}) because the next less restrictive treatment (20–30 µmol m^{-2} s^{-1}) permitted significant regeneration.

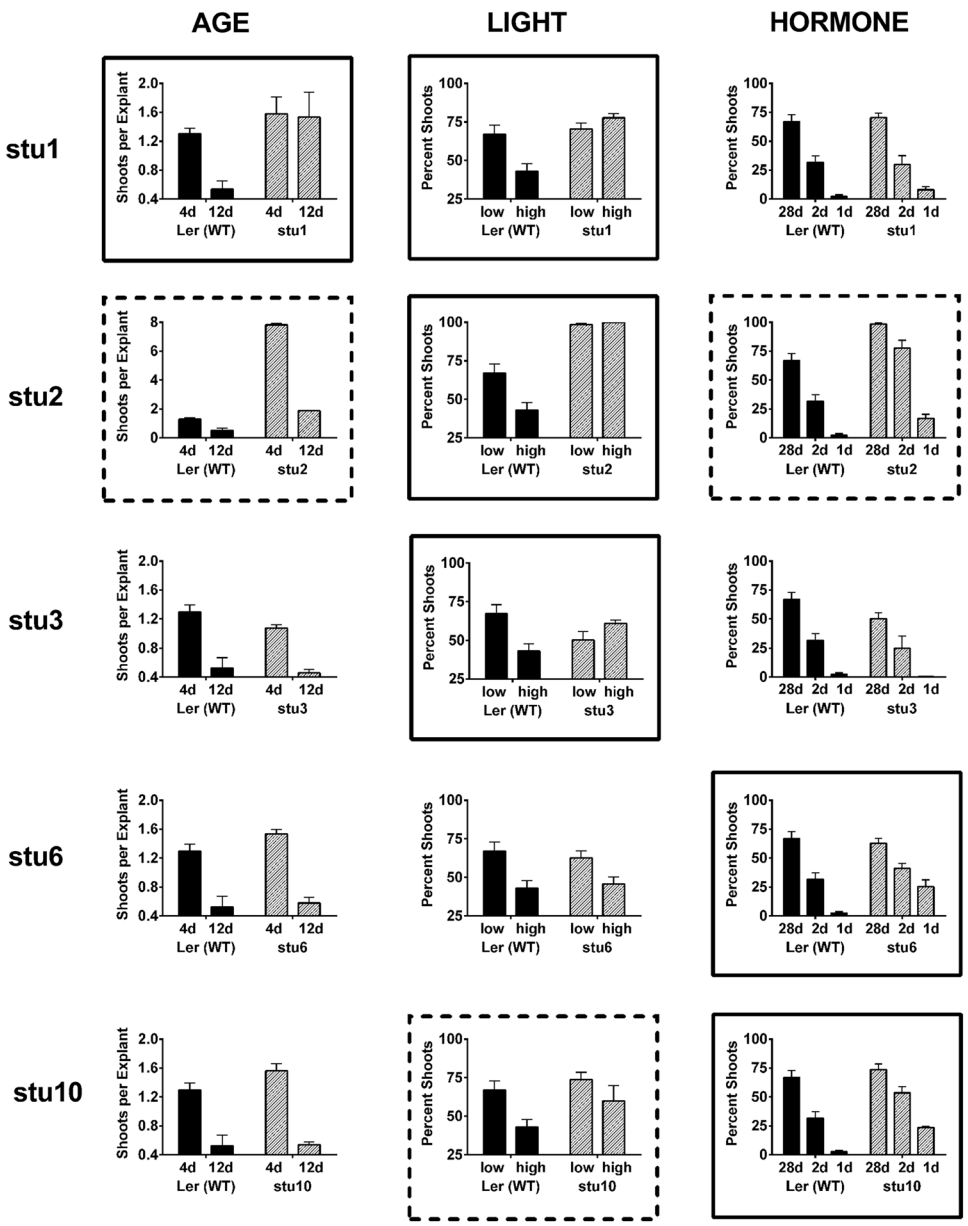

Fig. 3 Analysis of shoot regeneration phenotypes of pyramid screen-derived *stu* mutants compared to wild-type *Arabidopsis*, ecotype Landsberg *erecta* (Ler-0 WT). Shown is the number of regenerated shoots per explant (for age graphs) or the percentage of cotyledons that regenerated shoots (for light and hormone graphs) at 5 weeks after detachment. The primary pathway affected is outlined using a *solid line* and the presumptive secondary effect in a *checkered line*. To test the specificity of enhancement to a particular pathway (for example, age), two out of three conditions (for example, light, hormone) were kept optimal. Each histogram is the mean of five replicates (n = 26 per replicate). *Error bars* represent standard deviation (SD)

Mutant Screen

By having defined a non-permissive condition for regeneration using the pyramid approach described above, we screened approximately 18,000 EMS lines for shoot regeneration. We recovered 103 putative enhancer mutants (Fig. 2) of which 12 were confirmed to be genetically transmitted and able to regenerate both shoots and roots in the M3 and M4 generations. The mutants were named *SHOOTING UP* (*stu1–12*) of which five mutants (*stu1, 2, 3, 6, 10*) showed consistent phenotypes across generations.

Determining Whether the Enhancer Mutants were Specific to a Pathway

Mutants in the M4 generation were then subjected to a secondary screen to determine if we had found mutants specific to each of our three pathways, light, hormone and age, or whether we had simply recovered general regeneration mutants. In the secondary screen, shoot regeneration was tested under a single "stress" condition, keeping two of the three conditions as optimal. For example, to screen for age-specific enhancement, regeneration of very young (4 days old) versus very old (12 days old) cotyledons was compared, but under optimal low light intensity (20 μmol m^{-2} s^{-1}) and optimal hormone exposure (4 weeks

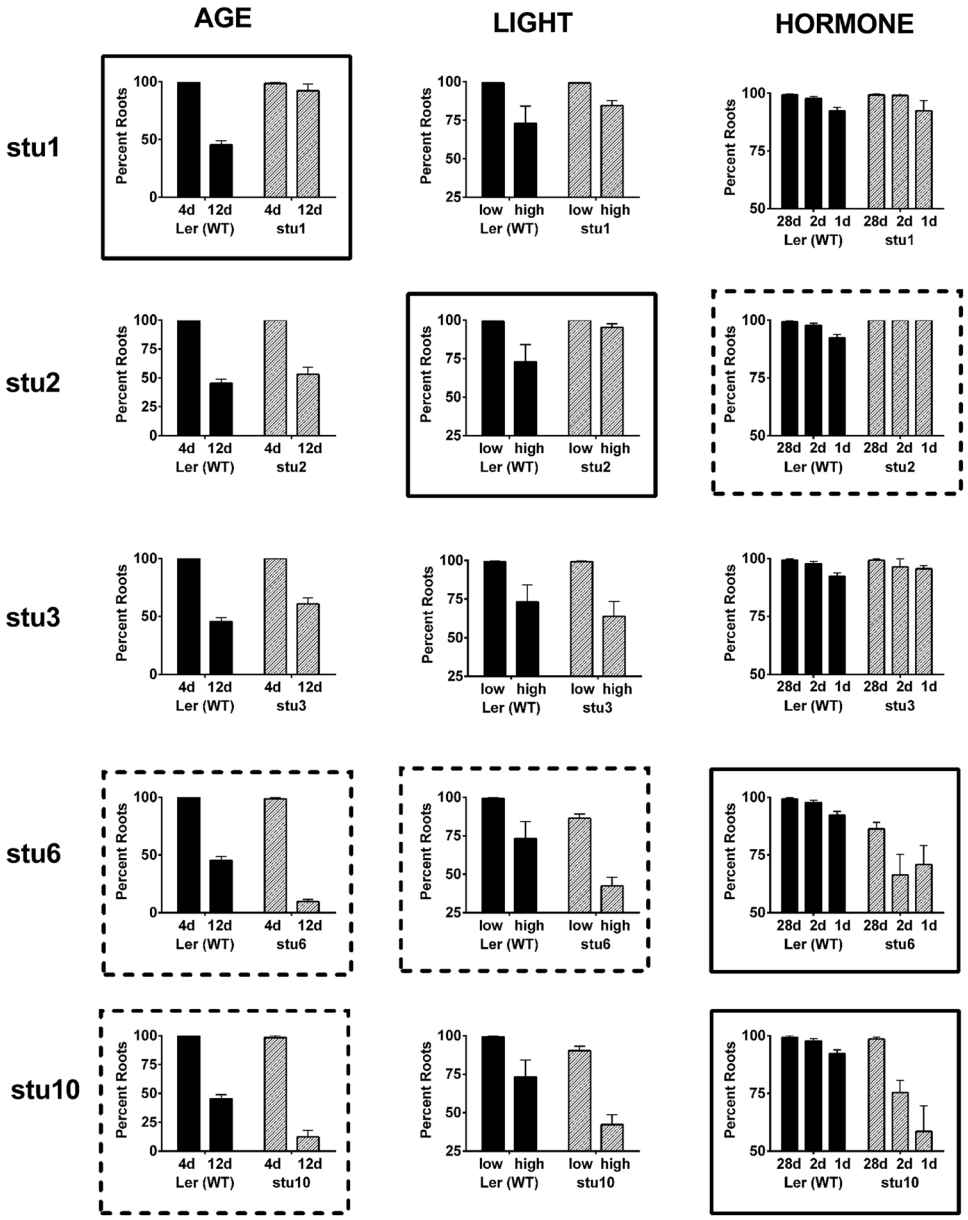

Fig. 4 Analysis of root regeneration phenotypes of pyramid screen-derived *stu* mutants compared to wild-type *Arabidopsis*, ecotype Landsberg *erecta* (Ler-0 WT). The percentage of cotyledon explants that regenerated roots was scored 5 weeks after detachment. The samples correspond to the tissues sampled for shoot regeneration (Fig. 3) and scoring occurred simultaneously. The primary pathway affected is outlined using a *solid line* and the presumptive secondary effect in a checkered line. Each histogram is the mean of five replicates (n = 26 per replicate). The *error bar* represents standard deviation (SD)

of auxin and cytokinin). Under these single stress conditions, the shoot regeneration phenotypes of five *stu* mutants are shown (Fig. 3); the corresponding root regeneration phenotypes are shown (Fig. 4). Compared to wild-type (WT), *stu1* was found to confer complete insensitivity to age inhibition of both shoot and root regeneration, and also insensitivity to high light intensity inhibition of shoot regeneration, but not root regeneration. The primary effect of *stu2* was to confer to Ler-0 tolerance to high light intensity inhibition of both shoot and root regeneration, with secondary shoot enhancement of old age and suboptimal hormone exposure. Mutant *stu3* was specific to overcoming high light intensity inhibition of shoot regeneration. Mutant *stu6* was specific to enhancing shoot regeneration under minimal hormone exposure (1 day), whereas *stu10* enhanced hormone sensitivity, but also conferred partial enhancement to high light intensity inhibition. In addition, other interesting phenotypes emerged from the screen: for example, both the hormone shoot-enhancement mutants *stu6* and *stu10* had root regeneration phenotypes that showed decreased tolerance to age, light and hormone stress (Fig. 4). All differences described (and those boxed in Figs. 3, 4) were statistically significant using Mann–Whitney non-parametric statistical analysis (Table S1). Therefore, in one mutant screen, we successfully screened for mutants that are both specific for a pathway and/or which enhanced multiple pathways.

Pyramid screening offers both advantages and disadvantages. The principal advantage of pyramid screening is economy: we saved on approximately 36,000 woundings,

thousands of media plates and about 160,000 tissue transfers. Another advantage of pyramid screening is that it can maintain specificity: in our screen, mutants *stu6* and *stu10* significantly increased shoot regeneration on 1 day of SIM hormones, but not more than 2 days of SIM (Fig. 3; Table S1); the original screen was to select enhancers of 1 day of hormone exposure. Rather than our concern of recovering only general regeneration mutants, we recovered gain-of-function mutants specific for a pathway. Our pyramid screen also recovered putative cross-talk alleles as we had hypothesized. A key disadvantage of pyramid screening is that significant preliminary experiments are required to generate a pyramided non-permissive stress condition.

Unlike the laboratory, nature selects for alleles that integrate multiple environmental conditions and/or which integrate multiple genetic pathways. With respect to the former, pyramid screening may permit a deeper exploration of genotype × environment (G × E) interactions; in this study, each genotype was studied under six complex environments. With respect to pathway integration, alleles emerging from pyramid screening may provide functional evidence (loss or gain of phenotype) for interactions between signaling pathways.

Conclusion

Rather than undertaking three separate mutant screens for shoot regeneration following injury in *Arabidopsis thaliana*, here we have described a single mutant screen methodology which resulted in mutants that were both specific for a pathway (light, hormones, age) or which enhanced multiple pathways. Such pyramid screening represents an economical method for the discovery of novel alleles underlying complex traits such as somatic organogenesis.

Acknowledgements This research was supported by an Ontario Premier's Research Excellence Award, a grant from the Ontario Ministry of Agriculture and Food (OMAF) and a Discovery Grant from NSERC Canada, to MNR.

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