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D2.2 – Cloning plant-based biosensor for heavy metal detection on soil

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D2.2 – Cloning plant-based biosensor for heavy metal detection on soil

Short summary: Deliverable D2.2 reports the work done by UR and CNR-IRET on WP2, in particular tasks 2.1 and 2.2. The document describes the strategies to clone multiple plasmid vectors that allowed to introduce in the model plant *Arabidopsis thaliana* the yeast transcription factor YAP8 and a novel synthetic promoter named YAPRE. These genetic elements when triggered by arsenic are able to activate the production of a red pigment thanks to the activation of the reporter RUBY in all areal tissues of *Arabidopsis thaliana*. This approach delivers a living biosensor capable of visibly indicating the presence of arsenic in soil thanks to plant's tissues colour shift (green→red in presence of arsenic). Moreover, the document details the genetic strategies employed to render the engineered *Arabidopsis thaliana* line reproductively sterile, thereby mitigating the risk of uncontrolled propagation in natural ecosystems.

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1. SUMMARY

This deliverable reports on the successful development and functional validation of a plant-based biosensor for environmental arsenic (As) detection (Task 2.1), integrated with a conditional sterility system designed to ensure robust biological containment during potential field applications (Task 2.2).

To establish the biosensing framework, we engineered a custom multigene construct in *Arabidopsis thaliana* leveraging the yeast transcription factor YAP8 and a novel synthetic promoter (YAPRE). Upon exposure to As, YAP8 activates the polycistronic RUBY reporter system, triggering a non-invasive, visually distinct phenotypic color transition from green to vivid red.

To maximize intracellular As accumulation and sensing sensitivity, the host background was optimized. Functional evaluation demonstrated that loss of EXPA1 (*expa1* mutant) mitigates arsenic-induced root growth arrest while preserving heavy metal uptake and root cell viability, outperforming alternative mutant lines. Functional in planta testing confirmed that transgenic 35S::YAP8-YAPRE::RUBY plants exhibit a robust red pigmentation specifically in response to 100 μ M As exposure.

To address environmental safety concerns and prevent unwanted gene flow or persistence in wild populations, the biosensing cassette was combined with a Tissue-Specific Knockout (TSKO) conditional sterility system. By placing the Cas9 gene under the control of a heat-shock (HS) inducible promoter targeting the PIN1 gene via two single-guide RNAs (sgRNAs), we established a controllable gene-disruption mechanism. Heat-shock treatment (40°C) of imbibed seeds successfully triggered the targeted *pin1* developmental phenotype in approximately 98% of evaluated plants, conferring conditional sterility.

In conclusion, tasks 2.1 and 2.2 of WP2 have successfully achieved their primary milestones: delivering a highly sensitive, visual plant biosensor for As monitoring while integrating a reliable heat-induced containment system to support safe, field-deployable biotechnological applications in compliance with European biosafety standards.





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2. MOBILES project short description

The National Technical University of Athens (NTUA) is working with another 14 partners from academia, research, and industry to develop prototypes of electronic and organism-based biosensors to monitor organic chemicals, antimicrobial-resistant (AMR) bacteria, and pathogens in water, soil, and air.

The MOBILES project is studying and developing biosensors for detecting heavy metals, antibiotics, pesticides, arsenic, microplastic and nanoplastic. It is also developing genetically modified plants and bacteria for detecting heavy metals, antibiotics, and pesticides, and the use of marine diatoms for monitoring bioplastic degradation.

MOBILES aims are to tackle chemicals, including persistent and mobile (PMCs) pollutants and contaminants of emerging concern (CECs), that degrade the environment. Another severe global health risk is associated with increasing AMR in bacteria. Foodborne pathogens, including *Listeria*, *Salmonella*, and *Campylobacter*, pose significant public health risks and are already monitored. However, current bacterial detection methods for environmental control are slow and require specialized laboratories with trained personnel. Similarly, conventional pollutant detection methods, such as chromatography and mass spectrometry, are accurate but time-consuming and require specialized equipment. State-of-the-art detection methods are unsuitable for constant on-site and real-time monitoring. The long time between sampling and detection reduces the efficiency of public health and environmental protection authorities in implementing effective countermeasures. To tackle this problem, several electrochemical biosensors will be developed within the MOBILES project.

Biosensors are devices that combine biological elements with electronic systems to detect specific pollutants. The MOBILES project enhances these sensors with advanced nanomaterials, significantly improving their sensitivity and reliability. All biosensors will have common basic electronics and functional principles (e.g., an organic ligand able to recognize target pollutants), but they will differ in the biological element employed: (i) aptasensors based on aptamers that recognize bacterial cells or spore surfaces, (ii) electronic noses for detecting and quantifying volatile organic compounds (VOCs) produced by bacteria, (iii) genosensors for detecting genes involved in antibiotic resistance, and (iv) interdigital capacitors functionalized with aptamers for estradiol, a member of CECs family.

Continual threats (such as industrial pollution and the overuse of drugs and pesticides) to sources of drinking water require real-time solutions for wide-ranging water monitoring systems to detect toxicants such as heavy metals, pesticides, and antibiotics. Conventional methods are limited in their ability to detect sub-lethal concentrations of active antibacterial compounds. The damage caused by the activity of an antibacterial agent or pesticide may stimulate different biological mechanisms of bacterial repair. Each antibiotic and/or pesticide triggers specific cellular pathways, mechanisms, and targets within the bacterial cell. This specific biological response, enabling the detection of antibiotics and pesticides using microorganisms, is being investigated in the MOBILES project through the use of genetically modified bacteria to detect toxic pollutants in water. For detecting heavy metals (cadmium, chromium, lead, mercury) in water, MOBILES is developing a flow-through





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device for continuous monitoring using biological systems (genetically modified bacteria) combined with an optical sensor and flow unit.

Highly toxic As pollution can come from various sources, including industrial activities, mining, and even natural processes. Water and food contaminated by As can cause serious health problems, including cancer and heart disease. For detecting As pollution in soil and groundwater, the MOBILES project is developing genetically modified plants that change colour when As is present in the soil or water used to grow them. The project will conduct safety evaluations to ensure that the genetically modified organisms and developed devices have minimal environmental impact. A specific work package (WP4) is dedicated to evaluate the effects of genetically modified organisms on other organisms and the environment. Safety tests and environmental impact of MOBILES organisms are performed in laboratory using EFSA guidelines.

Microplastic and nanoplastic pollution is raising concerns about its potential impact on human health. The transfer of very small plastics through the trophic chain is a potential source of contamination at all trophic levels. Understanding the distribution, degradation, and life cycle of micro- and nanoplastics in the marine environment is limited by the intrinsic difficulties of current techniques for detecting, quantifying, and chemically identifying small particles in liquids. The MOBILES project is addressing this challenge by utilizing marine diatoms. Diatoms are known for their resilience and adaptability, making them ideal candidates for studying the biodegradation of bioplastics in marine environments. Preliminary studies have shown promising results, indicating that diatoms not only survive in environments containing bioplastics but also contribute to their biodegradation.

In addition to the development of sensors, MOBILES will undertake comprehensive metagenomic analysis, profiling the microbiota of polluted areas across Europe. This work will uncover gene clusters and reveal genetic diversity, enabling a deeper understanding of microbial functions. These insights will provide genetic markers to facilitate rapid evaluation of soil and land health. Two annual sampling rounds are planned for at least two years, and sample collection will be conducted at different locations to target microbiota related to specific pollution types: Greece for urban wastewater contamination, Poland for heavy metal pollution, Cyprus for microplastics and plastics, France for agriculture and animal farming, Italy for As, and Germany for chemicals and heavy metals from former mining activities. Genomic and transcriptomic data will be analysed, visualized, and interpreted using bioinformatic tools and soil metagenomic web-based platform specifically realized by MOBILES partners. The project's data storage, located in Spain, will be connected to other well-known genomic databases in order to provide a wide range of information.

The biosensors will be rigorously tested with real-world samples from polluted sites to validate their environmental performance.

MOBILES workplan is organized in 6 WPs listed in Table 1. Management actions and collaboration with other EU funded projects are implemented within WP6 while disseminations, exploitation and communication (DEC) activities are grouped within WP5. WP1-WP2 deal with interconnected scientific and technological activities to develop electrochemical biosensors to detect specific





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pollutants, and organism-based biosensor to monitor other typology of selected pollutants. In WP3 an extensive metagenomic analysis will be performed in order to enable searches for diverse functionalities across multiple gene clusters in polluted and not polluted areas across Europe. In WP4 the safety of all genetically modified bacteria and plants will be tested using standard EU procedures (e.g., EFSA guidelines for genetically modified organisms), and a pre-industrial design of the various biosensors will be provided along with stability (shelf-life) tests.

Table 1. MOBILES WPs list

WP	Work Package Title	Lead Name	Start Month	End month
1	Electronic biosensors for environmental monitoring	INRAE	1	36
2	Detection of pollutants via biotic sensors	UR	1	36
3	Metagenomics database and fully-sequenced polluted soil microbiota	CNR- ISAFOM	1	42
4	Environmental performance and safety of developed organisms, and packaging of sensor devices	RICPA	10	42
5	Dissemination, exploitation and communication of project outcomes	GG	1	42
6	Project Management and Coordination	NTUA	1	42





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3. Introduction: arsenic pollutant

Arsenic is a widespread and persistent environmental contaminant that enters terrestrial and aquatic ecosystems through both natural geogenic processes and anthropogenic activities. In soils and waters, As occurs predominantly as the inorganic oxyanions arsenate [As(V)] and arsenite [As(III)], whose relative abundance and mobility depend strongly on environmental conditions, including pH and redox potential¹.

Contamination of groundwater represents a major environmental and public-health concern worldwide, particularly where As-rich water is used not only for drinking but also for agricultural irrigation². Continuous irrigation with contaminated groundwater can progressively transfer As to agricultural soils, creating a long-term reservoir of contamination and increasing its potential entry into the food chain.

Plants can absorb both As(V) and As(III) through their roots, and once inside plant cells, As(V) is rapidly reduced to As(III). Arsenic exposure can interfere with several physiological and metabolic processes, inhibit plant growth, and ultimately reduce crop productivity. Moreover, accumulation in edible plant tissues may contribute to human exposure through food consumption, linking water and soil contamination directly to food safety and human health^{3,4}. The persistence of As in soils, its transfer between water, soil, and plants, and the spatial and temporal heterogeneity of contamination therefore make effective environmental monitoring particularly challenging.

Conventional As determination relies primarily on highly sensitive and quantitative (Inductively Coupled Plasma) ICP-based analytical techniques. Although these methods provide accurate measurements, they are time-consuming and destructive analyses, limiting their suitability for rapid, repeated, and spatially distributed monitoring. More recently, X-ray-based techniques and hyperspectral imaging (HSI) have opened new opportunities for detecting and mapping As accumulation in plant tissues, potentially providing less invasive approaches to environmental assessment^{5,6}. Nevertheless, further technological development is still required before these methods can be routinely applied for rapid and cost-effective field monitoring. Consequently, there

¹ Pigna, M., Caporale, A. G., Cavalca, L., Sommella, A., & Violante, A. (2015). Arsenic in the soil environment: Mobility and phytoavailability. *Environmental Engineering Science*, 32(7), 551–563.

² <https://www.who.int/news-room/fact-sheets/detail/arsenic>

³ Allevato, E., Stazi, S. R., Marabottini, R., & D'Annibale, A. (2019). Mechanisms of arsenic assimilation by plants and countermeasures to attenuate its accumulation in crops other than rice. *Ecotoxicology and environmental safety*, 185, 109701.

⁴ Zhao, F. J., Ma, J. F., Meharg, A. A., & McGrath, S. P. (2009). Arsenic uptake and metabolism in plants. In *New Phytologist* (Vol. 181, Issue 4, pp. 777–794).

⁵ Antenzio, M. L., Capobianco, G., Costantino, P., Vamerali, T., Bonifazi, G., Serranti, S., Brunetti, P., & Cardarelli, M. (2022). Arsenic accumulation in *Pteris vittata*: Time course, distribution, and arsenic-related gene expression in fronds and whole plantlets. *Environmental Pollution*, 309.

⁶ Van Der Ent, A., De Jonge, M. D., Spiers, K. M., Brueckner, D., Montargès-Pelletier, E., Echevarria, G., Wan, X. M., Lei, M., Mak, R., Lovett, J. H., & Harris, H. H. (2020). Confocal Volumetric μ xRF and Fluorescence Computed μ -Tomography Reveals Arsenic Three-Dimensional Distribution within Intact *Pteris vittata* Fronds. *Environmental Science and Technology*, 54(2), 745–757.





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is a growing need for complementary strategies capable of translating the presence and biological availability of As in the environment into an easily detectable signal.

Plants are particularly attractive candidates for the development of environmental biosensors because they continuously interact with soil, water, and the atmosphere and integrate contaminant exposure over time. Unlike conventional analytical measurements, which provide information on contaminant concentrations at a specific sampling point, plant-based sensing systems have the potential to report the fraction of a contaminant that is effectively accessible to a living organism. Their root systems explore relatively large volumes of soil or water, while uptake, transport, and accumulation processes can integrate spatial and temporal variations in contaminant availability. Advances in plant synthetic biology have further expanded this concept by enabling endogenous or heterologous sensing mechanisms to be coupled to reporter systems that generate measurable outputs in response to specific environmental compounds.

Transgenic *Arabidopsis* plants capable of detecting target substances through engineered genetic circuits have already been developed, demonstrating the feasibility of using living plants as programmable environmental sensors^{7,8}. However, most plant biosensors reported so far have been demonstrated primarily under controlled in vivo laboratory conditions, and their translation into robust systems suitable for environmental applications remains limited.

A major challenge is therefore to combine the specificity of synthetic biological sensing systems with a well-established plant system such as *Arabidopsis thaliana*, in order to develop a reliable and tractable platform for biosensor engineering. In the case of As, this approach is particularly relevant because plants differ in their capacity to tolerate, accumulate, and translocate the metalloid. Although most plants are sensitive to As, accumulator and hyperaccumulator species can tolerate considerably higher concentrations. Among them, the Chinese brake fern *Pteris vittata* is an exceptionally efficient As hyperaccumulator, capable of taking up, translocating, and storing large amounts of As without showing severe toxicity symptoms^{9,10}. However, despite its physiological relevance for As accumulation, *P. vittata* is not yet a suitable base for synthetic biology applications. For this reason, *A. thaliana* is selected as the host system for the implementation of the As biosensor. Importantly, testing and validating the biosensor construct in *A. thaliana* is an essential step to ensure its functionality and robustness before any subsequent transfer or adaptation to a *P. vittata*-based biosensing system.

⁷ Antunes, M. S., Morey, K. J., Jeff Smith, J., Albrecht, K. D., Bowen, T. A., Zdunek, J. K., Troupe, J. F., Cuneo, M. J., Webb, C. T., Hellinga, H. W., & Medford, J. I. (2011). Programmable ligand detection system in plants through a synthetic signal transduction pathway. *PLoS ONE*, 6(1).

⁸ Park, S. Y., Qiu, J., Wei, S., Peterson, F. C., Beltrán, J., Medina-Cucurella, A. V., Vaidya, A. S., Xing, Z., Volkman, B. F., Nusinow, D. A., Whitehead, T. A., Wheeldon, I., & Cutler, S. R. (2024). An orthogonalized PYR1-based CID module with reprogrammable ligand-binding specificity. *Nature Chemical Biology*, 20(1), 103–110.

⁹ Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED. A fern that hyperaccumulates arsenic. *Nature*. 2001 Feb 1;409(6820):579.

¹⁰ Tu S, Ma LQ, Fayiga AO, Zillioux EJ. Phytoremediation of arsenic-contaminated groundwater by the arsenic hyperaccumulating fern *Pteris vittata* L. *Int J Phytoremediation*. 2004;6(1):35-47.





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Beyond the plant kingdom, remarkable tolerance to As is also observed in yeasts, due to their capacity to sense As and activate specific detoxification responses. In *Saccharomyces cerevisiae*, the AP-1-like transcription factor YAP8 is activated through interaction with As(III) and subsequently binds a unique 13-bp response element (Y8RE) in the shared promoter of ACR2 and ACR3, encoding a cytosolic As(V) reductase and a plasma membrane As(III) efflux protein, respectively¹¹. This highly specific As-sensing mechanism represents an attractive synthetic biology module that can be transferred to plants and coupled to reporter gene expression in *Arabidopsis*.

Traditional reporter genes are widely used to visualize gene expression in plants, but their application beyond laboratory-scale experiments is often constrained by invasive procedures, expensive substrates, or specialized detection equipment. The RUBY reporter offers a particularly suitable alternative for environmental biosensing because it produces a red betalain pigment that is directly visible to the naked eye. Betalain production is achieved through only three enzymatic steps starting from tyrosine, an amino acid naturally present in plant cells, with all three required enzymes encoded within a single artificial open reading frame¹². By coupling YAP8-mediated As sensing with transcriptional activation of RUBY in a synthetic transactivation system in *Arabidopsis thaliana*, it should therefore be possible to generate genetically engineered plants capable of converting As(III) perception into an easily detectable visual signal.

However, even in *Arabidopsis thaliana*, the successful implementation of such a synthetic biosensing system requires careful optimization of genetic constructs, regulatory elements, and transformation strategies. The development of an As-indicator *Arabidopsis* biosensor therefore relies on the assembly of robust molecular modules and their functional validation in planta.

Accordingly, the work conducted during the first year aimed to design and generate preliminary destination vectors containing the basic molecular components required for the synthetic sensing system in *Arabidopsis thaliana*. These preparatory steps provide the technological foundation for the subsequent engineering and functional validation of the As-indicator biosensor. The long-term objective is to generate a living, visually readable *Arabidopsis*-based biosensor capable of integrating As perception with a specific synthetic response, laying the foundations for a complementary tool for environmental monitoring of As contamination in water and soil.

¹¹ Kumar, N. V., Yang, J., Pillai, J. K., Rawat, S., Solano, C., Kumar, A., Grøtli, M., Stemmler, T. L., Rosen, B. P., & Tamás, M. J. (2016). Arsenic Directly Binds to and Activates the Yeast AP-1-Like Transcription Factor Yap8. *Molecular and Cellular Biology*, 36(6), 913–922

¹² He, Y., Zhang, T., Sun, H., Zhan, H., & Zhao, Y. (2020). A reporter for noninvasively monitoring gene expression and plant transformation. *Horticulture Research*, 7(1).





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4.Task 2.1: Generation of an “As-indicator” gene cassette

The primary objective of UR in task 2.1 is to develop a plant-based biosensor for As detection and to deliver it to CNR-IRET. The biosensor relies on the heterologous expression of the *Saccharomyces cerevisiae* transcription factor YAP8, which acts as an As sensor. Upon As exposure, YAP8 activates the transcription of an optically visible reporter gene driven by a custom synthetic promoter in the model plant *Arabidopsis thaliana*. This sensing mechanism triggers a clear phenotypic color change (from green to red) in plants growing in As-contaminated soil. To prevent gene flow and environmental cross-contamination with wild-type populations, this biosensing cassette will be introduced into a heat-shock-inducible sterile plant line.

4.1. Molecular design of As sensor

To construct the As biosensor, we utilized the GreenGate cloning system, enabling the assembly of complex multi-gene super-modules (up to 9 functional elements) within the T-DNA Left and Right Borders (LB/RB).

4.1.1. YAPRE promoter generation

We first engineered a novel synthetic promoter and designated YAPRE (YAP8-Responsive Element). This promoter harbours multiple copies (6 or 8 repeats, tables 1 and 2) of the consensus motif (5'-TGATTAATAATCA-3') specifically recognized by the yeast transcription factor YAP8, separated by 2–4 bp spacer sequences designed to avoid recognition by endogenous plant transcription factors. The synthetic operator repeats were fused upstream of a 35S minimal promoter (35S mini), a truncated TATA-box-containing promoter region that is transcriptionally inactive in the absence of upstream enhancer elements.

Table 2. YAP8-Responsive Element with 6 copies of consensus motif marked in red

6XYAPRE Repeat Variant::

AAGTGATTAATAATCAACGTTGATTAATAATCAAGTGATTAATAATCAAGTGATTAATAATCAAAA
GTGATTAATAATCAAGTGATTAATAATCACTGCAGCAAGACCCTTCCTCTATATAAGGAAGTTC
ATTCATTTGGAGAGGACAGCCCAAGCTTCGACTCTACAACACAACA

Table 3 YAP8-Responsive Element with 8 copies of consensus motif marked in red

8XYAPRE Repeat Variant::

TCTAGCCCATTTGATTAATAATCAAGTGATTAATAATCAAAGTGATTAATAATCAACGTTGATTAAT
AATCAAGTGATTAATAATCAAGTGATTAATAATCAAAAGTGATTAATAATCAAGTGATTAATAATC
ACTGCAGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTGAGAGGACAGCCCAAG
CTTCGACTCTACAACACAACA





Both synthetic fragments were synthesized into a modified pBluescript vector backbone (Figure 1) stripped of internal *BsaI* restriction sites to ensure compatibility with downstream Golden Gate assembly reactions.

Created by SnapGene

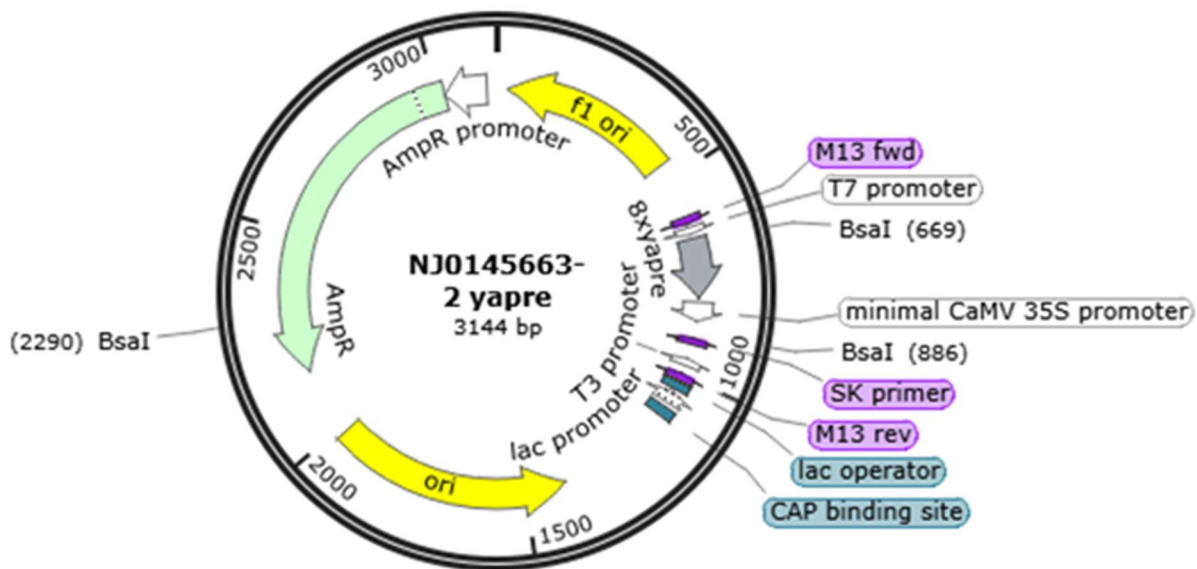


Figure 1. Plasmid (pBluescript vector) map containing 8xYAPRE promoter, the same vector was used to clone the 6xYAPRE element

4.1.2. YAP8 isolation: Codon Adaptation and Translational Compatibility Assessment

To evaluate the feasibility of expressing the *Saccharomyces cerevisiae* YAP8 protein in *Arabidopsis thaliana* without prior gene synthesis codon optimization, an *in silico* codon usage analysis (Table 4) was conducted using plant-specific codon frequency tables (*A. thaliana*, TaxID: 3702). The YAP8 coding sequence (885 bp) yields a Codon Adaptation Index (CAI) of approximately 0.69 relative to the *Arabidopsis* transcriptome, well above the standard threshold (> 0.60) necessary to prevent translational bottlenecks. Furthermore, the overall GC content (36.2%) aligns reasonably well with standard dicotyledonous coding regions, and no high-density clusters of rare codons or functional cryptic polyadenylation signals were identified. Consequently, the native yeast YAP8 sequence demonstrates sufficient translational compatibility to support robust heterologous expression driven by standard plant promoters (e.g., p35S), bypassing the mandatory requirement for codon optimization while preserving wild-type sequence integrity for downstream functional characterization.

Table 4. Silico codon usage analysis. N/A= not analysed

Parameter/Feature	<i>S. cerevisiae</i> YAP8 Value	<i>A. thaliana</i> Optimal Range/Target	Status & Compatibility Impact
Gene Length	885 bp	N/A	Full-length native coding sequence.
Protein Size	294 aa		Acceptable.
Codon Adaptation Index (CAI)	~0.69	> 0.80	Exceeds the minimum threshold (>0.60) required for reliable protein expression without severe translational bottleneck.
GC Content	36.2%	~44.0% (mean CDS)	Compatible. Moderate AT-bias remains within tolerable limits for dicotyledonous mRNA stability.
Rare Codons Frequency	< 3%	< 5%	Low Risk. Absence of rare codon clusters; negligible risk of ribosomal stalling or premature translation termination.
Cryptic Polyadenylation Signals	None detected	Absence required	Low Risk. No canonical plant polyadenylation motifs identified within the coding region.
Overall Expression Feasibility	High (Native CDS)	N/A	Suitable for functional expression under standard constitutive/inducible plant promoters without prior codon optimization.

S. cerevisiae YAP8 harbors a conserved N-terminal basic region leucine zipper (bZIP) domain that acts as an intrinsic Nuclear Localization Signal (NLS). Comparative sequence analysis indicates that this basic domain contains the canonical arginine/lysine-rich motifs recognized by the eukaryotic Importin- α /beta transport machinery. Consequently, the native yeast NLS is fully functional in plant cells, ensuring proper nuclear targeting of YAP8 in *Arabidopsis thaliana* without requiring an exogenous NLS tag.

4.2. Cloning of YAP8

Genomic DNA was extracted from *Saccharomyces cerevisiae* and used as a template for high-fidelity PCR amplification. The YAP8 coding sequence was amplified using Phusion High-Fidelity PCR Master Mix (Thermo Scientific) with the following specific primers:

Yap8_fw: 5'-AACAGGTCTCaGGCTctATGGCAAACCGCGTGGAAG-3'

Yap8_rev: 5'-AACAGGTCTCaCTGATTATAATTTTGACGAAAAGACCTTAATGAAAT-3'



These primers incorporate terminal *BsaI* restriction sites (Type IIS) flanking the YAP8 coding sequence (Figure 2). Upon cleavage, they generate specific 4-bp single-stranded overhangs (5'-GGCT at the 5' end and 5'-AATC at the 3' end) designed for seamless, directional Golden Gate assembly (Greengate standard).

BsaI site (Fw)	4-bp Overhang	4-bp Overhang	BsaI site (Rev)
-----	--	--	-----
5'-AACAGGTCTC A	GGCT CT	ATGGCAAAC...	[YAP8 CDS]...TTTCGTCAAAATTATAA TCA
3'-TTGTCCAGAG T	CCGA GA	TACCGTTTTGG...	[YAP8 CDS]...AAAGCAGTTTAAATATT AGT
			CACTCTGG GACAA -5'
^	^	^	^
Cut Top	Cut Bottom	Cut Bottom	Cut Top

Figure 2 Cloning sites generated by Yap8 primers, *BsaI* restriction site are indicated on top line

The resulting 944 bp amplified PCR product exhibits the following full sequence:

```
5'_AACAGGTCTCAGGCTCtATGGCAAACCGCGTGGAAGAAAAGGCGGCAGGAAG
CCTTCACTTACTCCACCTAAAAATAAGAGAGCTGCGCAACTTAGAGCATCCCAAAA
CGCATTTAGAAAACGAAAGTTGGAAAGATTAGAAGAACTAGAGAAGAAAGAAGCTC
AGCTAACGGTAACAAATGATCAAATTCACATATTAAAGAAGGAAAATGAACTTCTCC
ATTTTATGTTGAGGAGTCTTTTGAAGGAGAACATGCCTTCTGATGAACGGAAC
ATTAGTAAGGCCTGTTGTGAAGAAAAACCGCCCATGTAACACTCTCGATGGTAG
TGTGGTTCTTTTCTCCACATATAATTCCCTTGAAATACAACAATGCTACGTATTTTC
AAGCAACTGCTCTCAGTGTGTGTTGGAAAGAATTGTACAGTGCCGTCACCGTTGAA
TTCCTTTGATCGGTCCTTCTATCCCATAGGCTGTACGAATCTCTCAAATGACATACC
AGGCTATTCTTTTCTAAATGATGCAATGAGTGAGATTACATACGTTTGGTGACTTTAAT
GGGGAAGTTGATAGTACCTTTTTTGGAGTTTAGTGGGACTGAAATAAAGGAGCCAAA
TAATTTCACTTACTGAAAACACTAATGCAATAGAAACAGCGGCAGCGAGCATGGTGAT
TAGGCAGGGTTTCCATCCAAGACAGTATTATACAGTGGATGCTTTTGGAGGGGACG
TCCTATTAAGTGCGATGGATATTTGGAGTTTTATGAAAGTGATCCAAAGGTTAACA
CATTTGACCTTGAAATTTTGGGAACAGAACTTAAGAAAAGTGCTACGTGTTCTAATT
TTGATATTTTAATTAGCCTCAAGCATTTTCAATTAAGGTCTTTTCGTCAAAATTATAATCA
GTGAGACCTGTT_3'
```

This was subsequently subcloned into a PGGC000 vector (Figure 3) for subsequent reactions.

Recombinant plasmids were transformed into competent *Escherichia coli* cells. Transformants were initially screened via colony PCR using construct-specific primers to verify insert integration. Plasmid DNA was subsequently extracted from eight positive colonies using miniprep kits. To confirm sequence fidelity, junction alignment, and the absence of unwanted mutations across the entire construct, all eight isolated plasmids were subjected to full-length plasmid sequencing (Microsynth).



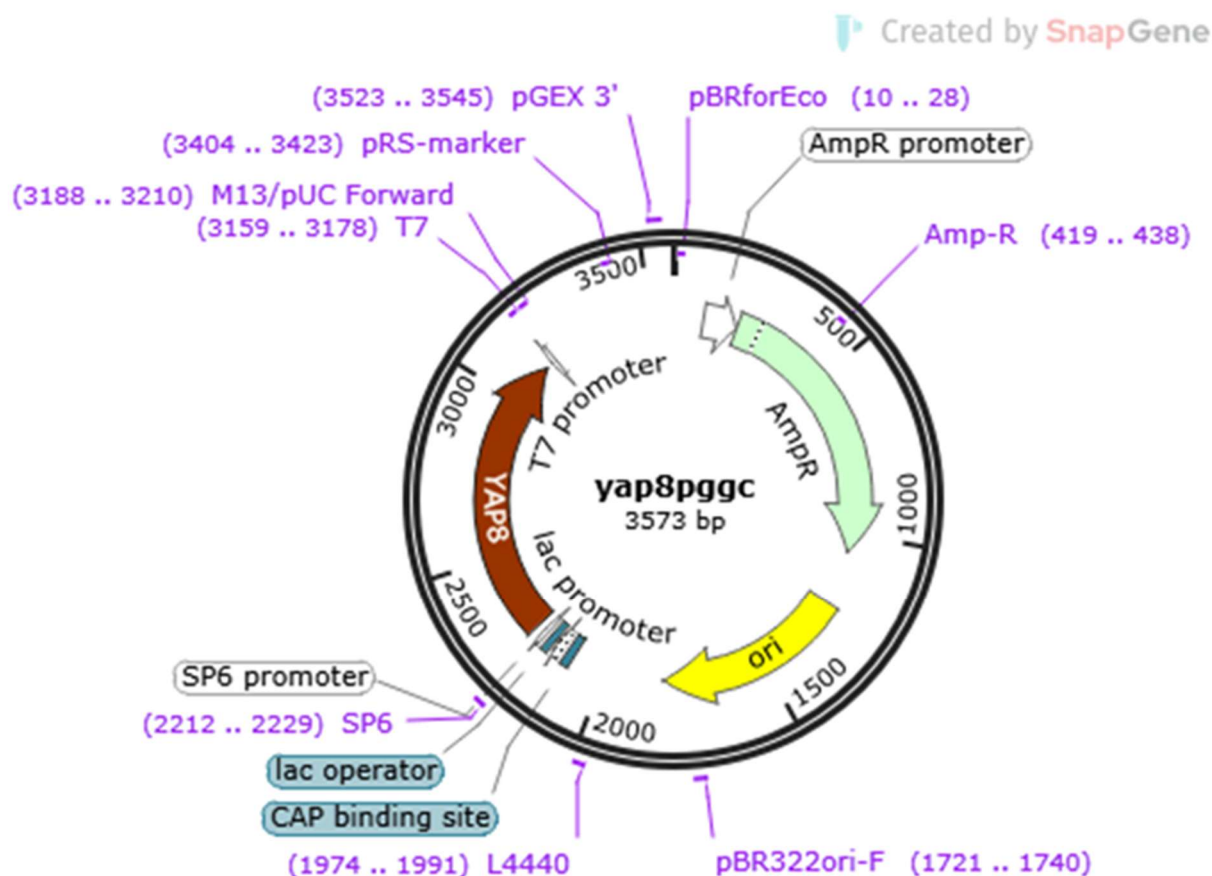


Figure 3. PGCC000 vector map containing the YAP8 gene

4.3. Cloning of RUBY: Visual Reporter System Selection

For visual monitoring of YAP8 activity in response to As contamination, the RUBY reporter system was selected as the optimal candidate. We consider RUBY more suitable than EsMY80 as reporter because it has been proven to be highly active in plants and to give a more robust red coloration. RUBY encodes a polycistronic cassette containing three biosynthetic enzymes derived from *Beta vulgaris* (beetroot)—CYP76AD1, DOPA 5-O-glucosyltransferase, and L-DOPA cytochrome P450 oxidase—which converts endogenous L-tyrosine into betalain, the vivid red pigment characteristic of beetroot. The CDS are separated by sequences for 2A self-cleaving peptides.

Unlike traditional reporters requiring exogenously supplied substrates (e.g., GUS assay) or specialized excitation hardware (e.g., GFP/RFP fluorophores), the RUBY system drives real-time, non-invasive visual detection under ambient light without compromising plant viability or requiring co-factor administration. The RUBY polycistronic reporter cassette was amplified using the plasmid *p35S::RUBY* (Addgene plasmid #160908) as a template.



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4.3.1. Primer Design & *Bsa*I Site Incorporation

Specific primers (listed below) were designed to append terminal *Bsa*I restriction sites (Type IIS) and standard Green Gate 4-bp overhangs flanking the full coding sequence:

ruby_fw_pggc: 5'-AACAGGTCTCAGGCTCTATGGATCATGCGACCCTCG-3'

(Contains a *Bsa*I recognition site, a 1-bp spacer, a 5'-GGCT 4-bp N-terminal overhang, and 20 bp annealing to the 5' end of RUBY starting at the ATG codon).

ruby_rev_pggc: 5'-AACAGGTCTCACTGATCACTGGAGGCTTGGCTC-3'

(Contains a *Bsa*I recognition site in reverse orientation, a 1-bp spacer, a 5'-AATC C-terminal overhang [rc: 5'-GATT], and annealing sequence to the 3' end of the cassette including the TGA stop codon).

Amplicon Sequence was evaluated with High-fidelity PCR amplification and yielded the following 3,966 bp DNA product:

```
5'_AACAGGTCTCAGGCTCTATGGATCATGCGACCCTCGCCATGATCCTCGCGATCTGGTTCAT
CAGCTTCCACTTCATCAAGCTGCTGTTCTCCAGCAGACCACCAAGCTGCTTCCGCCAGGAC
CAAAGCCGCTTCCGATCATCGGCAACATCCTTGAGGTGGGCAAGAAGCCGCATCGGTCTTC
GCCAACCTCGCCAAGATTCACGGCCCACTCATTTCCCTCAGACTCGGCTCTGTGACCACCATC
GTTGTGTCCTCTGCCGACGTGGCCAAAGAGATGTTCTCAAGAAGGATCACCCGCTCTCCAA
CCGCACGATCCCGAATAGTGTTACAGCCGGCGACCACCACAAGCTCACCATGTCTTGGCTCC
CGGTGTCTCCGAAGTGGCGCAACTTCCGCAAGATTACCGCCGTGCATCTGCTCTCCCCACAG
AGACTCGATGCCTGCCAGACATTCAGGCACGCCAAGGTGCAGCAGCTCTACGAGTACGTTCA
AGAGTGCGCCCAGAAAGGCCAGGCCGTGGATATTGGCAAGGCCGCCTTTACGACCAGCCTCA
ACCTCCTCAGCAAGCTGTTCTTCAGCGTCGAGCTGGCGCACCACAAGTCCCATACCAGCCAA
GAGTTCAAAGAGCTGATCTGGAACATCATGGAAGATATAGGCAAGCCGAAGTACGCCGACTAC
TTCCCGATTCTCGGCTGCGTTGACCCATCTGGCATTAGAAGAAGGCTCGCCTGCTCCTTCGAC
AAGCTGATCGCCGTGTTCCAGGGCATCATCTGCGAGAGACTCGCCCCAGATTCTCCACCAC
AACTACCACCACCACCGACGACGTGCTCGATGTGCTCCTCCAGCTGTTCAAGCAGAACGAGC
TGACGATGGGCGAGATCAACCACCTCCTCGTGGACATCTTCGACGCCGGCACCGATACCACA
TCCTCCACATTTCGAGTGGGTGATGACCGAGCTGATCCGCAATCCAGAGATGATGGAAAAGGCC
CAAGAGGAAATCAAGCAGGTCTCGGCAAGGACAAGCAGATCCAAGAGTCCGACATCATCAA
CCTGCCGTACCTCCAGGCGATCATCAAAGAGACACTCCGCCTCCATCCGCCGACCGTGTTCTT
GCTCCCAAGAAAGGCCGACACCGATGTGAGCTGTACGGCTACATCGTGCCGAAGGATGCC
AGATCCTCGTGAACCTCTGGGCCATTGGCAGGGACCCAAACGCCTGGCAGAACGCCGATATT
TTCAGCCCAGAGCGCTTCATCGGCTGCGAGATCGATGTTAAGGGCCGCGATTTTCGGCCTCCTT
CCATTTGGCGCTGGCCGCAGAATTTGCCAGGCATGAATCTCGCCATCAGGATGCTCACCCCTC
ATGCTCGCCACACTCCTCCAGTTCTTCAACTGGAAGCTCGAAGGCGACATCTCCCCGAAGGA
CCTCGACATGGACGAGAAGTTCGGCATTGCGCTCCAAAAGACCAAGCCGCTCAAGCTCATCC
CGATTCCGCGCTACGGTAGCGGAGCTACCAATTTTAGCCTCCTTAAGCAGGCAGGTGATGTAG
AAGAGAACCCCGGGCCTATGAAGATGATGAACGGCGAGGACGCCAACGACCAGATGATCAAA
GAGTCCTTCTTCATACCCACGGCAACCCGATCCTCACCGTCGAGGATACACATCCGCTCAGG
CCGTTCTTCGAGACATGGCGCGAGAAGATTTTCTCCAAGAAGCCGAAGGCCATCCTCATCATC
TCCGGCCACTGGGAGACAGTGAAGCCAACCGTGAACGCCGTGCACATCAACGACACCATCCA
CGACTTCGACGACTACCCAGCCGCCATGTACCAGTTCAAGTACCCAGCTCCAGGCGAGCCAG
AGCTTGCGAGAAAGGTGGAAGAGATCCTCAAGAAGTCCGGGTTTCGAGACAGCCGAGACAGA
CCAAAAGAGGGGCTTGATCACGGCGCCTGGGTTTCACTCATGCTCATGTATCCAGAGGCGG
ACATCCCGGTGTGCCAGCTCTCAGTTCAGCCACATCTCGACGGCACCTACCACTACAATCTCG
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GCAGAGCCCTCGCGCCGCTCAAGAATGATGGCGTGCTCATTATTGGCTCCGGCAGCGCCACA
CATCCACTCGATGAGACACCGCACTACTTCGATGGTGTGGCCCTTGGGCGGCTGCCTTCGAT
TCTTGGCTTAGGAAGGCCCTCATCAACGGCCGCTTCGAGGAAGTGAACATCTACGAGAGCAA
GGCCCCGAACTGGAAGCTCGCCCATCCATTTCCAGAGCACTTCTACCCGCTCCACGTTGTGC
TCGGCGCTGCTGGTGAAGAGTGAAGGGCCGAGCTGATCCACTCCTCCTGGGATCATGGCACA
CTTTGCCACGGCTCCTACAAGTTCACCTCCGCGGATCCGGAGCAACCAACTTTAGCCTGCTC
AAGCAAGCAGGAGATGTTGAGGAAAATCCTGGCCCCATGACCGCCATCAAGATGAACACCAAC
GGCGAGGGGCGAGACACAGCACATCCTCATGATCCCGTTCATGGCGCAGGGCCACCTCAGGC
CATTTCTCGAACTCGCCATGTTCTCTACAAGCGCTCCCACGTGATCATCACCTGCTCACAAC
TCCGCTCAATGCCGGCTTCTCAGGCACCTCCTTACCACCATTCTACTCCTCCAGCGGCAT
CAGGATCGTCGAGCTGCCATTCAACTCCACCAACCACGGACTCCCACCGGGCATCGAGAACA
CCGATAAGCTCACACTCCCGCTCGTGGTGTCCCTCTTCCATTCCACCATCAGCCTCGATCCGC
ACCTCCGCGATTACATCTCCAGGCATTTAGCCAGCCAGGCCACCACTCTGCGTGATCCATG
ATGTGTTCTCGGCTGGGTTGACCAGGTGGCCAAGGATGTGGGCTCTACAGGCGTGTTGTTT
ACAACAGGCGGCGCTTATGGCACATCCGCCTACGTGTCCATCTGGAACGATCTCCCGCACCA
GAACTACTCCGACGACCAAGAGTTCCCGCTGCCAGGCTTCCAGAGAACCATAAGTTCCGCA
GGTCCAGCTCCATCGGTTCTCAGATATGCCGACGGCTCCGACGATTGGTCCAAGTATTTCC
AGCCGCAGCTCCGCCAGTCCATGAAGTCTTTTGGCTGGCTCTGCAACTCCGTGGAAGAGATC
GAGACACTCGGCTTCTCCATCCTCCGCAACTACACCAAGCTGCCGATCTGGGGCATCGGCCC
ACTTATTGCTTCCCCAGTGCAGCACTCCTCCTCCGACAACAATTCAACAGGCGCCGAGTTCTGT
GCAGTGGCTCAGCCTCAAAGAGCCGGACTCCGTCTCTACATCTCCTTCGGCTCCGAGAACA
CGATCAGCCCGACGCAGATGATGGAAGTGGCTGCTGGCCTTGAGTCTCCGAGAAGCCATTC
CTCTGGGTGATCAGAGCCCCGTTTCGGCTTCGACATCAACGAAGAGATGCGCCCAGAGTGGCT
GCCAGAGGGCTTTGAGGAACGCATGAAGGTGAAGAAACAGGGCAAGCTCGTGTACAAGCTCG
GCCCGCAGCTTGAGATCCTCAACCATGAATCCATCGGCGGCTTCTCACCCACTGCGGATGGA
ACAGCATCCTTGAGTCTCTTCGCGAGGGCGTTCCGATGCTTGATGGCCACTTGCTGCCGAG
CAGGCCTACAACCTCAAGTACCTCGAGATGAGATGGGCGTCGCGGTTGAGCTTGCTAGAGGC
CTCGAAGGCGAGATCTCAAAGAGAAGGTCAAGCGCATCGTCGAGATGATCCTTGAGCGCAA
CGAGGGCTCAAAGGCTGGGAGATGAAGAATCGCGCCGTGGAATGGGCAAAAAGCTCAAG
GACGCCGTGAACGAGGAAAAAGAGCTGAAGGGCTCCTCCGTGAAGGCGATCGACGATTTCTT
CGACGCCGTCATGCAGGCCAACTTGAGCCAAGCCTCCAGTGATCAGTGAGACCCTGTT_3'
```

This amplicon was subsequently cloned into a PGEC000 vector for Greengate reaction (Figure 4). Resulting plasmid containing RUBY system were transformed into competent *Escherichia coli* cells. Transformants were initially screened via colony PCR using construct-specific primers to verify insert integration. Plasmid DNA was subsequently extracted from eight positive colonies using miniprep kits. To confirm sequence fidelity, junction alignment, and the absence of unwanted mutations across the entire construct, all eight isolated plasmids were subjected to full-length plasmid sequencing (*Microsynth*). All analyzed colonies showed no mutations and insertion of the fragment.



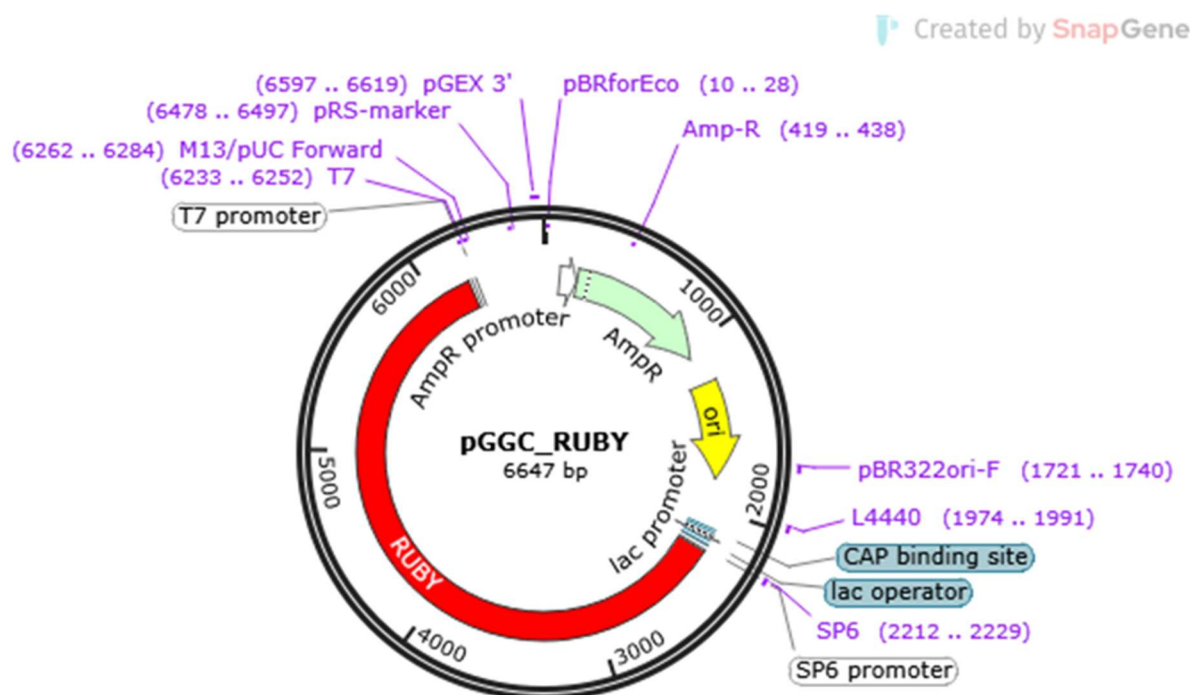


Figure 4. Plasmid map containing RUBY system

4.3.2. Assembly Strategy & GreenGate Cloning System of the 35S::YAP8::TER–YAP8RE::RUBY::TER–NPTR Supermodule

To construct the final multigene expression vector (35S::YAP8::TER–YAP8RE::RUBY::TER–NPTR), we employed the GreenGate modular cloning system¹³ (Addgene Kit #1000000036). The assembly was executed in a two-step hierarchical strategy: first generating two intermediate modules (pGGM and pGGN destination vectors) via standard Golden Gate cut-and-ligation reactions, followed by final supermodule assembly.

i. Intermediate modules

Simultaneous digestion and ligation cycles were conducted using *Bsa*I-HF v2 and T4 DNA Ligase with ~200 ng total plasmid input per reaction, according to the standard GreenGate protocol:

¹³ Lampropoulos A, Sutikovic Z, Wenzl C, Maegele I, Lohmann JU, Forner J. GreenGate - A novel, versatile, and efficient cloning system for plant transgenesis. PLOS ONE. (2013) 8: 12 doi.org/10.1371/journal.pone.0083043



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The intermediate Vector 1 (*pGGM-35S::YAP8::UBQ10ter*, Figure 5) was cloned into the pGGM intermediate destination vector using the following module configuration:

Slot A (pGGA): 2x35S Constitutive Promoter
 Slot B (pGGB): Dummy B adapter
 Slot C (pGGC): YAP8 Coding Sequence (CDS)
 Slot D (pGGD): Dummy D adapter
 Slot E (pGGE): UBQ10 Terminator (*AtUBQ10ter*)
 Slot F (pGGF): Neutral Linker / Adapter

The intermediate Vector 2 (*pGGN-YAP8RE::RUBY::RBCSter::NPTR*, Figure 6) was cloned into the pGGN intermediate destination vector using the following module configuration:

Slot A (pGGA): YAP8 Responsive Element (YAP8RE) Promoter
 Slot B (pGGB): Dummy B adapter
 Slot C (pGGC): RUBY Reporter CDS
 Slot D (pGGD): Dummy D adapter
 Slot E (pGGE): RBCS Terminator (*AtRBCSter*)
 Slot F (pGGF): NPT Resistance Cassette (*pNOS::NPT::HSPter*)

ii. Transformation, Selection, and Full-Length Sequencing Validation

Recombinant plasmids were transformed into chemically competent *Escherichia coli* cells (DH5α). Initial screening of transformants was carried out via colony PCR using module-junction-specific primers to verify correct insert integration.

Plasmid DNA was subsequently extracted from eight PCR-positive colonies per construct using a standard silica-column miniprep kit (Thermo Scientific). To confirm absolute sequence fidelity, scarless junction alignment, and the complete absence of point mutations or indels across the multi-cassette assemblies, all eight isolated plasmids were submitted for full-length plasmid sequencing (Microsynth Whole Plasmid Sequencing). Alignment analysis confirmed 100% sequence identity across all analyzed clones with no detectable mutations, validating the correct assembly of both intermediate vectors.

To construct the final multigene expression vector (*35S::YAP8::UBQ10ter–YAP8RE::RUBY::RBCSter–NPTR*), we employed the GreenGate modular cloning system (Lampropoulos et al., 2013; Addgene Kit #1000000036). The cloning strategy was executed in a two-step hierarchical workflow: a. Assembly of two intermediate modules into pGGM and pGGN destination vectors via Type IIS restriction/ligation; b. Supermodule assembly combining both intermediate cassettes into the final binary vector.



Created by SnapGene

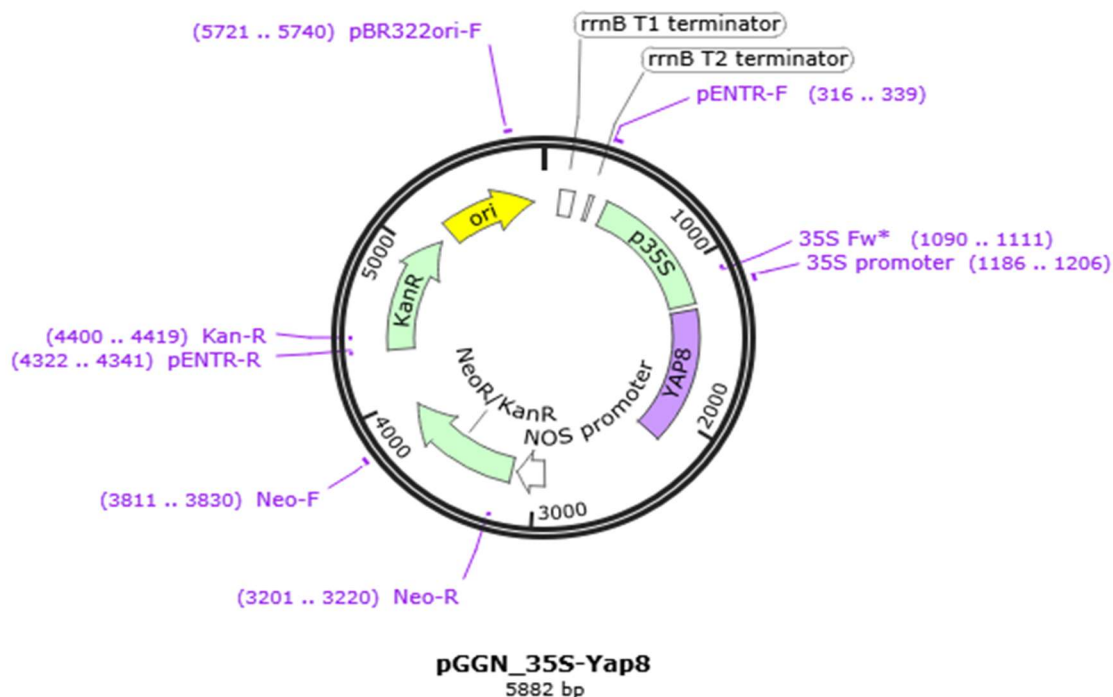


Figure 6. pGGN-YAP8RE::RUBY::RBCSter::NPTR plasmid map

Created by SnapGene

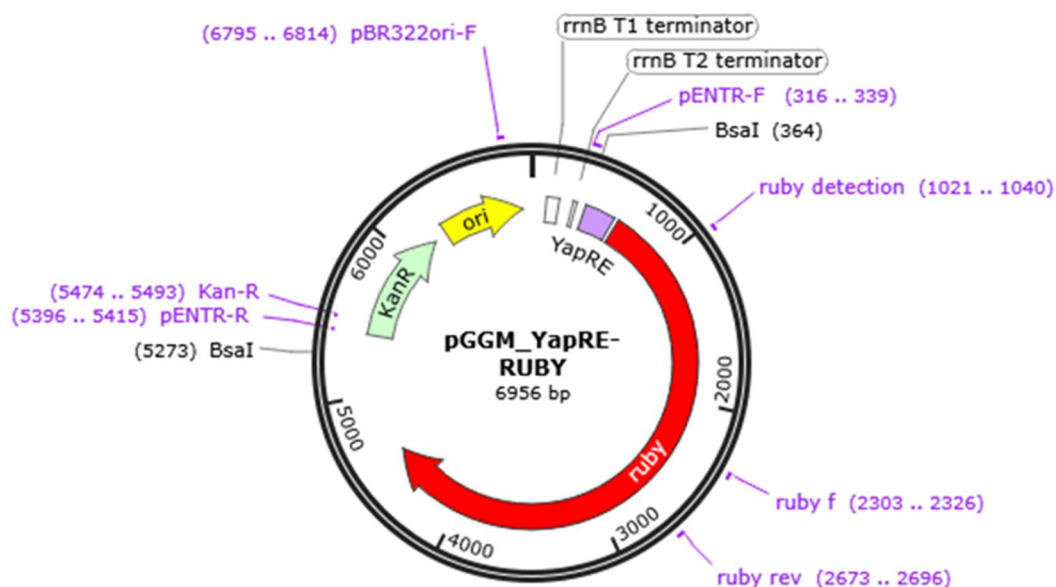


Figure 5. pGGM-35S::YAP8::UBQ10ter plasmid map



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Step a. Assembly of Intermediate Vectors (pGGM and pGGN) Reaction mixes contained ~200 ng total plasmid input per reaction, using BsaI-HF v2 and T4 DNA Ligase over 30 cycles of digestion/ligation (Figure 7A).

Step b. Supermodule Assembly. Equal molar ratios (~100 ng each) of purified pGGM-35S::YAP8::UBQ10ter and pGGN-YAP8RE::RUBY::RBCSter::NPTR intermediate plasmids were combined with the pGGS binary super-destination vector. A final GreenGate cut-and-ligation reaction using BsaI-HF v2 and T4 DNA Ligase drove the directional fusion of both transcription units and the plant selection marker into a single binary T-DNA backbone. Validation, selection, and sequence verification of recombinant plasmids were transformed into chemically competent *Escherichia coli* cells. Initial screening of transformants was performed via colony PCR using junction-spanning primers targeting the 35S–YAP8 and YAP8RE–RUBY boundaries. Plasmid DNA was isolated from eight PCR-positive colonies per construct using standard silica-column miniprep kits (Thermo Scientific). To guarantee complete sequence fidelity, precise junction alignments, and the absence of point mutations across the entire multigene construct, all isolated plasmids were validated by full-length plasmid sequencing (Microsynth Whole Plasmid Sequencing). Sequence alignment confirmed 100% sequence identity across all analyzed clones with no detectable mutations or indels, validating the structural integrity of the final supermodule vector for *Agrobacterium tumefaciens* (Strain GV3101)-mediated plant transformation (Figure 7).

A)

pGGA (35S) + pGGB (Dummy) + pGGC (YAP8) + pGGD (Dummy) + pGGE (UBQ10ter) + pGGF (Adapter)

↓ (BsaI Cut / Ligation)

pGGM-35S::YAP8::UBQ10ter

[Intermediate Assembly 2]

pGGA (YAP8RE) + pGGB (Dummy) + pGGC (RUBY) + pGGD (Dummy) + pGGE (RBCSter) + pGGF (BASTAR)

↓ (BsaI Cut / Ligation)

pGGN-YAP8RE::RUBY::RBCSter::BASTAR

↓ (BsaI Cut / Ligation into pGGS Super-destination Vector)



B)

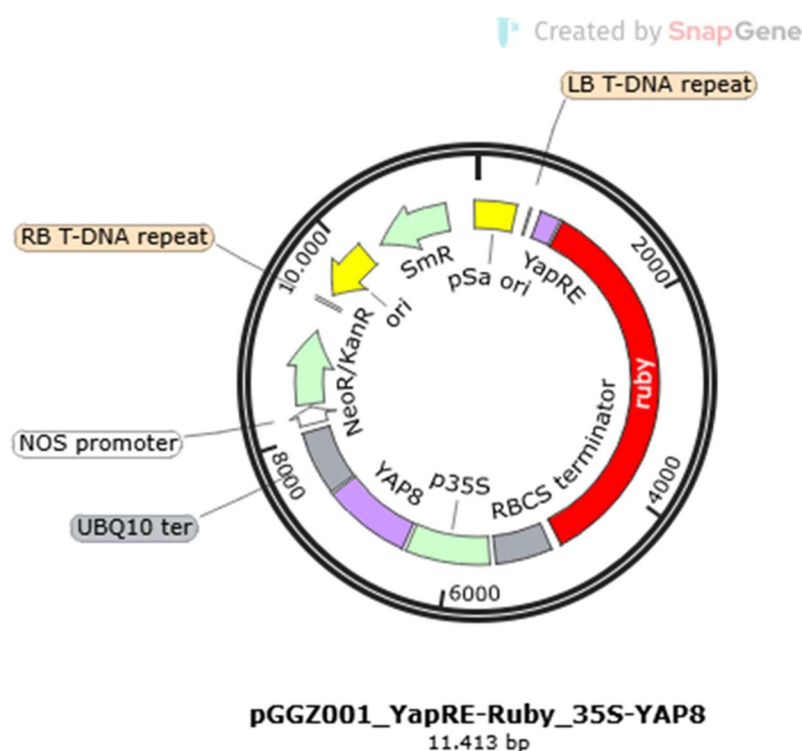


Figure 7. A) Scheme reporting the step for obtaining the final plasmid B)Final plasmid map containing the Arsenic sensor

4.4. Plant transformation

4.4.1. Scientific Rationale & Host Background Optimization

To establish a sensitive, high-performing plant-based biosensor for environmental As detection, choosing an optimal *Arabidopsis thaliana* genetic background is critical. The ideal host line must exhibit enhanced physiological tolerance to As contamination while maintaining robust root meristem activity and cellular accumulation to drive the synthetic YAP8 signaling cascade. Evaluation of Cytokinin Signaling Mutants (*arr1 arr12*). We initially evaluated cytokinin loss-of-function mutants, specifically the double mutant *arr1 arr12*, which has been reported to confer tolerance to As toxicity¹⁴.

¹⁴ Zhang, P., Liu, F., Abdelrahman, M., Song, Q., Wu, F., Li, R., Wu, M., Herrera-Estrella, L., Tran, L.-S.P. and Xu, J. ARR1 and ARR12 modulate arsenite toxicity responses in Arabidopsis roots by transcriptionally controlling the actions of NIP1;1 and NIP6;1. Plant J, (2024) 120: 1536-1551.



However, internal investigations conducted within MOBILES project revealed a key physiological limitation: *arr1 arr12* plants exhibit reduced intracellular As accumulation. Mechanistically, ARR1 and ARR12 directly promote the expression of the plasma membrane H⁺-ATPase proton pumps AHA1 and AHA2 in root cells¹⁵. Proton extrusion by AHA pumps acidifies the rhizosphere—a phenomenon demonstrated by Bromocresol Purple pH indicator assays, where wild-type (Col-0) and AHA1-overexpressing lines (*J2571-UAS::AHA1* and *J2921-UAS::AHA1*) drive strong rhizosphere acidification (yellow color transition), whereas *aha1-8* loss-of-function mutants exhibit significantly reduced proton export (purplish color; Figure 9). Figure 8 also shows the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) quantification of total As content in roots of wild-type (Col-0), *aha1-8*, and *aha2* loss-of-function mutant seedlings exposed to 100 μ M As contamination.

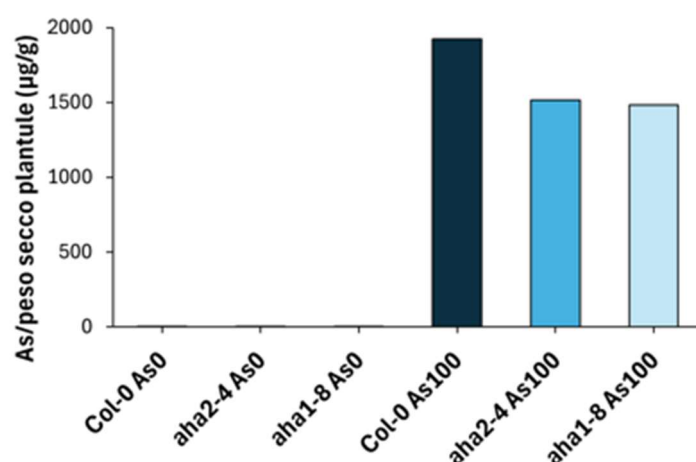


Figure 8. Alteration of AHA H⁺-ATPase expression modulates rhizosphere pH.

Loss of AHA1 or AHA2 proton pump activity leads to a significant decrease in intracellular As accumulation compared to wild-type control plants, confirming the requirement of H⁺-ATPase-mediated rhizosphere acidification for efficient heavy metal uptake. Rhizosphere acidification enhances the solubility and cellular import of arsenite [As(III)] and arsenate [As(V)]. Bromocresol Purple acidification assay illustrates rhizosphere pH variation mediated by *Arabidopsis thaliana* roots (Figure 9). Wild-type (Col-0) seedlings are compared alongside *aha1-8* loss-of-function mutants and AHA1 overexpressing lines (*J2571-UAS::AHA1* and *J2921-UAS::AHA1*). Bromocresol Purple functions as a pH indicator, displaying a purplish color under alkaline/neutral conditions and

¹⁵ Pacifici, E., Di Mambro, R., Dello Iorio, R., Sabatini S. Acidic cell elongation drives cell differentiation in the Arabidopsis root. EMBO J 37, (2018) EMBJ201899134.



transitioning to yellow under acidic conditions driven by root H^+ extrusion (Figure 8). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) quantitative analysis confirmed that loss-of-function mutants for these proton pumps (*aha1* and *aha2*) display lower internal As levels (Figure 9), explaining the reduced accumulation phenotype observed in *arr1 arr12*. Consequently, impairing uptake mechanisms makes these backgrounds suboptimal for a biosensor reliant on intracellular sensing.

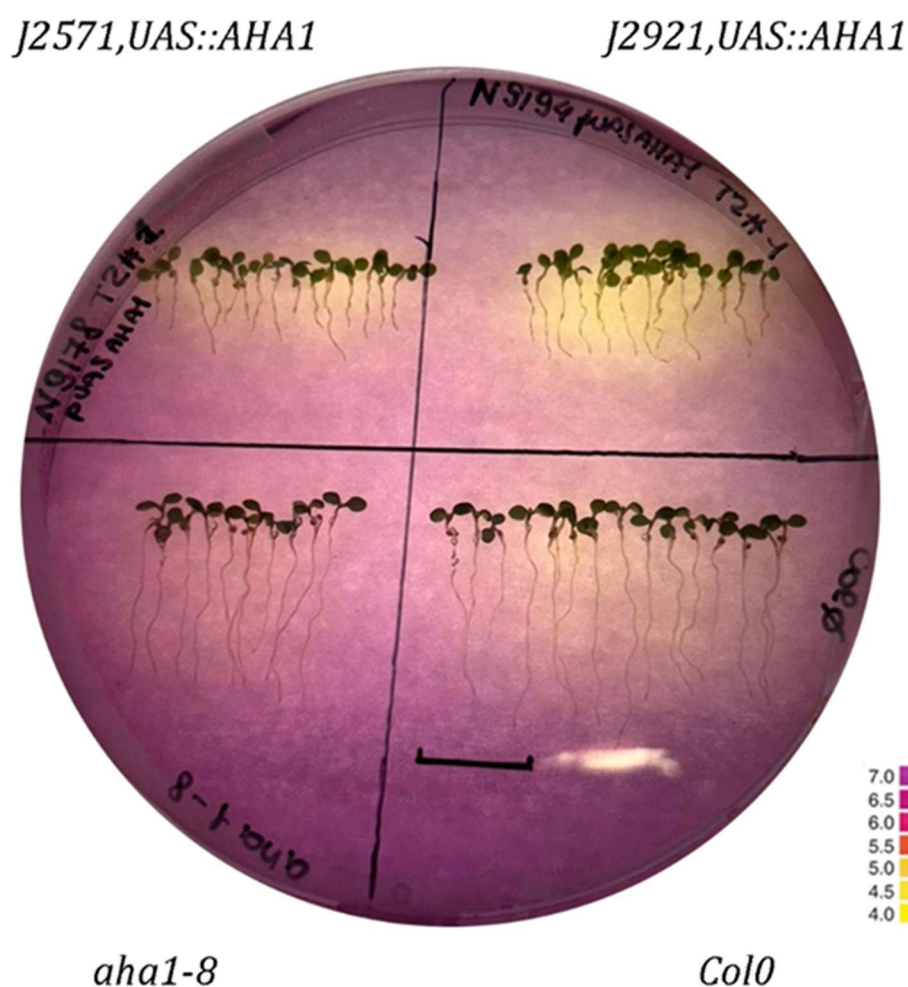


Figure 9 :As quantification in *aha1* and 2 mutants

4.4.2. Targeting Cell Differentiation downstream of Cytokinin (*expa1* and *expa10*)

To decouple As tolerance from reduced uptake, we focused downstream of the ARR1/ARR12 pathway. In the root transition zone (Figure 10), ARR1 and ARR12 drive cell differentiation by promoting the expression of cell-wall-loosening proteins, specifically *EXPANSIN A1* (*EXPA1*) and *EXPANSIN A10* (*EXPA10*)¹⁶. We hypothesized that targeting *expa* loss-of-function mutants could mitigate As-induced root growth arrest without compromising metal uptake. We analyzed root apical meristem (RAM) activity and primary root length under toxic As exposure (Figure 11). Remarkably, *expa1* single mutants demonstrated significant tolerance to As toxicity while maintaining normal growth and cellular uptake dynamics.

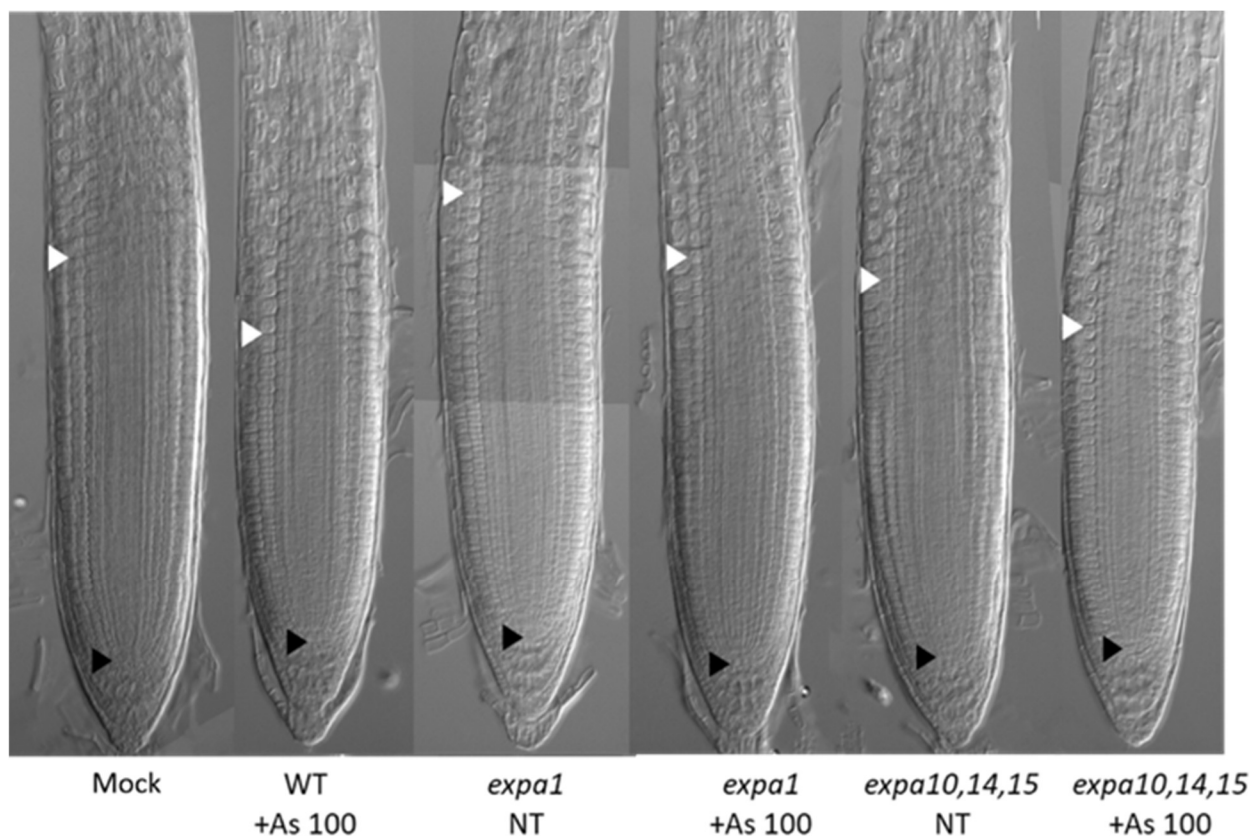


Figure 8. Representative confocal laser scanning microscopy images of primary root apical meristems from 8-day-post-germination (dpg) wild-type (Col-0), *expa1*, and triple *expa10 expa14 expa15* mutant seedlings grown under control conditions or exposed to 100 μ M As contamination. Black arrowheads mark the stem cell niche (SCN); white arrowheads delineate the transition zone (TZ) boundary.

¹⁶ Pacifici, E., Di Mambro, R., Dello Ioio, R., Sabatini S. Acidic cell elongation drives cell differentiation in the Arabidopsis root. EMBO J 37, (2018) EMBJ201899134.

Based on these findings, the *expa1* loss-of-function mutant was selected as the optimal host background for *Agrobacterium*-mediated transformation of our 35S::*YAP8::UBQ10ter-YAP8RE::RUBY::TER-NPTR* supermodule construct.

Seeds treated with As at germination

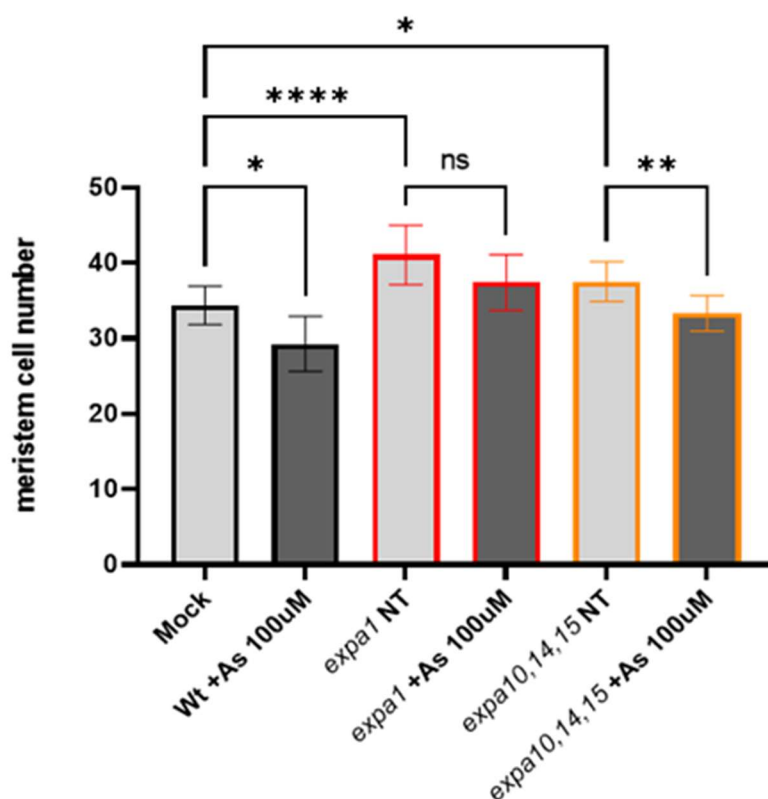


Figure 9. Quantitative analysis of root meristem size, expressed as cortex meristematic cell number in the cortical layer from the stem cell niche to the TZ (a proxy for meristematic activity and arsenic tolerance) in the genotypes shown in figure 10. Data represent mean $\pm$ SD ($N=3$ independent biological replicates, $n=20$ roots per genotype/treatment). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p<0.001$). Asterisks denote significant differences (* $p<0.05$, ** $p<0.01$, ns = non-significant).

4.5. Generation and Functional Validation of the Plant-Based Arsenic Biosensor

4.5.1. Agrobacterium-Mediated Transformation and Line Selection

To generate the plant-based As biosensor, Wt and *expa1* loss-of-function mutants were transformed with the *35S::YAP8::UBQ10ter–YAP8RE::RUBY::RBCSter–NPTII* supermodule construct via *Agrobacterium tumefaciens*-mediated floral dip transformation (strain GV3101). Primary transformants (T1) were selected on media containing kanamycin. A total of 12 independent T1 transgenic lines for each genotype were successfully identified. Genomic DNA was extracted from T1 leaves and screened via PCR using construct-specific primers targeting the RUBY reporter cassette and the YAP8 gene to confirm transgene integration. Three independent lines exhibiting robust PCR verification and mendelian segregation patterns were selected and propagated to subsequent generations (T2 and T3).

4.5.2. In Planta Functional Validation of Arsenic Sensing

To test the operational responsiveness of the biosensor, T2/T3 transgenic lines (*35S::YAP8::UBQ10ter–YAP8RE::RUBY::RBCSter–NPTII*) were grown on growth media supplemented with 100 μ M As (arsenate). Under control conditions, both untransformed *expa1* control plants and transgenic biosensor lines maintained a standard green phenotype. Upon exposure to 100 μ M As, untransformed control plants showed no induction of stress-induced pigmentation and remained green. In stark contrast, transgenic lines containing the YAP8-driven biosensor turned distinctly red due to the activation of the YAP8RE-promoter driving betalain accumulation (Figure 12).

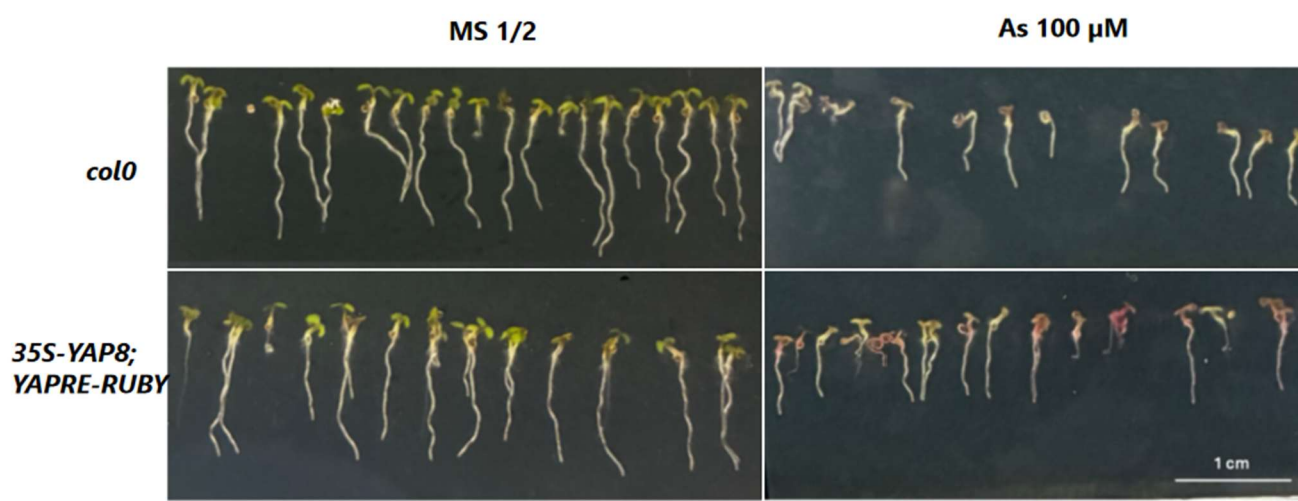


Figure 10. Plant harboring the *35S::YAP8-YAPRE::RUBY* construct acquire red color in presence of Arsenic. 10 days post germination seedling of Wt plants (*Col0*, upper panel) and *35S::YAP8-YAPRE::RUBY* T2 plants (bottom panel) exposed to 100 μ M of Arsenic (Arsenate)



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5. Task 2.2: Generation of conditional unfertile mutants of *Arabidopsis* As-indicator

The primary objective of task 2.2 was to generate conditionally sterile *Arabidopsis thaliana* mutants to enable the safe field cultivation of As biosensors while preventing environmental persistence or gene flow. To achieve this, we developed genetic constructs leveraging the Tissue-Specific Knockout (TSKO) framework, placing the Cas9 gene under the control of a heat-shock (HS) inducible promoter. Gene disruption was targeted to PIN1 using two single-guide RNAs (sgRNAs).

5.1. Generation of *pHs::CAS9-U6::2sgPIN1* plants with fluorescent seeds.

To obtain an inducible promoter, the *pFAST-GK_pHs::CAS9-U6::2sgPIN1* vector, two 20-nucleotide guide RNAs were amplified using specific primers designed with CCTop (<https://cctop.cos.uni-heidelberg.de/>) and ChopChop (<https://chopchop.cbu.uib.no/>) (Figure 13). The sgRNAs with the lowest predicted off-target effects were selected. The two sgRNAs were designed to cut the PIN1 gene 61 bp apart in order to generate a deletion into the gene.

```
ChopChop: GC 45%, Strand +, Efficiency 64,04, Rank 5
CCTop: Efficacy score 0,76 (High), Rank T2
ATGGGGATACCTCTTCTCAAAGG
Oligo pair fwd: ATTGATGGGGATACCTCTTCTCAA rev: AAAGTTGAGAAGAGGTATCCCCAT

ChopChop: GC 60%, Strand -, Efficiency 54,54
CCTop: Efficacy score 0,72 (Medium), Rank T3
TGCGGCTGAGTTTGCACCAGAGG
Oligo pair fwd: ATTGTGCGGCTGAGTTTGCACCAG rev: AAACCTGGTGCAAACTCAGCCGCA
```

Figure 11. Guide RNAs for the inducible promoter

The pEN-2xAtU6_template vector was used as a template for a subsequent PCR reaction with the two sgRNAs, generating a 575 bp amplicon. The PCR product was run on an agarose gel, and the corresponding band was excised and purified using a PCR purification kit (Geneaid, PD300). The sgRNA vector pGG-F-AtU6-26-AarI-AarI-G_2xgRNA was digested with the *AarI* restriction enzyme (Thermo Scientific, ER1581), and the digestion product was purified using the PCR purification kit (Geneaid, PD300). The purified fragments were then subjected to a digestion–ligation reaction with the pEN-2xAtU6_2sgPIN1 vector using BsaI-HF (NEB, R3733L) and T4 DNA ligase (NEB, M0202L). As destination vector the pFAST-GK has been chosen because it carries Fluorescence-Accumulating-Seed Technology with OLE1-GFP.

Following transformation into *Escherichia coli* DH5α competent cells and selection on LB medium containing ampicillin, colonies yielding a 670 bp amplification product using specific primers (pFAST-GK Fw and HS Rev) were selected, purified, and the full plasmid was verified by Sanger sequencing.



The pGG-F-AtU6-26-AarI-AarI-G_2sgRNA_PIN1 construct was then subcloned into the destination vector pFAST-GK together with pGG-A-pHs-B (providing the heat shock promoter), pGG-B003 (providing a default dummy GreenGate N-terminal tag module), pGG-D-P2A-GFP-NLS-E (providing GFP and a nuclear localization signal at the N-terminus), and pGG-E-NOST-F (providing a NOST terminator), to generate the *pFAST-GK_pHs::CAS9-U6::2sgPIN1* construct (Figure 14). After transformation of *E. coli* DH5α competent cells with the Golden Gate reaction and selection on LB medium containing spectinomycin, colonies were purified and validated by PCR (Table 5) and Sanger sequencing. Finally, the confirmed construct was used to transform *Agrobacterium tumefaciens* strain GV3031 and subsequently introduced into plants.

Table 5. PCR primers used to generate gRNA for PIN1

Primer name	Primer Sequence
PIN1 Fw sgRNA1	ATTGGACTTTCTGGAGAGAATCTG
PIN1 Rew sgRNA1	AAACCAGATTCTCTCCAGAAAGTC
PIN1 Fw sgRNA2	ATTGTCCAATCTAAAGAACCGTTG
PIN1 Rew sgRNA2	AAACCAACGGTTCTTTAGATTGGA

We selected 12 T1 independent lines and we analyzed zygosity and brought 3 of them to homozygosity. We exposed imbibed seeds to 40°C and then we placed them on soil. We grew them for 4 weeks and we found the typical pin1 phenotype in approximately 98% of plants (Figure 15).

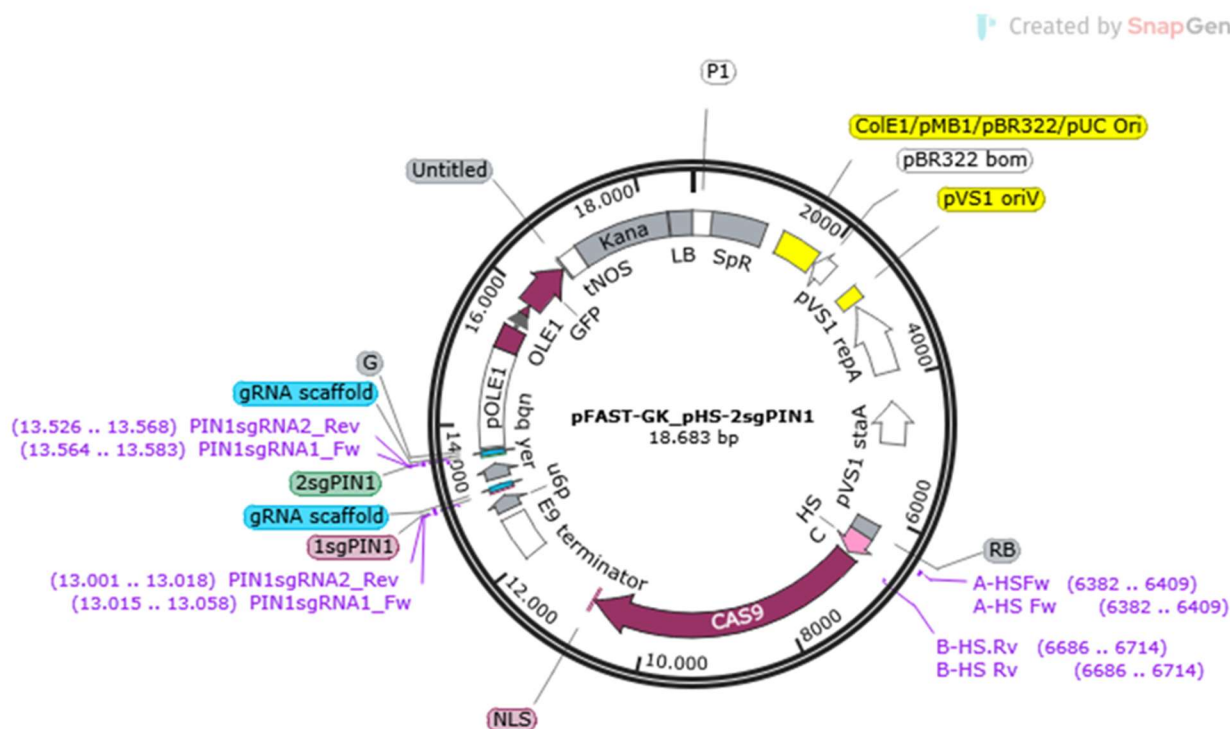


Figure 12. Final vector of *pFAST-GK_pHs::CAS9-U6::2sgPIN1* for conditional sterility.

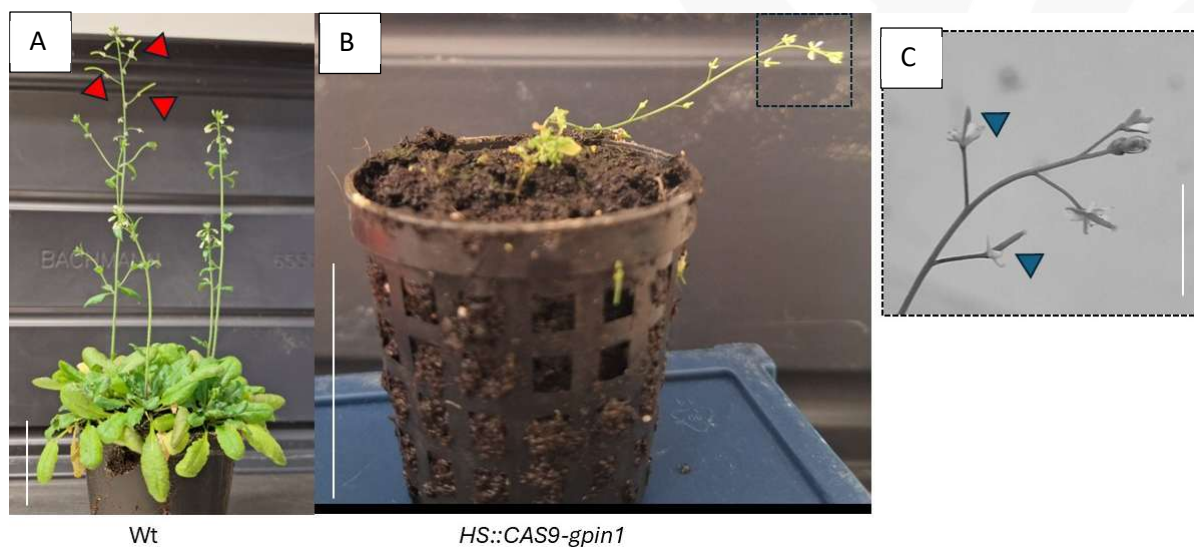


Figure 15: A) Arabidopsis Wt plant of 8 weeks. Red arrowheads indicates fertile siliques. Scalebar 5 cm. B) An Arabidopsis plant harbouring the pHs::CAS9-U6::2sgPIN1 construct after heat shock exposure. Scalebar 5 cm. C) Blowup of the inflorescence of pHs::CAS9-U6::2sgPIN1. Blue arrowheads indicates infertile siliques.



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6. Conclusion

Overall, the project has achieved proposed milestones, successfully providing a solid proof-of-concept for the plant-based biosensing platform. On a functional level, the biosensor performed well, triggering a distinct and visually unambiguous phenotypic response, a clear turning from green to red of the plant tissues upon As exposure. In parallel, to comply with strict biosecurity standards and address regulatory requirements regarding genetic containment, we successfully developed conditional infertile mutant lines, offering a reliable strategy to prevent transgene dissemination in open environments. A major scientific outcome of this reporting period lies in characterizing the genetic backgrounds best suited for deployment, which revealed key mechanistic differences in As handling. The *expa1* mutant line proved to be the most promising background for soil applications, as it efficiently accumulates As while maintaining high tolerance within the substrate. In contrast, the *arr1 12* double mutants display a high tolerance to As in liquid media through an exclusion mechanism. Our analysis showed that this exclusion is governed by the targeted regulation of NIP transporters alongside the modulation of plasma membrane proton pumps (H⁺ATPases), effectively minimizing toxic ion uptake. Those data will be described on a manuscript in preparation that will be submitted to New Phytologist. Moving forward, future efforts will focus on transitioning these validated lines from controlled laboratory environments to real-world operational scenarios, which will be reported in deliverable D4.3 due at M42. Key priorities include testing the biosensor performance in hydroponic setups and field conditions to evaluate overall robustness and response consistency. Furthermore, a crucial objective of the next phase will be monitoring long-term transgene stability to ensure that environmental fluctuations do not trigger post-transcriptional or transcriptional gene silencing, thereby guaranteeing sustained performance across operational cycles. Preliminary safety investigation is already schedule in MOBILES workplan and will be reported in D4.2 due at M42.





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Figure 1. Plasmid (pBluescript vector) map containing 8xYAPRE promoter, the same vector was used to clone the 6xYAPRE element

Figure 2 Cloning sites generated by Yap8 primers, Bsal restriction site are indicated on top line

Figure 3. PGGC000 vector map containing the YAP8 gene

Figure 4. Plasmid map containing RUBY system

Figure 5. pGGN-YAP8RE::RUBY::RBCSter::NPTR plasmid map

Figure 6. pGGM-35S::YAP8::UBQ10ter plasmid map

Figure 7. A) Scheme reporting the step for obtaining the final plasmid B) Final plasmid map containing the Arsenic sensor

Figure 8. Alteration of AHA H⁺-ATPase expression modulates rhizosphere pH.

Figure 9. As quantification in *aha1* and 2 mutants

Figure 10. Representative confocal laser scanning microscopy images of primary root apical meristems from 8-day-post-germination (dpg) wild-type (Col-0), *expa1*, and triple *expa10 expa14 expa15* mutant seedlings grown under control conditions or exposed to 100 μ M arsenic contamination. Black arrowheads mark the stem cell niche (SCN); white arrowheads delineate the transition zone (TZ) boundary.

Figure 11. Quantitative analysis of root meristem size, expressed as cortex meristematic cell number in the cortical layer from the stem cell niche to the TZ (a proxy for meristematic activity and arsenic tolerance) in the genotypes shown in figure 10.

Figure 12. Plant harboring the 35S::YAP8-YAPRE::RUBY construct acquire red color in presence of Arsenic. 10 days post germination seedling of Wt plants (Col0, upper panel) and 35S::YAP8-YAPRE::RUBY T2 plants (bottom panel) exposed to 100mM of Arsenic (Arsenate)

Figure 13. Guide RNAs for the inducible promoter

Figure 14. Final vector of pFAST-GK_pHs::CAS9-U6::2sgPIN1 for conditional sterility.

Figure 15. A) Arabidopsis Wt plant of 8 weeks. Red arrowheads indicates fertile siliques. Scalebar 5 cm. B) An Arabidopsis plant harbouring the pHs::CAS9-U6::2sgPIN1 construct after heat shock exposure. Scalebar 5 cm. C) Blowup of the inflorescence of pHs::CAS9-U6::2sgPIN1. Blue arrowheads indicates infertile siliques.





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Table 1. MOBILES WPs list

Table 2. YAP8-Responsive Element with 6 copies of consensus motif marked in red

Table 3 YAP8-Responsive Element with 8 copies of consensus motif marked in red

Table 4. Silico codon usage analysis. N/A= not analysed

Table 5. PCR primers used to generate gRNA for PIN1

9. List of Abbreviations

Abbreviation	Abbreviation for
aa	Amino acid
As	Arsenic
As(III)	Arsenite
As(V)	Arsenate
bp	Base Pair
bZIP	Basic region leucine zipper
CAI	Codon Adaptation Index
CDS	Coding Sequence
EXPA	EXPANSIN A
EXPA1	EXPANSIN A mutant 1
GC	Guanine Cytosine
HS	Heath Shock
HSI	hyperspectral imaging
ICP	Inductively Coupled Plasma





LB/RB	Left and Right Borders
MS	Mass Spectroscopy
NLS	Nuclear Localization Signal
NT	Not treated
PCR	Polymerase Chain reaction
sgRNAs	single-guide RNAs
TSKO	Tissue-Specific Knockout
Wt	Wild Type
YAPRE	YAP8 RESPONSIVE ELEMENTS





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10. Project Consortium



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www.eden-microfluidics.com/ *Terminated consortium membership effective June 2026*



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