What Is Stress? Dose-Response Effects in Commonly Used in Vitro Stress Assays* [OPEN]

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In vitro stress assays are commonly used to study the responses of plants to abiotic stress and to assess stress tolerance. A literature review reveals that most studies use very high stress levels and measure criteria such as germination, plant survival, or the development of visual symptoms such as bleaching. However, we show that these parameters are indicators of very severe stress, and such studies thus only provide incomplete information about stress sensitivity in Arabidopsis (Arabidopsis thaliana). Similarly, transcript analysis revealed that typical stress markers are only induced at high stress levels in young seedlings. Therefore, tools are needed to study the effects of mild stress. We found that the commonly used stress-inducing agents mannitol, sorbitol, NaCl, and hydrogen peroxide impact shoot growth in a highly specific and dose-dependent way. Therefore, shoot growth is a sensitive, relevant, and easily measured phenotype to assess stress tolerance over a wide range of stress levels. Finally, our data suggest that care should be taken when using mannitol as an osmoticum.

To study the effects of abiotic stress on plants, in vitro setups are often used as a proxy for the complex field environments in which plants are subjected to stress. These experimental setups are based on the addition of compounds to the growth medium. Drought, for instance, is simulated by adding osmotic, such as mannitol, sorbitol, or polyethylene glycol (Verslues et al., 2006), which lower the water potential of the medium. This makes it harder for plants to extract water, simulating what happens in drying soil. Similarly, NaCl is added to the medium to expose plants to salt stress, which is a combination of osmotic stress, as NaCl also lowers the water potential of the medium, and Na⁺ toxicity, mainly important at high NaCl concentrations (Munn and Tester, 2008). To simulate general oxidative stress, occurring for instance under high-light conditions, the medium is supplemented with hydrogen peroxide (H₂O₂) or paraquat/methyl viologen, which induce the formation of the toxic reactive oxygen species O₂⁻ in plant tissues (Slade, 1966). While these artificial setups are inherently imperfect, they offer practical advantages, such as tight control of stress level and onset, low variability, and the ability to grow many plants using limited space (Verslues et al., 2006; Lawlor, 2013). Consequently, much of our current knowledge on stress physiology in Arabidopsis (Arabidopsis thaliana) is based on the use of these types of artificial stress conditions, and this resulted in the identification of many genes that enhance stress tolerance (Munn and Tester, 2008; Gill and Tuteja, 2010; Deikman et al., 2012).

An aspect that is usually underestimated in studies of stress physiology is the great variety of stress levels. Often, stress is seen as a binary condition, comparing an arbitrary stress treatment with a control. However, in natural and field conditions, plants often experience a wide variety of stress levels, requiring a range of different response mechanisms. For instance, life-threatening drought is very different from a transient mild water deficit and thus elicits different responses (Claeyś and Inzé, 2013). Therefore, genes that confer tolerance to severe stress may not enhance growth under more mild stress conditions (Skirycz et al., 2011b). To address this issue, we performed a quantitative survey of the scientific literature on Arabidopsis stress physiology using in vitro assays, and for each relevant publication we determined which stress level and what types of phenotyping were used to assess stress responses. Based on the results of this survey, we exposed Arabidopsis seedlings to a wide range of salt, osmotic, and oxidative stress, tracking germination and growth over time and measuring the expression of marker genes. These experiments confirmed that there is a highly dose-dependent response of plants to stress, the nature of which depends on the type of stress, and

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that shoot growth is a very sensitive indicator of stress. Furthermore, current marker genes for stress are only induced at very severe stress levels, and novel mild stress markers are needed.

RESULTS

Most Studies Use Very High Concentrations of Stress-Inducing Agents

To assess which concentrations are commonly used in literature, PubMed abstracts and open-access articles from PubMed Central, available through the text-mining resource EVEX (Van Landeghem et al., 2013a, 2013b), were scanned for keywords related to salt, osmotic, and oxidative stress in Arabidopsis, and concentrations of NaCl, mannitol, sorbitol, and H$_2$O$_2$ used in stress assays were automatically extracted, followed by manual curation. Our analysis showed that most studies use very severe stress conditions, with median concentrations of 150 mM NaCl, 300 mM mannitol/sorbitol, and 10 mM H$_2$O$_2$ used in stress assays were automatically extracted, followed by manual curation. Often, a range of concentrations is used, but this range usually only starts at high concentrations (typically 50 mM for NaCl and 100 mM for mannitol/sorbitol). Accordingly, when all ranges are reduced to their average, the overall medians do not change (data not shown). For all papers in which stress tolerance was assessed, we also analyzed the phenotypes that were used to quantify this trait (Fig. 1B). These phenotypes were divided into three major categories: germination/survival tests, assessment of plant health (usually based on overall plant morphology and the appearance of bleaching), and growth measurements. This analysis showed that most studies (almost 60%) measure germination or survival after treatment with stress-inducing agents, while less than one-half look at growth parameters. When growth is assessed, in most cases this is on the basis of root length measurements. Our observations clearly show that most published studies expose plants to very high levels of stress-inducing agents and, accordingly, record phenotypes that are associated with very severe stress.

Different Parameters Show Varying Sensitivities to Abiotic Stress

In order to study the effects of low to high doses of common stress-inducing agents, we germinated Arabidopsis Columbia-0 (Col-0) seeds on a wide range of concentrations of mannitol, sorbitol, NaCl, and H$_2$O$_2$ and measured the germination rate, the extent of bleaching, the root length at 12 d after stratification (DAS), and the rosette area at 22 DAS. While inherently difficult to extrapolate, these ranges of osmotic and salt stress should be comparable to what plants experience in nature: we used 5 to 300 mM mannitol or sorbitol, corresponding to an osmotic potential of −0.08 to −0.8 MPa, respectively, while the usable soil water potential for plants ranges from −0.03 to −1.5 MPa (O’Geen, 2012). Similarly, concentrations ranging from 5 to 300 mM NaCl were used, while mild to severe salt stress in the field ranges from 40 to 160 mM NaCl (Abrol et al., 1988). For all stresses, it is clear that the four parameters we tested show very different sensitivities to the stress level (Fig. 2). The germination rate was not affected by the concentrations of mannitol, sorbitol, and H$_2$O$_2$ we tested (up to 300 mM mannitol and 2.5 mM H$_2$O$_2$), while it was strongly inhibited by NaCl concentrations of 200 mM and more. However, germination was delayed at concentrations starting from 150 mM mannitol or sorbitol, 100 mM NaCl, and 1.75 mM H$_2$O$_2$ (data not shown). Visible stress symptoms, such as bleaching and anthocyanin accumulation, were more sensitive to NaCl and H$_2$O$_2$ than germination, occurring from 75 mM NaCl or 1 mM H$_2$O$_2$ upward. Mannitol and sorbitol did not elicit these responses. Growth was the most sensitive parameter we tested, and for osmotic and salt stress, shoot growth (rosette area) was more strongly inhibited than root growth. These results indicate that shoot growth can be used as a very sensitive indicator of abiotic stress.

Mannitol, Sorbitol, NaCl, and H$_2$O$_2$ Induce Highly Dose-Dependent Growth Responses

To get a more accurate view of the effect of the different stresses on shoot growth, we tracked the rosette area over time, allowing the calculation of relative growth rates (RGRs), which are less sensitive to changes in germination time than absolute growth rates. While all compounds induce highly dose-dependent responses, the relationship between concentration and growth response differed significantly between different stress-inducing agents.

Mannitol had a drastic effect on the final rosette area, which was already apparent from 5 mM mannitol upward and leveled off toward high concentrations (Fig. 3A). Above 25 mM, plants exhibited aberrant and elongated leaf shapes. At very high concentrations, plants were very small, dark green, and compact, but they still had positive growth rates. At higher stress levels, rosettes also became more compact (Fig. 3A, inset). The strongest effects of mannitol on growth rates were seen at early time points (Fig. 3B). At the first time point, 8 DAS, the RGR behaved as a quadratic function of the mannitol concentration, illustrating the drastic decrease in growth rates with increasing mannitol concentrations. This relationship was relaxed at later time points for low mannitol concentrations (up to 50 mM), indicating the acclimation of growth to mannitol. Strikingly, the response of plants to sorbitol, an osmoticum that is structurally very similar to mannitol, was very different: low concentrations had little effect on shoot growth, and concentrations of 75 mM and higher were needed to significantly reduce the rosette area at 22 DAS (Fig. 3C). A similar trend was seen in the...
growth rates at early time points (Fig. 3D). Similar to mannitol, there again was a strong acclimation effect, as at 22 DAS the RGR was not affected by concentrations of up to 150 mM sorbitol (Fig. 3D).

Salt stress induced a response that was similar to that of sorbitol: low concentrations (up to 25 mM; equivalent to 50 mM sorbitol in osmotic equivalents) had little effect on growth (Fig. 3E). However, once the NaCl concentration exceeded 25 mM, growth was strongly inhibited and compactness increased sharply. These dose-dependent effects were confirmed when analyzing RGR of young seedlings: at low concentrations, RGRs were not affected, while at higher concentrations, the RGR quickly decreased as a quadratic function of the concentration (Fig. 3F). As for mannitol and sorbitol treatments, this quadratic relationship relaxed when seedlings got older, reflecting acclimation.

Finally, H$_2$O$_2$ had a very striking effect on plant health, as it induced bleaching (Fig. 3G). Bleaching seemed to be a binary trait with a threshold that was at 1 to 1.25 mM H$_2$O$_2$; at this concentration, some plants were fully bleached while others appeared to be normal. While bleaching strongly affected plant growth, growth was also inhibited when plants were exposed to low concentrations of H$_2$O$_2$ that did not induce bleaching (Fig. 3G, inset at bottom left). Interestingly, the bleaching-independent growth inhibition increased in a linear fashion with increasing H$_2$O$_2$ concentration.

By contrast, rosette compactness was only impacted by high H$_2$O$_2$ concentrations (Fig. 3G, inset at top right). Bleached plants showed a strong reduction in growth rates over time, as can be seen when looking at growth rates of plants grown on concentrations exceeding 1 mM H$_2$O$_2$ (Fig. 3H). A possible explanation for this lies in the fact that bleaching is progressive and abolishes efficient photosynthesis, and the Suc supplied in the medium can only sustain plant growth for a short period of time. This is different from osmotic and salt stress, which showed a relief of growth inhibition over time due to acclimation.

Gene Expression Shows Similar Dose-Dependent Responses

As many stress-responsive genes have previously been characterized and described, we analyzed whether their expression patterns show similar dose-dependent responses to those observed in our growth experiments. We performed these analyses on young seedlings, at the time when the effects of stress on their growth rate were most pronounced. For all types of stress, we measured gene expression levels of oxidative stress markers (NAC DOMAIN-CONTAINING PROTEIN32 [NAC032] and ALDO-KETO REDUCTASE 4C9 [AKR4C9]; Vanderauwera et al., 2005), abscisic acid (ABA) markers (CYTOCHROME P450 707A3 [CYP707A3] and NINE-CIS-EPOXYCAROTENOIDE DIOXYGENASE3 [NCED3]; Goda et al., 2008), dehydration...
markers (RESPONSIVE TO DESSICATION 29B [RD29B], DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A [DREB2A], and LATE EMBRYOGENESIS ABUNDANT-LIKE5 [LEA5]; Kilian et al., 2007), and mild osmotic stress markers (ETHYLENE RESPONSIVE ELEMENT-BINDING FACTOR5 [ERF5], MYB DOMAIN PROTEIN51 [MYB51], and WRKY DNA-BINDING PROTEIN33 [WRKY33]; Dubois et al., 2013; Fig. 4).

The oxidative stress markers were the only transcripts that responded to all four stresses, confirming that all stresses share an oxidative stress component (Gill and Tuteja, 2010), but this only occurred at high concentrations (100 mM or greater mannitol or sorbitol, 75 mM or greater NaCl, or 1.25 mM or greater H$_2$O$_2$). Very high levels of salt and osmotic stress also induced ABA markers and dehydration markers, although the expression of the latter was highly variable, but both were not induced by low stress levels. Finally, the mild osmotic stress markers were indeed confirmed to be specific for osmotic stress in young seedlings and already responded to low concentrations of mannitol and sorbitol, although, as for the growth response, higher concentrations of sorbitol were needed to achieve the same effect.

This analysis shows that, already with a limited set of stress markers, different stress levels induce unique combinations and expression levels of stress-responsive genes, indicating that the transcriptome response is strongly dependent on the stress level.
Figure 3. Effects of stress on rosette growth. A, C, E, and G, Projected rosette areas at 22 DAS (final time point), with representative images of plants for a range of mannitol, NaCl, and H2O2 concentrations. The top right insets show rosette compactness. For H2O2 treatment, the inset at the bottom left shows rosette areas (dots) and their averages (lines) grouped by the presence (red) or absence (black) of substantial bleaching. B, D, F, and H, RGRs as a function of the stress levels at 8 DAS (red), 15 DAS (blue), and 22 DAS (green). At NaCl concentrations greater than 150 mM (E and F), germination or seedling establishment was inhibited, so no growth data could be obtained for these concentrations, as represented by the dashed lines on the graphs. Error bars indicate SE. The letters above the error bars denote significance groups (ANOVA; $P < 0.05$; $n = 30–36$).
DISCUSSION

Shoot Growth as an Indicator of Stress Sensitivity

Many published studies about stress signaling expose plants to very high stress levels (median concentrations of 150 mM NaCl and 300 mM mannitol/sorbitol) and score very pronounced phenotypes such as germination rate, seedling survival, or bleaching. To illustrate this, the rosette area was reduced by more than 95% when we exposed plants to these high concentrations of mannitol, sorbitol, and NaCl, and salt-stressed plants showed additional symptoms of severe stress such as bleaching. However, plants often experience stresses that are not immediately life threatening but that do impact growth and productivity (Claeys and Inzé, 2013). Here, we confirmed that low stress levels can already severely limit shoot growth without leading to other visible stress phenotypes, as shown in previous reports (Granier et al., 2006; Harb et al., 2010; Skirycz et al., 2010, 2011a; Baerenfaller et al., 2012).

These findings are quite remarkable, in particular because all growth media contained 1% (w/v) Suc. We mainly chose to add Suc to the medium to replicate the experimental conditions that are often used to study abiotic stress in vitro, where Suc-supplemented medium is typically used because it reduces variation in growth. However, Suc is known to enhance stress tolerance and affect ABA signaling (Finkelstein and Gibson, 2002) and could counteract the negative effects of oxidative stress on photosynthesis that potentially limit growth (Ramel et al., 2007). The fact that we still see strong growth inhibition using low stress levels with Suc-containing media fits with the current view that stress-induced growth inhibition is an active process that is not dependent on carbon limitation (Claeys and Inzé, 2013). Our results thus indicate that shoot growth can be used as a sensitive indicator of stress tolerance, while root growth, which is more commonly measured, is less sensitive to abiotic stress (Hsiao and Xu, 2000; Verslues et al., 2006). Phenotypes such as germination rate and root growth are most commonly recorded, possibly because this type of measurement is perceived to be less labor intensive than measuring shoot growth. However, by regularly taking photographs of plates or pots containing plants, shoot growth can easily be tracked over time at the rosette level. Moreover, several experimental setups have been specifically designed to automatically track rosette growth under control and a range of mild stress conditions, both in soil and in vitro (Granier et al., 2006; Skirycz et al., 2011b; Dubois et al., 2013; Tisné et al., 2013). Additionally, at the end of the experiment, individual leaf areas and cellular parameters can be measured, providing very detailed information on the impact of stress on growth. This allows one to study responses to mild stress, at which point commonly recorded stress phenotypes are not yet affected.

Current Stress Marker Genes Are Indicators of Severe Stress in Young Seedlings

For all four stresses, the expression of a number of known stress-induced genes was measured in young seedlings. Salt and osmotic stress led to the induction of dehydration, oxidative stress, and ABA markers, but only at very high stress levels that caused visible stress phenotypes, indicating that there most likely already was cellular damage. The molecular response to mild osmotic stress is very different, as was shown previously (Skirycz et al., 2010, 2011a), and marker genes taken from these studies, such as ERF5, are indeed good indicators that are already induced by low concentrations of mannitol and sorbitol. However, these are specific for osmotic stress and are not induced by salt stress, although this stress also has an osmotic component.
Different Responses

Osmotic, Salt, and Oxidative Stress Elicit Very Different Responses

While some studies attempted to study molecular responses at different stress levels, this was usually done in progressive soil-drying experiments (Cramer et al., 2007; Harb et al., 2010; Bonhomme et al., 2012), where the time factor cannot be separated from the stress severity factor. To our knowledge, no dedicated controlled studies have been performed on the effects of stress severity on gene expression. Based on the data presented here for osmotic stress, we postulate that there may not be a simple linear response in which the expression of individual genes correlates with the stress level but that different stress levels to some extent switch on entirely different transcriptomes. This is similar to what is seen when different stresses are combined, resulting in transcriptome changes that are different from those induced by single stresses (Rizhsky et al., 2004; Rasmussen et al., 2013). Accordingly, there is no correlation between enhanced survival under severe stress conditions and improved growth under mild stress conditions, because different mechanisms are involved (Skirycz et al., 2011b).

What Is Stress?

The effects of plant growth and gene expression in response to stress are highly dose responsive, suggesting the existence of very sensitive machinery assessing the stress level and fine-tuning molecular responses. Our findings have important consequences for studying stress physiology. To assess whether plants are stressed, researchers often rely on strong visible stress phenotypes or the induction of established stress markers. This strategy can be especially misleading when used to assess whether the growth of mutant or transgenic lines is impacted by changes in stress signaling pathways, as we show here that growth can be strongly inhibited without classical signs of stress.

The study of stress responses in plants has thus far mainly focused on severe and acute stress. However, stress is traditionally defined as any adverse environmental parameter that limits plant growth and productivity (Boyer, 1982), and these effects can be very subtle. The current understanding of the molecular networks underlying growth and survival responses to stress is still limited, but this regulation is most likely highly complex and dependent on parameters such as stress severity, organ or cell identity, and developmental stage (Claeyssens and Inzé, 2013). By tracking sensitive parameters such as shoot growth over a range of stress levels, rather than traditional severe stress parameters such as germination, visible stress symptoms, or root growth, a more accurate picture may be obtained of the stress sensitivity of, for instance, an ecotype or a transgenic line. Since it is becoming increasingly clear that there is no magic bullet that will improve stress tolerance in all conditions (Tardieu, 2012; Claeyssens and Inzé, 2013), tools to study the resilience of growth in response to mild stress are needed to improve stress tolerance.
MATERIALS AND METHODS

Plant Growth

Seedlings of Arabidopsis (Arabidopsis thaliana) accession Col-0 were grown in vitro in one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) Suc for all experiments and different concentrations of D-mannitol (Sigma-Aldrich), sorbitol (Sigma-Aldrich), NaCl (VWR), or H₂O₂ (Merck) depending on the experiment. For most experiments, 12 seeds were equally distributed on a 150-mm-diameter plate, while for root growth experiments, eight seeds were equally distributed on a plate that was placed vertically. Plants were grown at 21°C under a 16-h-day (110 μmol m⁻² s⁻¹) and 8-h-night regime. Three biological replicates were performed for each experiment.

Measurement of Germination, Growth, and Development

Germination (including successful seedling establishment) and symptoms of severe stress, such as the presence of bleaching or purple spots, were scored at 22 DAS. At this time point, photographs were taken of each plate, and projected rosette areas were measured using ImageJ version 1.46 (National Institutes of Health; http://rsb.info.nih.gov/ij/). For root growth, photographs were taken at 12 DAS, and the primary root length was measured using ImageJ. The presented germination rates and proportions of healthy plants are averages over the three experiments. For rosette and root growth, ANOVA showed that the experiment effect was not significant. Therefore, experiments were combined, and the presented data are from 30 to 36 plants for rosette area and 20 to 24 plants for root length.

Rosette Growth Analysis

Photographs were taken of each plate at 8, 11, 13, 15, 18, 20, and 22 DAS. Projected rosette areas were measured using ImageJ. Rosette compactness was calculated by dividing rosette area by the area of the convex hull, which were both measured using ImageJ. Growth was modeled in SAS 9.3 using linear mixed models. Due to the lack of significant experiment effects, the experiment factor was excluded from the model. Different types of models were tested, and for all experiments, a linear model with random intercepts and slopes in which the mean natural logarithm-transformed rosette area was expressed as a second-order function of time, using the concentration as a factor, showed the best fit to the experimental data. RGRs were then calculated as the first-order derivative of the resulting function. All other statistical analyses were performed in R (version 2.10.1).

Gene Expression Analysis

At 8 DAS, 12 complete seedlings were harvested from one plate per biological replicate. RNA was extracted with TriZol (Invitrogen), followed by a clean-up step with the RNeasy Mini Kit (Qiagen) including on-column DNase I (Qiagen) treatment according to the manufacturer’s instructions. Complementary DNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions, starting from 1 μg of RNA. Primers were designed with QuantPrime (Arvidsson et al., 2008). Quantitative reverse transcription-PCR was performed on a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer’s instructions. Melting curves were analyzed to check primer specificity. Expression values of ATIG13320, AT2G32270, and AT2G28390 were used for normalization (Czechowski et al., 2005).

Text Mining

To facilitate our literature analysis of compound concentrations used in previously published studies, we implemented custom text-mining methods. These methods were applied on all 22 million PubMed abstracts and 460,000 PubMed Central open-access full-text articles available through the text-mining resource EVEX (Van Lundeheim et al., 2013a, 2013b). Tests were restricted to studies on Arabidopsis through a keyword search, and articles that mentioned abiotic stress, salt stress, osmotic stress, or oxidative stress were identified, performing case-insensitive matching throughout the article text and allowing for lexical variations such as hyphens. The resulting set of articles was processed with a novel rule-based text-mining algorithm that attempts to find patterns of the form `<QUANTITY MEASURE COMPOUND>` such as “100 μmol NaCl” or `<COMPOUND QUANTITY MEASURE>` such as “NaCl, 100 μmol,” ignoring punctuation marks such as commas and parentheses. To identify the four compounds of interest (salt, H₂O₂, mannitol, and sorbitol), a list of synonyms was compiled to be used during pattern matching, including the words “salt,” “NaCl,” and “sodium chloride.” Similarly, a list of candidate terms describing units of measurement, such as “μmol,” “millimolar,” “micromolar,” and “molar,” was applied. In a final step, all data were verified and corrected manually to ensure a high quality.

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LITERATURE CITED

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