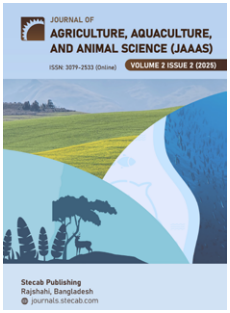

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Review Article

Phenotypic Analysis and Characterization Methods for Transgenic *Setaria viridis*: From Morphology to Molecular Markers: A Comprehensive Review

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About Article

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ABSTRACT

Setaria viridis (green foxtail) has become a principal C_4 model for functional genomics and translational crop research because its short life cycle and reliable transformation protocols enable rapid production of transgenic lines. Linking those genotypes to traits, however, demands a multilayered phenotypization strategy. This narrative review synthesizes current approaches that span traditional morphology, developmental staging, and precision measurement of vegetative and reproductive traits. It describes frameworks for assessing physiological performance, such as gas-exchange assays and chlorophyll-fluorescence imaging, that translate genetic changes into functional outcomes. Recent advances in automated, high-throughput phenotyping platforms are outlined, illustrating how time-series imaging accelerates large-scale trait discovery. The review also examines experimental designs for evaluating abiotic-stress responses, highlights biochemical assays and metabolite-profiling techniques that reveal underlying metabolic adjustments, and details molecular-marker systems that couple genotype with phenotype. By integrating these complementary methods, researchers can build comprehensive genotype–phenotype maps in *S. viridis*, thereby streamlining gene validation and informing next-generation plant-biotechnology applications.

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1. INTRODUCTION

Setaria viridis (L.) P. Beauv., commonly known as green foxtail, is a diminutive C_4 panicoid grass that has rapidly gained prominence as a genetic model for cereals and bioenergy crops (Acharya *et al.*, 2017). With a short life cycle (~6–8 weeks) and small stature (~30 cm), *S. viridis* offers practical advantages for lab cultivation, while its small diploid genome (~500 Mb) is now fully sequenced (Acharya *et al.*, 2017). Critically, efficient Agrobacterium-mediated transformation protocols (including callus-based and in planta methods) have been established (Acharya *et al.*, 2017), enabling routine generation of transgenic lines. These attributes position *S. viridis* as an attractive system for functional genomics studies in grasses, complementing major crops like maize, sorghum, and foxtail millet (Huang *et al.*, 2016). Indeed, *S. viridis* is interfertile with its domesticated relative Setaria italica and shares extensive synteny, making it a translational bridge to improve millets and other cereals (Huang *et al.*, 2016).

Early studies established *S. viridis* as a model to investigate C_4 photosynthesis and stress resilience in millets (Huang *et al.*, 2016). More recently, researchers have leveraged its genetic resources (RIL populations, mutant libraries, diversity panels) and transformation efficiency for trait discovery and gene validation (Huang *et al.*, 2016). Transgenic *S. viridis* lines are being used to test the functions of candidate genes (e.g., drought tolerance, plant architecture, and nutrient use) before deployment in larger crops (Huang *et al.*, 2016). However, realizing the full potential of this model requires robust phenotypic characterization methods. A given genetic modification may produce subtle or complex phenotypes, necessitating detailed analysis from the whole-plant level down to molecular markers.

Traditionally, phenotyping has been a bottleneck in linking genotype to phenotype (Fahlgren, 2015). In the context of transgenic *S. viridis*, this challenge is addressed by a spectrum of approaches, from careful morphological observations to automated high-throughput imaging and multi-omics profiling. Morphological and developmental characterization provides the first insight into how a transgene affects growth form or life cycle progression. Physiological performance assays (e.g., measuring photosynthesis, biomass accumulation, or yield components) are then used to quantify functional outcomes of the genetic change. Modern phenotyping platforms increasingly automate these measurements, using imaging sensors and machine vision to capture traits over time at scale (Huang *et al.*, 2016). In parallel, targeted stress evaluations reveal whether transgenics show improved tolerance or responsiveness under environmental challenges (Acharya *et al.*, 2017). Biochemical characterization, including enzymatic assays and metabolite profiling, can uncover metabolic alterations that underpin the phenotype (de Souza *et al.*, 2018). Finally, integrating molecular markers and genomic data allows researchers to correlate specific insertions or edits with observed traits and to extrapolate findings to crop breeding through marker-assisted selection or QTL mapping (Huang *et al.*, 2016).

In this review, we narratively synthesize phenotypic analysis methodologies for transgenic *S. viridis* across these levels. Emphasis is placed on practical frameworks and recent advances that enable a holistic "morphology-to-molecules" characterization. By surveying tools from classical morphology scoring to high-throughput phenomics and genotype–phenotype integration, we highlight how *S. viridis* can accelerate functional gene studies and translational plant biotechnology.

2. LITERATURE REVIEW

Scholars in urban sociology and environmental psychology converge on the understanding that biophysical processes both shape and are shaped by built environments. Lefebvre's production-of-space thesis positions urban form as a socio-spatial product whose design encodes power relations and everyday practices; vegetation thereby acquires symbolic and functional roles in what he terms "representational spaces" (Lefebvre, 1991). Environmental psychologists, in parallel, argue that contact with greenery mitigates attentional fatigue. Attention-Restoration Theory (ART) demonstrates that natural settings replenish directed attention, with laboratory and field studies consistently reporting cognitive-performance gains after exposure to foliage or even window views of trees (Kaplan, 1995). Wilson's biophilia hypothesis extends this restorative premise, positing an innate human affinity for life forms and natural patterns, a predisposition increasingly leveraged in biophilic urban-design guidelines (Wilson, 1984).

Positioned against this theoretical backdrop, the present study interprets transgenic *Setaria viridis* not merely as a laboratory model but as a potential contributor to emergent "green infrastructures" that embed high-performance C_4 grasses into rooftops, bioswales, and micro-parks. By integrating Lefebvre's socio-spatial lens with ART's cognitive-restoration model, the review frames phenotypization methodologies as a bridge between molecular plant science and the well-being-oriented design of urban nature. Specifically, traits such as rapid biomass accumulation, drought resilience, and enhanced photosynthetic efficiency become relevant metrics for assessing how engineered grasses might sustain ecosystem services, shade, cooling, and psychological restoration within dense cityscapes. Thus, the methodological repertoire surveyed (morphological scoring, physiological assays, high-throughput imaging) is recast as a toolkit for evaluating plant genotypes that align with both the socio-spatial logic of urbanism and the restorative imperatives highlighted by environmental psychology.

3. METHODOLOGY

This article is a narrative review of phenotypic characterization methods in transgenic *S. viridis*. We focused on peer-reviewed research articles and reviews (primarily 2010–2025) that introduce or utilize various phenotyping techniques in *S. viridis* or closely related model grasses. Literature was identified through database searches (e.g., Web of Science, Scopus) using keywords such as "*Setaria viridis* phenotyping," "transgenic Setaria characterization," "plant high-throughput phenotyping," and "genotype–phenotype integration millets." Relevant references cited within these sources were also included. Unlike a systematic review, no formal PRISMA flow or meta-analysis was performed; instead, representative studies were selected to illustrate key methodologies. Given this narrative scope, we did not calculate effect sizes or aggregate quantitative

data. Rather, the review qualitatively synthesizes findings and techniques, with in-text citations to original sources. All information is drawn from published works, and any synthesis or interpretation is presented to emphasize general trends and best practices in phenotypic analysis. No new experiments were conducted for this review.

4. RESULTS AND DISCUSSION

Our literature survey identified six major categories of phenotypic characterization methods applied to transgenic or mutant *S. viridis*: (1) morphological and developmental characterization frameworks, (2) physiological performance assessment and functional validation, (3) high-throughput phenotyping technologies with automation, (4) stress tolerance evaluation under controlled environmental challenges, (5) biochemical characterization and metabolic profiling, and (6) integration of molecular markers and multi-modal data for correlating genotypes with phenotypes. Each category addresses a distinct facet of plant phenotype, and collectively they provide a comprehensive toolkit for analyzing transgenic lines as shown in Figure 1. For example, standardized growth assays have been developed to quantify *S. viridis* seedling and adult morphology (Acharya *et al.*, 2017), while conveyor-based imaging systems now enable time-resolved tracking of traits like plant height, leaf area, water status, and fluorescence signals in hundreds of plants simultaneously (Fahlgren, 2015; Huang *et al.*, 2016). Studies consistently emphasize that no single method suffices; instead, multi-scale approaches are essential to capture pleiotropic effects of genetic modifications (Junqueira *et al.*, 2025; Rahaman *et al.*, 2015). Below, we discuss each category in detail, highlighting representative techniques and their relevance to transgenic *S. viridis* research.

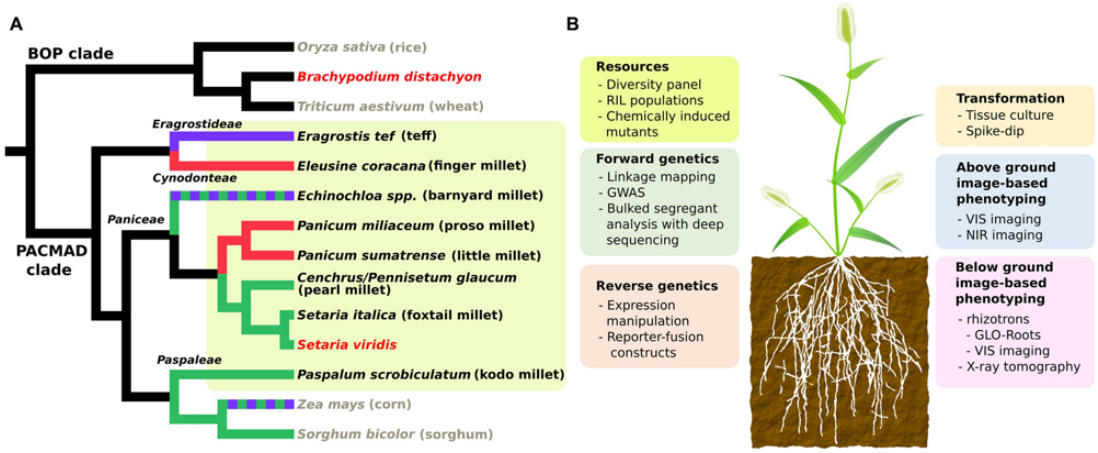

Figure 1. (A) Phylogenetic placement of Setaria viridis among grasses and (B) summary of genetic resources/phenotyping tools. *Source: Huang et al. (2016)*

(A) Phylogenetic placement of Setaria viridis among grasses and millets. Cladogram highlights *S. viridis* (red) within the Paniceae tribe of the PACMAD clade (green background), closely related to foxtail millet (Setaria italica) and other small-grain millets

(B) Schematic summary of resources and methods available for *S. viridis*. The model's genetic tools, like diversity panels, RIL populations, and mutants, help with forward genetics (like linkage mapping, GWAS, and bulked segregant analysis) and reverse genetics (like transgene overexpression, CRISPR knockouts, and reporter constructs) (Huang *et al.*, 2016).

Transformation techniques (tissue culture and floral spike-dip) enable transgenic line creation. High-throughput phenotyping platforms capture above-ground traits via visible (VIS) and near-infrared (NIR) imaging and below-ground traits via rhizotrons, GLO-Roots luminescence imaging, or X-ray tomography (Huang *et al.*, 2016). These integrated capabilities allow rapid genotype-to-phenotype studies in Setaria.

4.1. Morphological and developmental characterization frameworks

Morphological evaluation is a foundational step in characterizing transgenic *S. viridis*. Even subtle alterations in plant form or development can reflect the influence of an introduced transgene or edited gene. Researchers, therefore, employ systematic frameworks to capture traits such as plant height, leaf morphology, tillering (branching), flowering time, panicle architecture, and overall growth habit of transgenic lines (Acharya *et al.*, 2017). Standardizing these observations is critical. For example, Acharya *et al.* (2017) established a set of optimized growth assays at the seedling, juvenile, and adult stages of *S. viridis*, with precise protocols for measuring germination rates, root and shoot lengths, leaf number, tiller number, and reproductive traits under controlled conditions (Acharya *et al.*, 2017). Such assays enable reproducible comparison of morphological phenotypes across laboratories and studies. Transgenic lines can thus be screened for developmental deviations (e.g., dwarfism, altered leaf count or size, delayed flowering) against wild-type controls with statistical rigor (Acharya *et al.*, 2017).

A formal developmental staging system further strengthens morphological characterization. In this regard, a recent study codified the phenology of *S. viridis* using the BBCH (Biologische Bundesanstalt, Bundessortenamt, und Chemische Industrie) scale (Junqueira *et al.*, 2025). This scale defines key growth stages (germination, leaf development, tillering, inflorescence

emergence, anthesis, seed set, and senescence) with standardized codes, providing a common language to describe when phenotypic measurements are taken. Employing the BBCH-scale in Setaria ensures that transgenic phenotypes (for instance, "early flowering" or "reduced tillering") are referenced to comparable developmental milestones, allowing meaningful comparisons among studies (Junqueira *et al.*, 2025). Indeed, identifying mutant or transgenic phenotypes often "brings challenges, and a systematic and detailed morphological characterization throughout development is fundamental" (Junqueira *et al.*, 2025). By documenting traits at successive stages, researchers can distinguish genuine developmental effects of a genetic modification from benign variation or environmental influence.

In practice, morphological data on transgenic *S. viridis* are gathered through both direct measurements and imaging. Traits like plant height, internode length, and panicle length are measured with rulers or calipers (Acharya *et al.*, 2017). Counting tillers or leaves is done manually at set time points, sometimes aided by photography for later counting. High-resolution images of whole plants or specific organs (leaves, inflorescences) enable digital analysis of shape and size. For example, Acharya *et al.* included representative images of transgenic and control plants side-by-side to qualitatively illustrate morphological differences (Acharya *et al.*, 2017). In some cases, mutants or transgenics may exhibit dramatic morphology, such as bristless inflorescences or altered leaf orientation, that is readily visible (Kaggwa *et al.*, 2021). In others, differences might be subtle (a slight reduction in leaf blade width or delayed panicle exertion), which illustrates the importance of careful measurements over time.

Notably, forward-genetic screens in *S. viridis* have pinpointed the value of meticulous morphological phenotyping. For instance, chemical mutagenesis yielded sparse panicle mutants with reduced panicle branching; through detailed measurements of inflorescence traits, these were mapped to an auxin1 transporter gene, which affected both inflorescence development and root gravitropism (Kaggwa *et al.*, 2021). The discovery of pleiotropic phenotypes (shoot and root) for the SvAUX1 mutation highlights why comprehensive morphological profiling, across above- and below-ground organs, is recommended. In transgenic studies, even if the introduced gene is expected to affect, say, only inflorescences, it is prudent to assess whole-plant morphology in case of unanticipated effects on root or vegetative traits.

In summary, morphological and developmental characterization in transgenic *S. viridis* relies on standardized staging and measurement protocols to capture trait differences accurately. By anchoring observations to frameworks like the BBCH scale and using uniform assay conditions (Acharya *et al.*, 2017), researchers can ensure that any phenotypic divergence in transgenic lines (however subtle) is detected and attributed to the genetic modification with confidence. This information forms the basis for downstream functional assessments.

4.2. Physiological performance assessment and functional validation

Morphological data alone often do not reveal the full impact of a transgene on plant function. Hence, physiological performance assays are integral to characterizing transgenic *S. viridis*. These assessments quantify how a genetic modification influences the plant's functional outputs, such as photosynthetic capacity, growth rate, biomass accumulation, water-use efficiency, or yield, under given conditions. By comparing transgenic lines to wild-type, researchers can validate whether the introduced gene confers the expected physiological changes (often the ultimate goal of trait engineering).

A primary target of physiological phenotyping is photosynthesis and related growth metrics. Many transgenes in *S. viridis* are aimed at improving photosynthetic efficiency or stress resilience of photosynthesis (given *S. viridis*'s model role in C_4 photosynthesis research) (Huang *et al.*, 2016). Gas exchange measurements using portable photosynthesis systems (e.g., Li-Cor LI-6800) allow direct assessment of net CO_2 assimilation rates and stomatal conductance in transgenic plants (Danforth Center, n.d.). Chlorophyll fluorescence imaging provides complementary data on photosystem II efficiency and non-photochemical quenching (Danforth Center, n.d.). For example, a phenotyping platform at the Danforth Center routes Setaria plants through an imaging station that records whole-plant fluorescence to detect differences in photosynthetic performance (Fahlgren, 2015). In a drought experiment on this platform, *S. viridis* transgenic lines could be evaluated for their ability to maintain higher fluorescence (indicating sustained photosynthesis) under water stress relative to controls (Fahlgren, 2015). Similarly, near-infrared imaging is used to infer plant water content and canopy temperature differences, proxies for transpiration and water-use efficiency (Huang *et al.*, 2016). Fahlgren *et al.* (2015) employed NIR imaging to show strong water content differences between well-watered vs. drought-treated Setaria plants, capturing a physiological phenotype that might not be apparent from morphology alone (Fahlgren, 2015).

Growth rate and biomass production are another key aspect of physiological performance. Transgenic *S. viridis* intended to enhance yield or biomass (for biofuel traits) must be tested for actual gains in these outputs. In practice, plants are grown to maturity and final biomass (shoot dry weight) is measured, along with seed yield per plant if relevant (Acharya *et al.*, 2017). For instance, Acharya *et al.* found that *S. viridis* grown in an optimal greenhouse environment produced over double the seed yield per plant compared to growth chamber conditions (Acharya *et al.*, 2017). They concluded that physiological experiments on adult Setaria require near-optimal growth conditions to accurately gauge performance (Acharya *et al.*, 2017). When characterizing transgenics, this insight is crucial: any performance advantage conferred by a transgene (e.g., faster growth or greater yield) can be masked if baseline growth conditions are suboptimal. Therefore, physiological assessments are typically done under both ideal conditions (to see maximum performance) and challenging conditions (to test resilience, as covered in the next section).

Another dimension of functional validation is demonstrating that a transgene's hypothesized role manifests in measurable plant function. For example, if a transgene is expected to enhance nutrient uptake, one would measure tissue nutrient contents or growth under low-fertility conditions to validate improved nutrient use efficiency. If a gene is inserted to alter hormone sensitivity, experiments might track physiological

responses (such as growth inhibition by ABA or shoot elongation by GA) in transgenic vs. wild-type plants across hormone treatments (Acharya *et al.*, 2017). In *S. viridis*, a well-known example of testing gene function is when scientists increased the activity of stress-related genes from millets: boosting a Late Embryogenesis Abundant protein gene (SiLEA14) in foxtail millet greatly helped the plant grow better when facing salt and drought stress, showing that the gene helps protect the plant (Huang *et al.*, 2016). Although that example is in S. italica, the concept translates to *S. viridis*: researchers can introduce similar genes into *S. viridis* and then measure, say, its biomass retention or photosynthetic rate under salinity to see if the anticipated tolerance phenotype occurs. If the transgenic line maintains higher growth and slower leaf wilting than controls at, for instance, 100 mM NaCl, that physiological outcome validates the gene's function in planta.

It is also common to measure *S. viridis* transgenics' performance over time. Time-series data, enabled by automation, can reveal dynamic physiological differences. For instance, continuous imaging showed that *S. viridis* grows more rapidly early in development than S. italica, even if final biomass converges (Huang *et al.*, 2016). A transgene affecting growth rate might be detected through such temporal analysis when endpoint measurements alone might miss it. Figure 2 (later in this review) outlines how multiple levels of phenotypic analysis feed into an integrated genotype–phenotype understanding.

In summary, physiological performance assays translate the presence of a transgene into concrete functional readouts like photosynthetic efficiency, growth and yield, water and nutrient use, and overall vigor. These metrics are essential to determine whether a genetic modification simply causes a cosmetic change or truly enhances plant function. By validating functional outcomes, researchers ensure that promising transgenic lines in *S. viridis* have demonstrable benefits (or predictable trade-offs), informing decisions on advancing those genes toward crop improvement.

4.3. High-throughput phenotyping technologies and automation integration

Conventional phenotyping of dozens of plants by hand is labor-intensive and may miss subtle or time-dependent traits. In recent years, high-throughput phenotyping (HTP) platforms have revolutionized how researchers characterize transgenic plants, and *S. viridis* has been at the forefront of adopting these technologies (Huang *et al.*, 2016). High-throughput phenotyping combines automated imaging, environmental control, and data analytics to measure a multitude of traits on large plant populations nondestructively and objectively. The integration of HTP is especially powerful for *S. viridis*, given its small size (allowing many plants in a compact space) and uniformity, which suits automated handling.

A prime example is the Bellwether Phenotyping Platform at the Danforth Center (Danforth Center, n.d.). This system consists of a large growth chamber equipped with conveyor belts that shuttle up to ~1,140 potted Setaria plants through imaging stations daily (Fahlgren, 2015). At designated stations, multiple imaging modalities capture each plant: high-resolution red-green-blue (RGB) images from the side and top for morphology and color; near-infrared (NIR) images for water status; and fluorescence images (often chlorophyll fluorescence) for photosynthetic activity (Fahlgren, 2015; Huang *et al.*, 2016). The result is a quantitative visual diary of each plant's growth. Fahlgren *et al.* (2015a) utilized this platform to monitor transgenic *S. viridis* and wild-types under drought. The continuous imaging revealed nuanced differences, for instance, *S. viridis* plants initiated growth faster than foxtail millet and responded more rapidly to water deficit, as determined by changes in projected leaf area and NIR-based water content over time (Huang *et al.*, 2016). Such temporal response patterns would be difficult to discern without automation.

High-throughput platforms also enable three-dimensional (3D) phenotyping. While 2D images give valuable trait proxies (e.g., side-view area correlating to biomass), 3D reconstructions yield direct measurements of volume and architecture. Scanner-based systems (using multiple camera angles or laser scanners) can create 3D models of *S. viridis* plants (Huang *et al.*, 2016). Vadez *et al.* (2015) employed 3D scanning on millet canopies to compare leaf area development, an approach equally applicable to *S. viridis* transgenics for capturing traits like leaf inclination, canopy density, and tiller angles (Vadez *et al.*, 2015). Although full 3D phenomics for many small plants is computationally intensive, ongoing advancements are making it more feasible (Gaillard *et al.*, 2020). Even without full 3D, rotating plants in front of cameras (e.g., 360° imaging) can approximate multi-view phenotyping.

Root phenotyping has historically lagged due to the difficulty of observing roots without excavation. Automation is changing this as well. *S. viridis* has been used in innovative root imaging systems; for example, the GLO-Roots platform involves transgenic lines expressing luciferase in roots grown on transparent media so that root growth can be imaged via luminescence (Rellán-Álvarez *et al.*, 2015). Sebastian *et al.* (2016) used GLO-Roots with *S. viridis* to see how the roots reacted to not having enough water, discovering that drought slowed down the growth of crown roots, which they recorded using time-lapse images of luciferase signals in the roots (Sebastian *et al.*, 2016). Additionally, X-ray computed tomography (X-ray CT) has been trialed on Setaria (and pearl millet) to reconstruct root architecture in soil noninvasively (Huang *et al.*, 2016). While X-ray CT throughput is lower, it provides true 3D root structure data. Rhizotrons (large plates or tubes with clear sides) offer another semi-automated root phenotyping method; roots grow against a transparent surface and are imaged at intervals (A. Li *et al.*, 2022). Passot *et al.* (2016) used rhizotron imaging in millets to measure root growth rates; similar setups can screen transgenic lines of *S. viridis* for root length, branching, and angle differences under various treatments (Passot *et al.*, 2016).

Crucial to HTP is the software that translates images into data. Open-source tools like PlantCV have been specifically optimized for Setaria phenotypes (Huang *et al.*, 2016). PlantCV pipelines can process thousands of *S. viridis* images to extract traits such as leaf area, plant height (via pixel height), color indices (greenness or anthocyanin levels), and even estimate seed head size (Gehan *et al.*, 2017; Huang *et al.*, 2016). The pipeline is customizable; for example, it can identify individual leaves for growth analysis or detect early senescence by color changes. Notably, PlantCV and similar software incorporate

machine learning to improve trait detection accuracy across diverse plant architectures (Danforth Center, n.d.). This functionality is particularly beneficial as transgenic *S. viridis* may have novel forms (e.g., extra tillers or altered leaf angles) that require flexible image analysis approaches.

The integration of high-throughput phenotyping means that transgenic lines can be screened in unprecedented detail and quantity. A researcher could grow hundreds of independent T1 transgenic events and automatically track their growth and health, quickly flagging any outlier phenotypes for closer study. This capacity is invaluable given the push toward large-scale mutagenesis and gene editing projects in *S. viridis*. For instance, a recent advance enabled CRISPR/Cas9 multiplex editing (Junqueira *et al.*, 2025). HTP is arguably the only practical way to comprehensively phenotype such populations and identify which edited lines show interesting phenotypes.

In summary, high-throughput phenotyping technologies bring speed, objectivity, and breadth to transgenic plant characterization. In *S. viridis*, automated platforms capturing multi-angle images and employing sophisticated analysis are uncovering dynamics of growth and physiology that manual methods would overlook. The result is a data-rich phenotype profile for each genotype, strengthening the links between a transgene, its intermediate traits, and eventual performance. As HTP adoption grows, it is helping transform *S. viridis* into a high-resolution model for systems-level plant biology (Huang *et al.*, 2016).

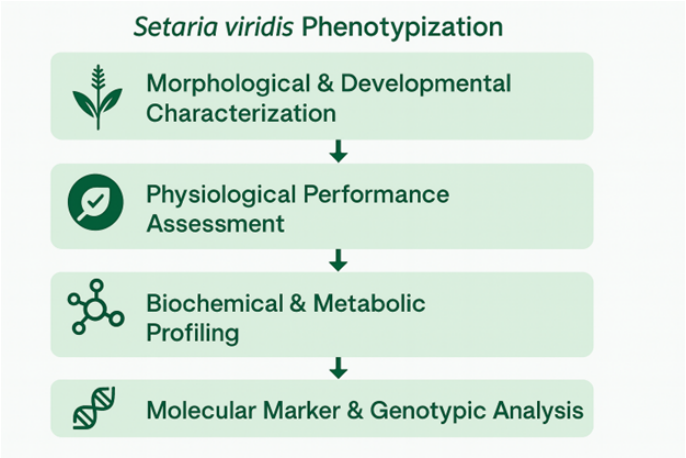

Figure 2. Multi-scale phenotypic characterization workflow for transgenic *Setaria viridis*.

Transgenic lines are evaluated sequentially through key analytical stages.

(i) Morphological & developmental characterization: Plants are observed from seedling to maturity, recording traits like germination rate, leaf number, plant height, tiller, and panicle development, using standardized growth-stage frameworks (Acharya *et al.*, 2017).

(ii) Physiological performance assessment: Functional metrics (photosynthetic rate, transpiration, biomass accumulation, yield components) are measured under optimal and stress conditions to validate gene function (Danforth Center, n.d.).

(iii) Biochemical & metabolic profiling: Tissue samples are analyzed for biochemical changes—e.g., metabolite levels, enzyme activities, cell wall composition—that elucidate the molecular impact of the transgene (de Souza *et al.*, 2018; Guedes *et al.*, 2023).

(iv) Molecular marker & genotypic analysis: DNA/RNA-based assays (PCR, sequencing, marker genotyping) confirm transgene insertion or mutation and allow correlation of genotype with phenotype across populations (Huang *et al.*, 2016).

(v) Integration: Data from all levels are combined to build a comprehensive genotype–phenotype map, strengthening the evidence for causal links and guiding translational research.

4.4. Stress tolerance evaluation and environmental response characterization

A significant motivation for developing transgenic lines is often to enhance stress tolerance, be it drought, salinity, extreme temperature, or nutrient deficiency. *S. viridis* is inherently adapted to relatively harsh, resource-limited environments (a trait shared with many millets) (Huang *et al.*, 2016), yet it still exhibits distinct stress-response phenotypes that can be quantified. Evaluating transgenics under controlled stress conditions is therefore a critical component of phenotype characterization.

Researchers have designed a range of stress assays for *S. viridis*. At the germination and seedling stage, plate-based assays are common: sterilized seeds are germinated on agar media supplemented with stress-inducing agents (e.g., high salt, mannitol for osmotic stress, abscisic acid for drought mimicry). Acharya *et al.* (2017) reported that *S. viridis* seeds are susceptible to exogenous ABA, even 1 µM ABA nearly halted germination (Acharya *et al.*, 2017). Germination is significantly delayed by moderate salt (50–100 mM NaCl). These assays, which record germination percentage and early root/shoot growth under stress, are a quick way to screen transgenic seeds for improved stress tolerance or sensitivity. For instance, a transgenic line overexpressing a candidate drought-tolerance gene might show higher germination rates than wild-type on 150 mM NaCl or maintain longer radicles on osmotic media.

At later growth stages, soil-based stress trials are employed. *S. viridis* transgenics are typically grown in pots under controlled environments where variables like water supply, nutrient availability, and temperature can be manipulated. Acharya *et al.* conducted a comprehensive stress experiment by imposing two single stresses, low water (drought) and low nitrogen, and their combination on *S. viridis* from one-week-old seedlings through maturity (Acharya *et al.*, 2017). The phenotypic outcomes were revealing: drought alone caused only modest reductions in growth and panicle size (consistent with *S. viridis*' reputation as a drought-tolerant species) (Acharya *et al.*, 2017). Water-stressed plants did not drastically differ in final height or panicle number from well-watered controls, though they had fewer seeds per panicle, resulting in a measurable yield drop (Acharya *et al.*, 2017). In contrast, nitrogen deficiency induced pronounced effects, leaves turned pale (chlorosis), plants were stunted, panicles were thinner and fewer, and seed yield plummeted (Acharya *et al.*, 2017). The combined stress (low water + low N) exacerbated these symptoms. These observations illustrate how *S. viridis* responds phenotypically

to nutrient vs. water stress, setting a baseline to judge transgenic improvements. If a transgenic line (say, expressing a high-affinity nitrate transporter) maintains greener leaves and higher biomass under low-N conditions than wild-type, it provides evidence of enhanced nutrient-stress tolerance.

Beyond drought and nutrition, *S. viridis* can be used to assess tolerance to heat, cold, or high light, though fewer studies have been published on those in this species. One can subject transgenic and control plants to heat waves in growth chambers or to chilling temperatures and monitor survival, recovery, growth, or physiological parameters (electrolyte leakage, F_v/F_m fluorescence ratio as a stress indicator). Given *S. viridis*'s C_4 nature, it is inherently efficient under heat and light; thus, transgenics aiming to tweak those traits might require stringent testing. For instance, one could hypothesize that a transgenic line with an altered membrane lipid composition would exhibit improved photosynthetic function at 42 °C. Such a hypothesis would be tested by measuring gas exchange at high temperatures or observing leaf damage after heat stress.

Biotic stress (pathogen or pest resistance) is another arena for phenotyping, though less documented in *S. viridis*. Brutnell and colleagues noted that *S. viridis* could be useful for studying grass-pathogen interactions and identifying resistance genes (Huang *et al.*, 2016). A few studies have used *S. viridis* for disease research (e.g., rust fungi), but routine phenotyping methods (like inoculation protocols and scoring of lesion development) remain to be standardized in this model. Nonetheless, if a transgene for disease resistance is introduced, classic pathology assays, inoculating plants with the pathogen and scoring disease severity or pathogen load, would constitute the phenotypic evaluation.

Transgenic *S. viridis* lines often leverage stress-related genes from other species. Validation in *S. viridis* provides a rapid proxy for how those genes might perform in crops. Huang *et al.* (2016) highlighted that an ABA-responsive DREB transcription factor from foxtail millet, when overexpressed, increased drought and salinity tolerance (Huang *et al.*, 2016). By creating a transgenic *S. viridis* with the analogous gene (or the millet gene itself, since *S. viridis* can be transformed with heterologous genes), one could rapidly observe if similar stress-tolerance phenotypes manifest, such as delayed wilting, sustained chlorophyll, or higher seed set under stress. They suggest that, as reverse genetics tools advance in *S. viridis*, genes from millets (like finger millet NAC and bZIP factors) can be functionally tested by introducing them into *S. viridis* and characterizing the stress response (Huang *et al.*, 2016). This strategy accelerates the identification of promising genes for crop improvement.

In executing stress phenotyping, it is essential to include proper controls and to quantify the responses. Traits commonly measured include survival rate (for severe stress), relative growth rate or biomass reduction, photosynthetic rate maintenance (under stress vs. control), leaf relative water content, stress-induced proline or other osmolyte accumulation (linking to biochemical profiling), and yield under stress. Many of these parameters cross over with physiological assays, reinforcing that stress testing is an extension of physiological characterization under adverse conditions.

Overall, stress tolerance evaluation in transgenic *S. viridis* involves subjecting plants to defined environmental challenges and measuring how well they perform relative to non-transgenic plants. The ability to impose these stresses in growth chambers or greenhouse settings with replication and control makes *S. viridis* a powerful system for dissecting stress-response phenotypes. If a transgene confers an advantage, *S. viridis* will show quantifiable improvement in one or more stress performance metrics. Those results then guide further development, for instance, advancing the transgene to crop trials or focusing on the underlying mechanisms (which often involves biochemical and molecular analyses, our next topics).

4.5. Biochemical characterization and metabolic profiling integration

Genetic modifications can lead to changes in the biochemical makeup of the plant that are not immediately evident from morphology or basic physiology. Therefore, a thorough phenotypic characterization often includes biochemical assays and metabolite profiling to capture the molecular phenotype of transgenic *S. viridis*. These methods bridge the gap between genotype and high-level phenotype by identifying changes in metabolites, proteins, or other cellular components induced by the transgene.

One important biochemical aspect is the composition of structural polymers like cell wall components. *S. viridis* has been used to study biomass quality traits relevant to biofuel or forage. A striking example comes from de Souza *et al.* (2018), who suppressed a single BAHD acyltransferase gene (SvBAHD01) in *S. viridis* via RNA interference. Biochemical analysis showed a ~60% decrease in cell wall feruloylation in the transgenic lines, with no reduction in total lignin content (de Souza *et al.*, 2018). Ferulic acid cross-linking in grass cell walls is a major factor in recalcitrance to enzymatic digestion. Correspondingly, the SvBAHD01-silenced plants exhibited a 40–60% increase in cell wall saccharification efficiency (sugars released), a desirable phenotype for biofuel feedstock (de Souza *et al.*, 2018). Notably, this biochemical phenotype (reduced ferulate) would not have been detectable by eye; the plants looked normal and even had unchanged total biomass (de Souza *et al.*, 2018). Only targeted biochemical assays (in this case, quantification of ester-linked hydroxycinnamates in cell wall extracts) revealed the effect of the genetic modification. This study underscores how biochemical characterization is crucial for validating that a transgene has achieved its intended molecular effect. In transgenic *S. viridis*, one might measure a variety of compounds depending on the gene's expected role: cell wall polysaccharides and lignin (for cell wall genes), specific amino acids or secondary metabolites (for metabolic genes), or hormone levels (if hormone pathways are targeted).

Metabolite profiling, especially using high-throughput techniques like GC-MS or LC-MS, allows an unbiased look at changes in the plant's metabolic network. Guedes *et al.* (2023) performed a comprehensive primary metabolite profiling of *S. viridis* under drought stress (Guedes *et al.*, 2023). Even though this was an abiotic stress study (not a transgenic study per se), the approach and findings are instructive. They sampled roots, leaves, and panicles of *S. viridis* under well-watered vs. water-limited conditions and analyzed metabolites via GC-MS

(Guedes *et al*., 2023). Each organ showed a distinct metabolic profile; for instance, drought caused accumulation of sugars and osmoprotectants like proline in leaves, whereas roots and panicles had different signature changes (Guedes *et al*., 2023). Out of 36 key drought-responsive metabolites identified, only four (including sucrose and glycerol-3-phosphate) were common to all organs (Guedes *et al*., 2023). This finding indicates organ-specific metabolic adjustments to stress. In a transgenic context, creating an *S. viridis* overexpressor of a gene involved in osmolyte biosynthesis allows metabolomic profiling to confirm whether expected compounds (such as proline or certain sugars) are elevated in the transgenic plants. Similarly, untargeted metabolomics can detect unexpected off-target effects of a transgene, revealing, for example, if altering one pathway triggers compensatory changes in another.

Combining metabolite data with phenotype data can provide mechanistic insights. In crop research, metabolite markers have been used to predict complex traits (Wei *et al*., 2023). A frontier study in foxtail millet built a metabolite–phenotype correlation network, associating 381 annotated metabolites with 63 agronomic traits (Wei *et al*., 2023). They found, for instance, that flavonoid and lignin pathway metabolites clustered with certain yield and growth traits (Wei *et al*., 2023). While that study was not in *S. viridis*, the concept applies: in transgenic *S. viridis*, if one profiles a broad array of metabolites, correlations might emerge (e.g., a line with higher biomass might consistently show higher levels of particular amino acids or sugars). This kind of integrated analysis can hint at why a transgene has the effect it does. For example, an *S. viridis* line overexpressing a transcription factor might show an altered metabolic fingerprint (perhaps elevated stress metabolites) that correlates with its improved drought tolerance phenotype, suggesting the transcription factor indeed upregulates those protective metabolites.

Enzyme assays are another facet of biochemical characterization. If a transgene encodes an enzyme or affects an enzyme's activity, directly measuring that activity in plant extracts is informative. For instance, a transgenic *S. viridis* with an engineered photorespiratory pathway enzyme would be assayed for that enzyme's activity (and possibly lower photorespiration rate in vivo). In practice, researchers often use *S. viridis* leaf or panicle extracts in colorimetric or fluorometric enzyme assays to confirm functional expression of the transgene at the biochemical level.

Proteomic analysis (e.g., via SDS-PAGE and immunoblot or mass spectrometry) can also fall under biochemical characterization, though it is less frequently reported for *S. viridis* specifically. If antibody tools are available, confirming the presence and amount of the transgene-encoded protein by Western blot adds a layer of evidence that any observed phenotype is due to that protein's action. Additionally, proteomics could reveal downstream protein expression changes triggered by the transgene.

In summary, biochemical and metabolic profiling provide a fine-grained view of the transgenic phenotype in *S. viridis*. They can confirm that the intended metabolic pathway is altered (as in the BAHD feruloylation example (de Souza *et al*., 2018), and detect broader metabolic shifts indicative of stress responses or growth changes (as with drought-induced metabolites (Guedes *et al*., 2023). By integrating these analyses, we can complete the phenotype picture, linking genes, molecules, and whole-plant performance. Moreover, such data are invaluable for genotype–phenotype integration and for translating findings to crops, since they identify biomarkers and pathways to target in breeding or engineering.

4.6. Molecular marker integration, data integration, and genotype–phenotype correlation analysis

The last component of phenotypic characterization connects the observed traits to the genetic level. Molecular marker integration refers to using DNA-based markers and genomic tools to confirm the presence and behavior of transgenes, as well as to map and associate genetic variations with phenotypic variations. In *S. viridis*, which benefits from a sequenced genome and rich genetic resources, genotype–phenotype correlation analysis is a powerful approach to validate and extend findings from transgenic studies.

At a basic level, molecular analysis of transgenic *S. viridis* lines involves confirming the transgene's insertion and expression. PCR is routinely used to verify transgene integration by amplifying parts of the T-DNA from genomic DNA. More sophisticated methods, like Southern blot or sequencing, can determine transgene copy number and insertion sites, important if positional effects might influence expression. For gene-edited lines, genotyping by targeted sequencing (amplicon sequencing of the edited locus) is done to identify mutations. These steps ensure that any phenotype being characterized can be confidently attributed to the intended genetic change and not to unintended background mutations or absent inserts.

Beyond individual lines, *S. viridis* serves as a model for mapping quantitative trait loci (QTLs) and performing genome-wide association studies (GWAS) for complex traits. The same marker technologies can be used to integrate transgenic phenotype data with natural genetic variation. For instance, if a transgene produces a phenotype of interest (say, increased tillering), one might ask, do natural accessions of *S. viridis* with higher tiller numbers carry certain alleles in related pathways? With a variety of *S. viridis* samples available (Huang *et al*., 2016), researchers can study these samples to measure tiller number and use SNP markers throughout the genome to find connections. Indeed, numerous QTLs have been identified in Setaria for agronomic traits like plant height, flowering time, and drought tolerance (Huang *et al*., 2016). Mauro-Herrera and Doust (2016) mapped QTLs for tillering and flowering in S. italica/*S. viridis* populations, for example, revealing chromosomal regions controlling these traits (Mauro-Herrera *et al*., 2013). The knowledge of such loci can inform transgenic strategies (e.g., choosing a target gene that lies in a major QTL for drought tolerance). Conversely, transgenic results can validate those associations, if a GWAS highlights a candidate gene, making a transgenic *S. viridis* knockout or overexpressor of that gene provides a direct genotype–phenotype test of its function.

Bulked Segregant Analysis (BSA) combined with next-generation sequencing is a particularly potent method in *S. viridis*, thanks to its short life cycle and simple genome. BSA involves crossing a transgenic or mutant line with wild-type (if needed) and then pooling progeny that show the extreme

phenotype vs. those that do not and sequencing their DNA. The method pinpoints the genetic locus causing the trait by identifying allele frequency skews between bulks (Huang *et al.*, 2016). In *S. viridis*, BSA-seq can map mutations within a few months and at high resolution because many offspring can be generated and the genome is modest in size (Huang *et al.*, 2016). For example, in foxtail millet (close enough to *S. viridis*), Li *et al.* (2016) used BSA-seq to map a mutant with yellow-green leaves to a chlorophyll biosynthesis gene (Li *et al.*, 2016). Similarly, if a transgenic *S. viridis* insertion caused an unexpected mutant phenotype, BSA could be applied to rapidly confirm if the T-DNA insertion co-segregates with the trait (essentially a way to map the transgene's effect if multiple insertions or background mutations are present).

Data integration goes beyond just genetic markers. Modern phenotypic datasets (from morphology, physiology, metabolomics, etc., as described above) are often combined with genotypic data in systems biology approaches. For instance, multi-omics data integration might involve linking a list of differentially abundant metabolites with differently expressed genes (from RNA-seq) in a transgenic vs. wild-type comparison to build a network of affected pathways. In the context of *S. viridis* as a model, researchers have begun to develop such integrative frameworks. The goal is to achieve a holistic genotype⟶phenotype mapping: every layer from DNA to phenotype is connected.

A concrete demonstration of genotype–phenotype data integration is provided by recent studies in millets that incorporate metabolite QTL (mQTL) mapping. In foxtail millet, Zhao *et al.* (2022) identified over a thousand mQTLs for leaf metabolites and built correlations to agronomic traits (Wei *et al.*, 2023). They discovered candidate genes for both metabolite variation and agronomic trait variation, including a case where a known dwarfing gene (Sd1 for GA synthesis) was associated with variation in certain metabolites and plant height (Wei *et al.*, 2023). Applying a similar approach in *S. viridis* transgenic research, one could, for example, map metabolite changes in a transgenic line and see if they overlap with known QTL regions or if they predict phenotypic outcomes. If a transgenic line accumulates high flavonoid levels and also shows a robust stress tolerance phenotype, and if natural variants with those flavonoids are also stress-tolerant; that triangulates evidence that flavonoids (and the genes controlling them) are key to the phenotype.

Furthermore, many molecular markers developed for other grasses cross-hybridize or are conserved in Setaria. Rajput *et al.* (2014) showed that 62% of tested microsatellite markers were shared between switchgrass and proso millet (Rajput *et al.*, 2014). Because of such conservation, researchers can use marker information from better-studied crops to inform *S. viridis* experiments. For example, if a particular SNP in maize is linked to a stress response, one might check *S. viridis* genomes for the orthologous gene and any variation or expression change when that pathway is perturbed. Additionally, since *S. viridis* can be crossed with S. italica, it's possible to introgress *S. viridis* alleles into millet and track them with markers (Huang *et al.*, 2016). Similar work has been done to create dense marker populations for mapping traits like flowering time and drought tolerance (Huang *et al.*, 2016). In essence, *S. viridis* can act as a conduit for favorable alleles to enter crops, and molecular markers are the tools enabling this transfer by confirming the genotype in hybrids.

As transgenic and genome editing studies progress, maintaining an association with molecular data ensures that findings are not "one-offs" but contribute to the broader genetic knowledge. For instance, if a CRISPR knockout in *S. viridis* produces a notable phenotype, sequencing of that gene across diverse Setaria accessions can reveal if natural loss-of-function or allele variation correlates with similar phenotypic variation. If the answer is yes, this strengthens confidence in the gene's role and highlights it as a candidate for breeding selection.

In summary, molecular marker integration and genotype–phenotype correlation analysis aim to complete the cycle by confirming and utilizing the genetic foundation of the phenotypes seen in transgenic *S. viridis*. Researchers use tools such as PCR confirmation, QTL mapping, GWAS, BSA, and multi-omics integration to link the phenotypic traits to specific genes and alleles (Huang *et al.*, 2016, 2016; Michelmore *et al.*, 1991). This procedure not only validates that the transgene is the cause of the phenotype but also situates that knowledge in the wider context of plant genetics and breeding. Ultimately, these approaches enhance the translational impact of *S. viridis* research, as discussed next.

4.7. Implications for plant-biotechnology research

The multi-faceted phenotypic characterization of transgenic *S. viridis* has direct implications for plant biotechnology and crop improvement. Each aspect of analysis, from morphology to molecular markers, yields insights that de-risk and inform the translation of genetic innovations to agronomically important species. Table 1 summarizes how the discussed phenotyping dimensions contribute to biotechnological advancement.

Firstly, detailed morphological frameworks in *S. viridis* ensure that growth or developmental abnormalities are identified early in the pipeline. In biotechnology, a candidate gene might improve one trait but inadvertently stunt growth or delay flowering. By screening transgenics for such issues in the model plant, researchers can prioritize genes that confer desirable traits without unacceptable developmental penalties. For example, a drought-tolerance gene that causes severe dwarfing in *S. viridis* might be less attractive for crop engineering, even though it offers stress benefits. Conversely, identification of transgenic lines with enhanced vigor or yield-related architectural traits can (Acharya *et al.*, 2017). In essence, morphological analysis in *S. viridis* acts as an early warning and discovery system for growth-related effects of genetic modification.

Physiological performance assessments translate those morphological observations into metrics relevant to crop productivity (photosynthesis, biomass, yield). This is crucial for biotechnology because it quantifies the potential agronomic value of a gene. A transgene that marginally increases photosynthetic efficiency or harvest index in *S. viridis* could have a big impact when scaled to field crops, but such subtle enhancements would be missed without careful physiological measurement. Validating function in *S. viridis* (e.g., improved water-use efficiency or nutrient uptake) builds a strong case for

field-testing that gene in crops. It also helps biotech researchers triage candidates: many genes may improve stress tolerance in a lab assay, but those that do so while maintaining or boosting yield under stress are the real winners (Acharya *et al.*, 2017). By using *S. viridis* to measure both stress resistance and growth/yield, one can select genes that optimize the trade-off between the two, a classic challenge in crop improvement.

The incorporation of high-throughput phenotyping (HTP) adds speed and precision to these endeavors, which is transformative for biotechnology pipelines. Instead of testing one gene at a time in a few transgenic events, HTP allows simultaneous phenotyping of hundreds of events or gene variants. This approach is aligned with the emerging paradigm of testing large mutant libraries or multiple gene edits to find optimal allelic variants. For instance, genome editing can create a range of loss-of-function and partial-loss alleles for a gene of interest in *S. viridis*. HTP can rapidly phenotype this allelic series for growth and stress traits, enabling selection of the variant with the best performance profile (a surrogate for trait optimization) (Junqueira *et al.*, 2025). The data richness from HTP (such as continuous growth curves and multi-trait correlations) also feeds into machine learning models that can predict performance, a cutting-edge approach in digital agriculture. By first developing these models on *S. viridis*, where many genotypes can be tested quickly, researchers can refine algorithms that might later apply to crop breeding programs.

Stress tolerance evaluations in *S. viridis* carry obvious implications for breeding climate-resilient crops. A key advantage is that *S. viridis* permits rapid generation testing: multiple generations of transgenics can be grown and stressed in a single year, compressing the time needed to assess a gene's effect. Moreover, *S. viridis*'s relative stress hardiness means that truly effective stress-mitigating genes must stand out to show additional benefit (Acharya *et al.*, 2017). These findings can raise confidence that any positive result is robust. When a transgenic *S. viridis* line significantly outperforms the wild-type under severe drought or low nutrients, it provides proof-of-concept that the genetic intervention can create more resilient phenotypes (Acharya *et al.*, 2017). Such proof-of-concepts are often required before advancing transgenes to regulated crop trials. Additionally, *S. viridis* can serve as a testing ground for stacking traits; for example, researchers can combine a drought tolerance gene with a nitrogen-use efficiency gene and then subject the resulting double transgenic to combined stress, similar to what Acharya *et al.* did with low water and low nitrogen (Acharya *et al.*, 2017). This data informs biotech strategies on whether gene stacks interact positively, negatively, or neutrally, guiding how multiple traits should be combined in future crop varieties.

Implications extend to biochemical traits as well. The BAHD feruloylation example demonstrates that *S. viridis* can be used to improve biomass degradability, a trait important for biofuel feedstocks and forage digestibility (de Souza *et al.*, 2018). The stability of that trait across generations in *S. viridis* and the lack of yield penalty were critical pieces of evidence that silencing BAHD could be beneficial in sorghum or maize cell walls. Generally, when metabolic engineering is pursued (e.g., boosting vitamin content, altering oil composition, or reducing anti-nutritional compounds), *S. viridis* offers a fast biofactory to prototype these changes and analyze their biochemical outcomes in planta. It helps answer questions like, Does diverting carbon to a new product compromise growth? Does a novel metabolite cause any toxicity or feedback inhibition in the plant? Without a model such as *S. viridis*, we might only learn about these issues after conducting extensive crop trials. Thus, metabolic phenotyping in *S. viridis* derisks metabolic engineering efforts by highlighting unintended consequences early or, conversely, by confirming that a desired biochemical trait is achievable and stable.

Finally, genotype–phenotype integration in *S. viridis* directly strengthens molecular breeding. The accumulation of genotypic and phenotypic data in this model creates a reference that breeders and biotech companies can exploit. For example, if *S. viridis* research identifies a particular allele of a gene that enhances root growth under drought, molecular markers for that allele (SNPs, etc.) can be developed. Breeders can then rapidly screen crop germplasm collections for analogous alleles using those markers, accelerating marker-assisted selection. Indeed, *S. viridis* is increasingly seen as a "fast-forward" platform for gene discovery that can be channeled into millets and other cereals (Huang *et al.*, 2016). By adopting high-throughput phenotyping strategies in *S. viridis*, researchers also create protocols that can be adapted to crop breeding programs (many of which are now incorporating drones, field imaging, etc.). *S. viridis* not only serves as a testing ground for genes but also for phenotyping methodologies that apply to crop improvement. Table 1 encapsulates these themes by matching each phenotyping aspect with its key implication for plant biotechnology.

Table 1. Phenotypic characterization aspects in transgenic *S. viridis* and their implications for plant-biotechnology research.

Phenotypic Analysis Aspect	Implications for Plant Biotechnology
Morphological & Developmental Characterization	Reveals growth/developmental impacts of transgenes early. Ensures only genes without deleterious morphology (e.g., stunting, altered flowering) are advanced and highlights traits like architectural changes that could improve yield (Acharya *et al.*, 2017). Provides standardized phenotypic descriptors (e.g., via the BBCH scale) to compare and select candidates across studies (de Souza *et al.*, 2018).
Physiological Performance Assessment & Functional Validation	Quantifies the functional benefits (photosynthesis, biomass, and yield) of transgenes under normal and stressful conditions. Identifies genetic modifications that truly enhance productivity or efficiency, guiding focus to those with agronomic merit (Acharya *et al.*, 2017). Validates gene function in planta (e.g., improved WUE or nutrient uptake), building evidence before field trials.

High-Throughput Phenotyping Technologies Integration	Accelerates screening of large gene libraries or multiple events with minimal bias. Increases precision and data volume, enabling discovery of subtle or temporal phenotypes undetectable by manual methods (Huang *et al.*, 2016; Schneider *et al.*, 2012). It enables swift iterations in trait engineering, such as testing numerous CRISPR edits, thereby reducing the duration from gene discovery to application.
Stress Tolerance Evaluation Environmental Response Tests	Demonstrates resilience conferred by transgenes (drought, salinity, etc.), a critical trait for climate-smart crops. De-risks investment by showing a gene improves survival or yield under stress in a model system (Acharya *et al.*, 2017). Enables testing of gene stacks for combined stress (multi-trait) tolerance before deploying complex traits in crops.
Biochemical Characterization & Metabolic Profiling	Confirms target biochemical pathway modifications (e.g., metabolite production, cell wall composition) and detects off-target metabolic effects (de Souza *et al.*, 2018). Supports nutritional biofortification and quality traits by showing trait feasibility in planta. Guides metabolic engineering by identifying key metabolites or biomarkers linked to desirable phenotypes (informing marker development for breeding).
Molecular Marker Integration & Genotype–Phenotype Correlation	Links phenotypes to specific genes/alleles, facilitating marker-assisted selection and genomic prediction in crops (Huang *et al.*, 2016; Mauro-Herrera & Doust, 2016). Leverages *S. viridis* as a fast testbed to validate gene-trait associations (QTL/GWAS hits), accelerating gene discovery for breeding. Ensures that knowledge from transgenics is anchored in transferable genetic markers and conserved pathways for crop improvement.

5. CONCLUSION

Setaria viridis has firmly established itself as a versatile model for dissecting and validating gene functions relevant to crop improvement. By employing a comprehensive phenotypic characterization pipeline, spanning classical morphology, rigorous physiology, high-throughput imaging, stress trials, biochemical profiling, and molecular correlation analyses, researchers can obtain a 360° view of how a genetic modification shapes plant performance. This "from morphology to molecular markers" approach in *S. viridis* is enabling the rapid identification of candidate genes and traits that are both biologically interesting and agronomically promising.

The collective evidence surveyed in this review underscores several key points. First, robust phenotyping is not one-dimensional: it requires integrating multiple layers of information. A transgenic phenotype may manifest subtly in one dimension but strongly in another (for example, negligible visual differences yet significant metabolic changes or stress resilience). Only by stitching together observations across scales can we confidently attribute phenotypic outcomes to the underlying genotype. Second, the tools and methods available for *S. viridis* phenotyping have advanced remarkably, echoing the progress in genomic tools. High-throughput platforms and data-driven analyses now match the speed of gene editing and transformation, helping to overcome the phenotyping bottleneck in plant science (Fahlgren, 2015). As a result, *S. viridis* can keep pace with, and even accelerate, the increasing throughput of genetic experiments. Third, the insights gained in this model are proving readily translatable. Traits and genes characterized in *S. viridis* are being transferred to relative crops or used to guide marker development, fulfilling its role as a bridge between Arabidopsis-like models and large cereals (Huang *et al.*, 2016).

In closing, the phenotypic characterization methods for transgenic *S. viridis* reviewed here form a template for how we might approach any model-to-crop system. By thoroughly vetting gene functions in a rapid-cycle surrogate, plant biotechnologists can de-risk and refine their strategies before committing to slow and costly field trials in crops. *S. viridis*, with its genetic tractability and expanding phenomic toolkit, exemplifies this principle. Continued innovation in phenotyping, coupled with community data-sharing and standardized practices, will further enhance the power of *S. viridis* to drive discoveries. As we face the challenge of improving crops for a changing climate and growing population, the meticulous phenotyping of tiny green foxtail plants in growth rooms and imaging chambers across the world is quietly laying the groundwork for the next generation of climate-resilient, high-yielding crops (Huang *et al.*, 2016). The small stature of *S. viridis* belies its outsized impact: by illuminating the path from gene to trait, it is helping to usher in a new era of informed and precise plant biotechnology.

FUTURE RECOMMENDATIONS

As *S. viridis* continues to mature as a model, several future directions can further enhance its utility in plant biotechnology research. One recommendation is to extend phenotyping to more field-like conditions and complex environments. While growth chambers and greenhouse assays are invaluable for control, eventually testing transgenic *S. viridis* lines in semi-field settings (outdoor pots or field plots, where feasible) could reveal phenotype–environment interactions (e.g., fluctuating diurnal stresses, competition effects) that controlled conditions smooth over. Even though *S. viridis* is small, pilot field trials have been done for population studies; similar trials for transgenics, coupled with drone imaging or field phenotyping carts, would help ensure that lab-observed trait benefits translate to field scenarios.

Integration of multi-omics data in a more routine, automated way is another frontier. In the coming years, a transgenic *S. viridis* experiment might simultaneously collect phenomics data (imaging), transcriptomics (RNA-seq of key tissues),

metabolomics, and even ionomics (elemental composition). Developing pipelines that integrate these layers will allow constructing gene network models that predict phenotype from genotype with higher accuracy. For instance, a phenotype-to-gene association network could be built for stress responses, converging data from *S. viridis* mutants, transgenics, and natural variants. This aligns with the concept of systems phenotyping, where advanced computational models link genotype, intermediate molecular states, and phenotype. *S. viridis* is well-suited as a platform for such integrative modeling because of its manageable scale and growing data resources (Rahaman *et al.*, 2015).

Another recommendation is to emphasize data sharing and standardization. High-throughput phenotyping generates massive datasets, so establishing public repositories (e.g., image databases, phenotype matrices) for *S. viridis* experiments would accelerate progress by enabling meta-analyses and machine learning applications. The 79,000 images from the Bellwether drought study were made publicly available, setting a precedent (Fahlgren, 2015). Expanding such open data efforts, alongside standardized phenotype ontologies (like the BBCH phenology codes for *S. viridis*), will allow researchers globally to compare results and train robust predictive models. AI-driven phenotype prediction (e.g., using convolutional neural networks on *S. viridis* images to predict biomass or stress status) is an emerging area that would benefit immensely from large, diverse training datasets.

On the genetic engineering front, multiplexed and precision gene editing in *S. viridis* will likely produce complex genotypes (e.g., knockouts of gene families or cis-regulatory edits for fine-tuned expression). Phenotyping methods must adapt correspondingly. We recommend developing in vivo reporters and non-destructive assays for physiological processes in *S. viridis*. For example, sensors for real-time monitoring of hormone levels or reactive oxygen species in transgenic plants could be employed to phenotype dynamic stress signals. Such devices would enrich the functional data gathered and allow phenotypic screening of subtler internal traits (like hormonal fluxes) that currently require destructive sampling.

Given *S. viridis*'s role in translational research, closer alignment with crop breeding programs is also advised. Breeders could incorporate *S. viridis* as a pre-breeding evaluation tool: before attempting a gene introgression or editing in a crop, test it in *S. viridis* for one or two generations to flag any issues. Formalizing this pipeline requires effective communication of *S. viridis* results in breeding-relevant terms. One idea is to use *S. viridis* to generate "smart datasets" that link directly to crop performance metrics, for instance, correlating *S. viridis* drought survival at the seedling stage with yield stability indices in millet field trials. If strong correlations exist, *S. viridis* could serve as a surrogate screening system for breeding lines (not just for transgenes but also conventional lines, given its interfertility with S. italica).

Lastly, we recommend broadening the trait diversity studied in *S. viridis*. Much focus has been on abiotic stress and C_4 photosynthesis. But *S. viridis* has potential in areas like symbiosis (e.g., mycorrhizal or rhizobial interactions), phytoremediation traits, or, as noted, disease resistance. Developing phenotyping protocols for these (such as root colonization assays, heavy metal uptake measurements, and pathogen infection scoring) will expand the model's relevance. In particular, plant-microbe interaction phenotypes could be quantified with some of the same imaging tools (e.g., luminescent reporters for pathogen spread and spectral imaging for nutrient deficiency or toxin accumulation). *S. viridis* could thus also become a model for sustainable agriculture traits, for example, analyzing how transgenes affect its performance under low-input conditions or its interactions with beneficial soil microbes.

In summary, the future of *S. viridis* phenotypic analysis lies in greater realism (field and complex environments), deeper data integration (multi-omics and AI), enhanced data sharing, adaptive methods for novel genetic techniques, closer crop model coupling, and diversification of trait focus. These steps will guarantee that *S. viridis* stays at the forefront of plant biotechnological innovation, consistently enhancing our understanding of the relationship between genes, traits, and real-world crop outcomes.

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