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Sentinel plants to improve nutrient use efficiency: living tools for nondestructive analysis under field conditions

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DEDICATIONS

I dedicate this thesis to my father ***Hedi*** *and my mother* ***Souad*** *for their endless love, unlimited sacrifices, and support in the most difficult times*

To my brother ***Anis****, his wife* ***Mouna*** *and their daughters* ***Shahd*** *and* ***Shaima***

To my brother ***Zied****, his wife* ***Wiem*** *and their sons* ***Tarak*** *and* ***Adam***

To all my friends and all those who supported me in this work

To all those I love and who love me

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SUMMARY

Sulfur is an essential element for plant growth which availability affects both quantitative and qualitative traits of crop yield. Our aim was to generate specific bioassays based on the use of "sentinel plants" to quickly determine sulfate bioavailability and/or crop nutritional status which may represent a reliable and efficient strategy to obtain valuable, timely and low-cost information about changes in sulfate availabilities and nutritional requirements in a crop system.

The characterization of two *Arabidopsis* gene-trap lines (FLAG and 718 lines) allowed the identification of two portions (440 and 1331-bp respectively) of the intergenic region (between *At1g12030 and At1g12040*), controlling as a bidirectional promoter the expression of both *At1g12030* and *GUS* under sulfur limitation. Thus, these lines are able to provide information about the sulfur nutritional status of the plant and/or the sulfate concentration in the growing medium. For this purpose, the two lines were grown in agar plates under a continuous sulfate gradient ranging from 0 to 150 μM in order to describe the growth of both roots and shoots as a function of sulfate external concentration and to determine the critical concentration of sulfate (i.e. the minimum concentration of sulfate necessary to achieve maximum biomass) in the growing medium. The main results indicate that the pilot lines are able to correctly indicate the critical concentration of sulfate in the external medium also in the presence of interfering metal ions (such as cadmium) able to increase the plant metabolic demand for sulfur. Others experiments were done to better characterize the pilot bioindicators. Firstly, experiments were conducted in order to evaluate if the lines sense the lack of sulfate in the growth medium or indicates an alteration of the S nutritional status in the plant. For this purpose the response of the lines was analyzed growing the plants in the presence of cysteine or glutathione as sole sulfur source. The presence of cysteine as sole sulfur source in the agar medium did not produce any *GUS* activity in both shoots and roots, differently from glutathione which instead induced *GUS* activity only in the shoots. The results indicate that the promoters of the Arabidopsis pilot bioindicators sense the metabolic effect produced by sulfur starvation and not the presence/absence of sulfate in the growing medium. Moreover, *GUS* expression was studied in lines grown in hydroponic media containing different sulfate concentrations. Interestingly, both lines showed *GUS* expression when grown in agar media in sulfur starved conditions, whereas in hydroponics, a significant *GUS* expression was detectable only in the FLAG line, for sulfate concentrations $\leq$ 5 μM. To better analyze this effect we tested the effect of sucrose, only present in agar media, on *GUS* expression and results show that expression level of *GUS* is influenced not only by sulfur but also by the presence of sucrose in the external medium. Finally, we tested the bidirectional promoter-

function of the 440-bp intergenic region shared by the divergent genes *At1g12030* and *GUS* using *GUS* reporter gene in both orientations in stably transformed transgenic Arabidopsis. The sulfur responsive functional nature of the bidirectional promoter was evaluated in independent transgenic Arabidopsis lines. The 440-bp bidirectional promoter in both orientations shows *GUS* expression under sulfur limitation which was detected in the leaves and the root tissues indicating that 440-bp fragment is able to modulate the expression of *GUS* in two orientations under sulfur starvation. Then, to confirm the response of 440-bp promoters to sense the metabolic effect produced by sulfur starvation, transgenic Arabidopsis plants were grown in complete agar medium in the presence of cadmium. Results of this experiment showed that also Cd was able to induce a strong GUS activity in both leaves (vascular tissues) and root tips.

In conclusion, we identified putative bidirectional promoters involved in modulating the expression of *GUS* reporter gene in response to sulfur deficient conditions and suitable for developing specific bioindicators to monitor plant S nutritional status.

INTRODUCTION

MONITORING PLANT NUTRITIONAL STATUS

Mineral nutrient availability is one of the most important factors determining yield in agriculture. Conventional farming requires a continuous and large supply of fertilisers to the soil in order to replace the nutrients removed with plant harvest. In the predicted scenario of a rising demand for food and energy for the expected world population of about 9 billion in 2050 (Godfray et al. 2010) a dramatic increase in the use of fertilisers in the crop systems is foreseen. Since fertiliser production and distribution have a high demand for energy, in the last decade the price of fertilisers, although fluctuating, have been burgeoning and it is highly improbable that this trend will change in the next few years (http://faostat3.fao.org/home/index.html).

Only a fraction of the nutrient provided to the soil as fertiliser is taken up from the crops. This fraction, expressed as a percentage, is defined as the crop *Apparent Recovery* (*AR*; Craswell and Godwin 1984):

$$AR = 100\ (N_F - N_{nF})/F$$

where N_F and N_{nF} are the total amount of the nutrient absorbed by the crop, if fertilised or not, respectively, and F is the amount of the nutrient added to the soil with the fertiliser. For the main crops quite low average values of *AR* are reported: about 35% for N (Raun and Johnson 1999), 10-30% for P (Malhi et al. 2002) and seldom higher than 50% for K (Rengel and Damon 2008). The low capacity of crops in removing the nutritional elements added to the soil has significant environmental implications and reflects limits in the management of the fertilisation practices, the existence of constraints due to both chemical-physical and microbiological soil properties and plant intrinsic biological limits. *AR* does not consider yield traits. On the contrary, the *Agronomic Efficiency* (*AE*) of the fertiliser, defined by the ratio dY/dF, where dY is the infinitesimal yield (Y) and dF is the infinitesimal increase in the amount of the nutrient in the soil (F) due to the fertiliser application, considers such traits. *AE* can in turn be expressed considering two components: the crop *Removal Efficiency* (*RE*), defined as the ratio dN_F/dF, where dN_F is the infinitesimal incremental amount of the nutrient taken up by plant after the fertilisation, and the *Physiologic Efficiency* (*PE*), defined as the ratio dY/dN_F:

$$AE = RE \times PE = dN_F/dF \times dY/dN_F = dY/dF$$

In the field, both *RE* and *PE,* and then *AE,* depend on the interaction between genetic and environmental factors. In other chapters of this book the molecular and genetic aspects determining *AE* for some essential elements are extensively reviewed. Here, in a perspective of precision farming (Pierce and Nowak 1999), some strategies to improve *AE* by optimising dN_F throughout the use of plant-based sensor systems are presented and discussed.

Fertiliser best management practices

Definitions

In order to limit the intrinsic risks of diffuse pollution due to intensive agriculture, both local and supranational authorities are committed to the fine tuning of methods or techniques found to be the most effective and practical means in achieving yield objective optimisation and preventing contaminations of soils, water resources and air. As a whole, these recommended measures are defined as agronomic Best Management Practices (BMPs) and include: the choice of variety, planting date, row spacing, seeding rates, integrated pest management, weed control, disease control, and nutrient management. Focusing on the input into the field of inorganic nutrients, BMPs are considered the management practices that foster the effective and responsible use of fertiliser matching nutrients supply with crop requirements and minimise their losses from the fields.

Toward a fertiliser precision management

Fertiliser BMPs include the identification of the: a) the *right product* by matching the fertiliser characteristics to the crop site specific needs and soil properties; b) the *right time* by synchronising the presence of the nutrient with the moment of crop maximum demand and uptake capacity; c) the *best rate* by matching the amount of fertiliser input to crop needs in order to avoid over-input leading to nutrient leaching and other losses to the environment, as well as starvation conditions; d) the *right place* by making sure the presence of the nutrients where plants can efficiently take up them.

Although in a field a relatively high spatial variability in the crop requirements of a specific nutrient could exist, fertilisers are uniformly applied, to avoid yield gaps and

considering the less fertile portion of soil, where the crops have the maximum demand. The amounts of nutrient provided in excess with the fertiliser can be absorbed by the crops without resulting in any benefit in term of yield or leached towards the underground water becoming a concern for the quality of the environment.

If spatial and temporal information about crop needs of nutrients were available site- and time-specific inputs of the nutrient could be planned, resulting in a precision fertilisation approach, and in a win-win option to increase fertiliser nutrient efficiency and to improve the economic and environmental sustainability of the crop systems. In other words, the optimisation of dN_F term, in the equation defining *AE*, requires detailed information for decision support systems, allowing farmers to adopt the minimal nutrient input for maximal return, according to a Fertiliser Best Management Practices (FBMPs) approach.

Evaluating plant nutritional status

Soil and leaf analyses

Within a field the spatial variability of the soil chemical-physical and biological characteristics, including the amount of bioavailable forms of the mineral nutrients essential for crops, can be pronounced. Mapping this variability and plant nutritional status at high temporal and spatial resolution represents the first step towards the setup of site-specific FBMPs. In the last years, several sensor-based techniques to assess parameters indicative of the nutritional status of soil-plant systems have been proposed to replace, or support traditionally used physical measures and chemical analysis. Results on soil properties obtained from electromagnetic induction sensors along with those derived from the use of ground conductivity meters and radiometers analysing canopy reflectance, appear to provide data which can be used to target N-fertilisation to specific field conditions (Adamchuk et al. 2011). However, soil and plant chemical analyses are still widely used in developing methods for the evaluation of the nutritional status of the crops. In particular, the evaluation of elemental concentrations in plant tissues can be helpful in diagnosing nutrient deficiency. This strategy is currently used to assess nutrient availability and guide fertility programmes for many fruit tree crops. Nevertheless, the usefulness of this approach in order to develop FBMPs for herbaceous crops is rather debatable, since the concentration of a specific element may change among the leaves of a single plant, and may also change over time within a single

leaf (Barker and Pilbeam 2007). Thus, plant sampling, in term of timing and tissues to choose, is the most critical step (Kalra 1998). Moreover, elemental analysis detects only severe and long-term deficiency since a plant's initial response to nutrient limitation is to activate mechanisms aimed at maintaining the ionic homeostasis of their cells (Schatchtman and Shin 2007; Gojon et al. 2009). Finally, conferring a diagnostic value to the concentration of a single element could be misleading since complex cross-talk connections between the regulatory mechanisms controlling the ionic homeostasis in plants exist (Rouached et al. 2010).

The reduction in sulphur dioxide emissions in Europe over last decades and changes in fertilizer practices have resulted in a widespread increase in the occurrence of sulfur deficiency in agricultural crops (McGrath et al. 1996). Sulfur deficiency is considered as a problem for agriculture resulting in decreased crop quality parameters and yield (McGrath et al. 1996). Appropriate applications of fertilizer can remedy deficiencies; however, there remain considerable uncertainties concerning timing and type of S-application, which in turn influence the persistence of S in the soil and the availability to the plant (Hawkesford 2000). Thus, it is necessary to develop an adequate system of diagnosis for S deficiency (Blake-Kalff et al. 2000) using soil and plant analysis as possible tools (Scherer HW 2001). The amount of sulfate present in the soil solution can be evaluated by different soil-testing methods but it gives information not always correlated to plant yield and the actual needs of the crop (Zhao et al. 1999). Moreover, the concentration of the S available to the plant present in the soil is characterized by seasonal variations (Hawkesford 2000) and thus soil-testing methods do not provide the actual bioavailability of the nutrient. Therefore, more knowledge is required in order to define the best diagnostic indicators for S deficiency. Plant analysis is preferred because S status in plant tissue may better reflect the amount of S available at the time of sampling. However, doubts about the time of sampling and, first of all, about which parameter are better remaining (Blake-Kalff et al. 2000). The concentration in the tissues of total sulfur, sulfate or glutathione, as well as the percentage of sulfate with respect to the total sulfur or the value of the N:S ratio are the parameters measured and considered as relevant indicators for S deficiency (Blake-Kalff et al. 2000; Scherer HW 2001). However, the different growth stages, the different parts of the plant sampled, the analytical methods used and how the analyses are carried out cause considerable variations of the critical values proposed for these indicators (Blake-Kalff et al. 2000). It has been suggested that in wheat and oilseed rape the best diagnosing of S deficiency in order to prevent yield losses is the evaluation of the ratio

malate: sulfate peak area ratio from ion chromatography. At early growth stages, a value of the ratio greater than 1 indicates S deficiency (Blake-Kalff et al. 2000).

Nutrient-critical concentration and dilution curve

The *critical concentration of a nutrient* (n_c) stands for the concentration of the nutrient in the shoots above which, in the absence of other growth limiting factors, the plant is sufficiently supplied with the nutrient to achieve its maximum potential yield. In other words, when the n_c is achieved and maintained, further supplies of the nutrient will not influence the growth of the plants and, in the absence of any sort of demand-driven negative-regulation of its uptake, it could be uselessly accumulated in the plant tissues. For some nutrients it is possible to define the so-called *toxicological value*, which indicates the concentration above which further nutrient accumulation induces damage on cell metabolism and structure. All the concentrations of the nutrient between its critical and toxicological values define the so-called *luxury range* (Annex. 1), which depends on the chemical properties of the element and on its biochemical roles. The fine-tuning of the application of fertiliser to maintain the concentration of the mineral nutrients in the plant tissues as close as possible to their critical values represents a FBMP approach.

The critical concentration of a nutrient is not a constant value since it depends on genotype, environmental conditions, and the developmental stage of the plants (Lemaire and Gastal 2009; Greenwood et al. 1986). For instance, in the above-ground tissue of cereals the value of n_c (as % on the DW basis) changes during the plant developmental stages according to the dilution function:

$$n_c = a\, W^{-b}$$

where W is the maximum above-ground biomass in a specific stage of the plant cycle, a represents the concentration of n_c in the shoot when the crop mass is 1 Mg DM ha^{-1}, and b represents the dilution coefficient.

The dilution curve of a specific cultivar is obtained by plotting the n_c values, at a given developmental stage and field situation determined by a set a fertilisation experiments *versus* the accumulated plant shoot biomass (Annex. 2). Once the dilution curve for a specific nutrient is known, it is possible to evaluate the nutritional status of the crop by evaluating its

nutritional index (*NI*) defined as the ratio between the actual nutrient concentration in the shoots and the corresponding value of n_c. Indeed, if *NI* is lower than 1 the crop is in a suboptimal nutritional status for a given nutrient and needs to be fertilised; if the *NI* is higher than 1 the crop is in the luxury range.

The actual feasibility of such an approach for a sustainable management of N fertilisation is conditioned by the limited availability of easy and low cost methods to rapidly estimate the value of *W* in the field and, mainly by the actual concentrations of the nutrient in the shoots over a representational cropping area. The value of *W* can be extrapolated by the crop leaf area index (LAI) currently evaluated through remote sensing approaches (Zheng and Moskal 2009). The evaluation of the actual concentration of the nutrient, without adopting the traditional chemical analyses, is the most difficult challenge to overcome.

Since nitrogen is the nutritional element that most often affects crop production and the current world use of N fertilisers is approximately 90 million metric tons (with an estimated cost of about $50 billion), it is reasonable that several research efforts have been focused on the fine tuning of non-invasive methods for the determination of N levels in shoots throughout the entire growth cycle of crops as a guide to N-FBMPs (for an exhaustive review see Samborsky et al. 2009). Assuming nitrogen as an example, in the next paragraph we briefly summarize the advances on the non-destructive approaches developed or under investigation for monitoring the nutritional status of a crop.

Non-destructive monitoring of crop nutritional status: the example of nitrogen

Nitrogen availability affects chlorophyll content in leaves (Schlemmer et al. 2005) and as a consequence the level of this pigment is considered a good indicator of the nitrogen nutritional status of a crop (Samborski et al. 2009 and references therein). Instruments analysing the spectral properties of leaf tissue to estimate their chlorophyll content (optical chlorophyll meters) have been developed to evaluate the need for agricultural N applications. Due to the pigment's light absorption properties (in the visible wavelength range), the higher the chlorophyll content, the higher the reflectance of the leaf (in the 525-680 nm range) and consequently, the higher the amount of red light absorbed. Combining light absorbance measures at 660 nm and near-infrared (NIR) light transmittance at 940 nm, which in turn depend on leaf moisture content and thickness, a good estimation of chlorophyll per unit area

has been obtained in the major crop leaves. It has been proved that the chlorophyll meter can detect the early signs of N stress not yet detectable by visual analysis using a leaf colour chart (Debaeke et al. 2006).

Recently, it has been proposed a hand-held instrument (Dualex®), exploiting chlorophyll as an internal sensor of photons, enables the user to contemporaneously assess the level of both photosynthetic pigments in the mesophyll and flavonoids in the epidermis of the leaf. Briefly, comparing the amount of chlorophyll fluorescence emitted under UV excitation (λ_{380}) with that emitted under visible light (λ_{660}) whether absorbed or not, the instrument is able to evaluate the level of flavonoids absorbing in the UV range. Contemporaneously, by comparison of the light transmittance at λ_{720}, in the range of chlorophyll absorption, and at λ_{840}, in the range influenced by leaf structural properties but not by chlorophyll, a reliable evaluation of the levels of the photosynthetic active pigment is obtained. Dualex® thus allows measurement of a Nitrogen Balance Index (NBI®), which indicates the ratios of both chlorophyll and flavonoids units and as a result is related to leaf N content (Cartelat et al. 2005) since leaf flavonoids can be considered an indicator of N availability. Indeed, in N-starved plants the concentration of carbon-based secondary metabolites increase (Hamilton et al. 2001) and in particular, due to the enhanced expression of specific transcription factors involved in controlling their biosynthetic pathway, those of anthocyanin and flavonols (Lea et al. 2007). Several experimental evaluations suggest that in the case of wheat and corn Dualex® seems to furnish more reliable information about the N status of the plants with respect to other hand-held optical systems (Tremblay et al. 2012).

Since leaf N status influences the quantum yield of PSII electron transport and then the chlorophyll fluorescence parameters (Lu and Zhang 2000), canopy fluorescence quenching analyses could be considered suitable for sensing crop N status (Tremblay et al. 2012). In particular, the recent introduction of a hand-held fluorimeter (Multiplex®) equipped with LEDs generating four wavelengths (λ_{375}, λ_{450}, λ_{530}, λ_{630}) and detectors monitoring fluorescence at three wavelengths (λ_{447} or λ_{590} if the excitation at λ_{450} is used or not, respectively, λ_{665} and λ_{735}) seems to be quite promising for the in-season assessment of crop N status (Trembleay et al. 2012 and references therein). Combining different excitation and emission bands the instrument provides independent parameters related to chlorophyll, flavonoids and N content of the plants (Tremblay et al. 2012).

The devices described above determine optical parameters for individual leaves or, in the case of Multiplex®, at a typical distance of a few centimeters thus monitoring circular canopy surfaces of not more than 10 cm in diameter. Consequently, they are not particularly suitable in evaluating the N status of a crop at field scale. Sensors, analysing canopy reflectance properties and thus its N status and needs are also available (Erdle et al. 2011). They are classified as passive (Yara N-Sensor®/Field Scan and FiledSpec® Portable Spectroradiometer) or active (GreenSeeker® and Crop Circle™) non-contact sensors depending on the sunlight reflected by the canopy or on their own specific light sources in the visible (650 or 590 nm) and NIR (770 or 880 nm) range, respectively. Spectral data collected by these devices allow the calculation of the so-called normalised differences vegetation index (NDVI) according to the formula:

$$NDVI = (NIR - Vis)/(NIR + Vis)$$

where *NIR* and *Vis* stand for the spectral reflectance measurements acquired in the *NIR* or visible (red) regions, respectively. The *NDVI* value is about 0.5 when the vegetation chlorophyll content and thus, in the absence of any other stress factors, plant N status is optimal; conversely in sub-optimal conditions the value of *NDVI* is much lower.

Several examples of the use of these portable proximal sensors (which can also be mounted on tractors) in the fine-tuning of variable-rate technology for site-specific N fertilisation exist (Solari et al. 2008; Diacono et al. 2013). The possibilities to easily and efficiently translate the information on N crop status, obtained by the hand-held or proxy sensor approaches described as above, in site- and time-specific recommendations for FBMPs can be invalidated by a plethora of biotic and/or biotic stressors, including a non-optimal availability of nutrients other than N, which influence the chlorophyll content of the leaves. Therefore, the parameters and the vegetation index obtained are usually validated by setting up standardisation procedures providing for plots of the same cultivar in the same environment at different N availability. In this way genetic, environmental and agronomical factors can be eliminated as potential sources of error and making the data obtained by the sensor-based approaches more reliable (Samborski et al. 2009; Diacono et al. 2013).

Hyperspectral radiometers providing contemporaneous reflectance measurements over a relatively narrow wavebands (<10 nm), should make it possible to identify specific regions of the spectrum which could be used to develop new indices, highly sensitive to plant N status and unaffected by other exogenous factors (Hansen and Schjoerring 2003). Indeed, an

increasing number of studies suggest that field as well as airborne or spaceborne hyperspectral canopy radiometric data can be useful for estimating plant nitrogen concentration in cultivated or natural environments (Ollinger et al. 2008, Stroppiana et al. 2009), although recently some criticisms about the remote sensing of leaf tissue constituents by hyperspectral data have been raised (Knyazikhin et al. 2013).

Leaf chlorophyll concentration is also an indirect diagnostic symptom for N status of the crop. However, it is important to take into account that reduced chlorophyll biosynthesis is a relatively late response to N starvation which only becomes evident after the plant has initiated other molecular and physiological responses for maintaining N homeostasis (Schatchtman and Shin 2007; Gojon et al. 2009).

Unfortunately, non-destructive reliable monitoring approaches comparable with those above described for N have not been developed for the other mineral nutrients whose availability affects crop yield (in particular P, K and S). Thus, for these nutrients the chance to adopt FBMPs is limited to the classic chemical evaluation of plant tissues and soils.

Plant bioindicator for nutritional status

Bioindication and biomonitoring

The development of quick and inexpensive methods to determine changes in nutrient bioavailability is required in order to monitor soil nutrient dynamics for better fertiliser management for a variety of crops in different environmental conditions. Developing bioassays based on the use of specific plant sentinels or biondicators, may represent a reliable and efficient strategy to obtain quick, accurate and low-cost information about nutrient availability changes in a given crop system. Thus, the use of these modern biotechnologies could allow the non-destructive analysis of plants under field conditions. Development of these kinds of tools represents a new and challenging area of research.

Plants respond to nutrient supply or shortage through a complex of physiological, morphological, and developmental responses, which are under the control of several gene pathways. Microarray technology is a convenient tool for rapid analysis of plant gene expression patterns under a variety of environmental and nutritional conditions. Genome-wide microarray analyses showed extensive changes in the expression of several genes involved in

primary and secondary metabolism, nutrient transport, protein synthesis, regulation of gene expression and cellular growth processes (Maruyama-Nakashita et al. 2003; Wang et al. 2003; Bi et al. 2007; Li et al. 2010; Kant et al. 2010; Ma et al. 2012). Such studies not only improved our general understanding on plant responses to nutrient availability but also provided a reliable data from which to develop new molecular strategies for real-time monitoring of plant nutritional status.

Recently, Yang et al. (2011) used multiple whole genome microarray experiments to identify gene expression biomarkers capable of assessing plant responses under limiting and sufficient nitrogen conditions. Using logistic regression statistical approaches, they identified a common set of genes in maize whose expression profiles quantitatively assessed the extent of plant stress under different nitrogen conditions. Interestingly, such a biomarker gene set is independent of maize genotype, tissue type, developmental stage, and environment (including plants grown under controlled conditions and in the field), and thus has the potential to be used as an agronomic tool for real-time monitoring and to optimise nitrogen fertiliser usage.

The gene fusion concept enables to define a new class of transgenic bioindicators

The existence of gene pools, which specifically respond to the nutritional status of the plant, has introduced a new class of bioindicators, based on the concept of gene fusion (Annex. 3). A generic nutrient-responsive gene is formally considered as consisting of two parts: the promoter or controller that senses the nutritional status of the plant and directs the synthesis of a new product from the second component, the responder. By replacing the original sequence of the responder gene with a new and easily studied gene, called a reporter gene, it should be possible to obtain valuable information about the activity of the promoter. Such a molecular manipulation should provide information about the nutritional status of the plant by simply measuring the activity of the reporter protein.

Plant biologists to study how a particular gene is controlled when measurement of the gene product is too difficult have extensively used the gene fusion concept. The elective tool used in this type of studies is the GUS gene fusion system, which uses *uidA* from *E. coli* as a reporter gene. This gene encodes a β-glucuronidase able to hydrolyse a wide range of β-D-glucuronide substrates producing coloured or other compounds in amounts proportional to enzyme activity (Jefferson 1989). Assays for testing the activity of GUS in genetically

modified plants are carried out on plant material or plant extracts under laboratory conditions. A variety of glucuronides is commercially available and can be used for fluorometric, spectrophotometric, luminometric and histochemical GUS analyses, qualitative as well as quantitative (Gallagher 1992).

Unfortunately, reporter systems based on the activity of β-glucuronidase (GUS) have severe intrinsic limitations that preclude their application under field conditions since they require the enzyme (GUS) and its substrate be brought together to produce the hydrolytic products necessary for the analysis. Likewise, luciferin–luciferase imaging systems have been used in plants, but their application in the field is hampered by the low level of light emission and the need for sensitive photon-counting cameras to detect signal (de Ruijter et al. 2003). Thus, new tools to perform non-destructive analysis are essential to develop specific sentinel plants to be directly used under field conditions.

In a pioneering paper, Jefferson (1993) listed some criteria useful to develop new reporter systems suitable for agricultural molecular biology. Some of the key criteria, such an *in vivo* reporter system are that it should be: i) non-destructive; ii) non-disruptive to avoid physiological alteration of crop performance; iii) useful and functional in most crop species; iv) inexpensive and capable of being used everywhere; v) simply to detect with little or no instrumentation; vi) easy to use under field conditions.

Naturally fluorescent proteins could offer a valuable alternative to the use of GUS reporter systems since, in contrast to GUS, the detection of their expression does not require the addition of a substrate. Green fluorescent protein (GFP), a spontaneously fluorescent protein, was initially isolated from the luminescent marine jellyfish (*Aequorea victoria*). GFP emits a highly and stable bright green fluorescence after absorbing blue light (Tsien 1998)**.** The wild type *Aequorea* protein has a major excitation peak at 395 nm which is about three times higher in amplitude than a minor peak at 475 nm. In normal solution, excitation at 395 nm gives emissions peaking at 508 nm, whereas excitation at 475 nm gives a maximum at 503 nm (Heim et al. 1994). Since its discovery GFP from *Aequorea victoria* has become a frequently used tool in plant biology. The first studies on transgenic plants expressing wild type GFP proved the usefulness of this protein as an *in vivo* and real-time visible marker and encouraged researchers to modify it in order to obtain new variants that could be more effectively synthesised in plant cells and macroscopically detectable at the whole plant level (Stewart 2001). One of these modified versions of GFP is the mGFP5er variant that produces

a stable protein targeted to the endoplasmic reticulum as the result of the addition of a N-terminal *Arabidopsis* basic chitinase fusion and a C-terminal HDEL fusion (Haseloff et al. 1997). The coding sequence contains three mutations that enhance the folding of mGFP5er at higher temperatures and allows excitation of the protein using either ultraviolet (395 nm) or blue (473 nm) light (Siemering et al. 1996). In addition, new fluorescence colours have been created through mutagenesis of the natural protein giving longer excitation and emission wavelengths and to enhance the fluorescence brightness. The new colours range from blue and cyan (EBFP and ECFP) to yellow (EYFP); such new proteins have excitation/emission peaks at 383/474, 434/472 and 514/527 nm, respectively (Spiess et al. 2005; Mena et al. 2006).

Fluorescent proteins have been largely used as visual genetic labels at the whole plant, tissue and cell levels, since they offer a fast and easy-to-use non-destructive tool with which the efficiency and timing of gene expression can be evaluated. Detection and quantification of fluorescence at the whole-plant level normally requires the use of complex and expensive laboratory instruments (scanning laser and fluorescence imaging systems). Portable instruments, such as fibre optic probe fluorometers, have recently been designed to assess GFP fluorescence under field conditions (Harper and Stewart 2000; Millwood et al. 2003). However, because biondicators need to be disseminated over a wide area, a successful field application of these plants also requires a cost-effective remote monitoring system providing real-time information about the nutritional status of a whole crop system. Recently Adams et al. (2011) proposed an alternative method for crop monitoring in which sentinel plants and sensing units are deployed in tandem at specific locations. Ideally, such a system integrates biological and sensory technologies with communication technologies to provide a practical field-deployable telemetry system.

The gene fusion concept could be used to measure complex phenomena, even in the absence of mechanistic knowledge of how that phenomenon works (Jefferson 1993). This technology is completely general and could be exploited to develop transgenic bioindicators providing signals whose intensity is proportional to the concentration of a given analyte in growing environment (*i.e.* mineral nutrients, pollutants, water, etc.) or to the intensity of a biotic or abiotic stress that plants could experience during their growth. Potential targeted traits to be monitored are only limited by the availability of specific promoters (or controllers) driving the reporter expression under a specific condition.

Recently these technologies have been applied in plants to develop model transgenic bioindicators of the nutritional status to be used for laboratory purposes. To date, reporter gene activity has been used to assess the phosphate, sulfate and magnesium status in *Arabidopsis* and also to detect the level of nickel in the growing medium (Hammond et al. 2003; Krizek et al. 2003; Maruyama-Nakashita et al. 2006; Kamiya et al. 2012). In all these studies GUS, GFP and LUC have been successfully used as reporter genes to indicate nutritional status under the control of promoter sequences indirectly identified by microarray analyses.

Hammond and co-workers (2003) first proposed the creation of an *Arabidopsis* transgenic bioindicator, able to monitor plant phosphorous status. They fused GUS with the promoter of the phosphate starvation responsive gene *SQD1* (a gene involved in the synthesis of sulfolipids), obtaining an *Arabidopsis* transgenic line in which GUS activity increased following P starvation. Interestingly, the reporter responses to P withdrawal were much more rapid and quantitative than phenotypic observations, showing this approach is particularly suitable for developing efficient systems for monitoring plant P status. More recently Kamiya et al. (2012) used a similar approach to establishe a novel monitoring system for magnesium in plants. In particular they obtained an *Arabidopsis* transgenic line that expressed luciferase (LUC) under the control of the Mg deficiency-inducible *CAX3* promoter. The transgenic lines showed a clear response under low Mg conditions and the degree of luminescence reflected the accumulation of endogenous *CAX3* mRNA. However, *CAX3* induction does not seem to be specific to low Mg, since the levels of other ions (Ca^{2+} and Na^{+}) or P starvation may influence transcription (Shigaki and Hirschi 2000).

Notwithstanding some limitations *Arabidopsis* 'smart' plants could also be used as tools in basic research aimed at isolating novel mutants disrupted in nutrient homeostasis or identifying plants with enhanced nutrient use efficiency. For instance, the key transcription factor, SLIM1, regulating the sulfur assimilatory pathway has recently been identified by screening *Arabidopsis* mutants carrying a fluorescent reporter gene under the control of the sulfur limitation-responsive promoter of the SULTR1;2 sulfate transporter (Maruyama-Nakashita et al. 2006). Using this approach it is possible to identify all the potential genes involved in controlling the expression of SULTR1;2 under sulfur shortage, since in this condition the relative mutants will display altered fluorescence emissions as compared to the wild type bioindicator.

Strategies to enhance the specificity of bioindicators

In the future, the use of smart plant technology in crops would provide rapid bioassay methods to obtain valuable information about nutrient availability in the soil solution and/or the nutritional status of the plants allowing efficient temporal and special application of fertilisers and the development of decision-making systems for precision farming. To date the exploitation of these technologies is limited, not only by the lack of telemetry systems suitable for plant monitoring across a large area, but also by the lack of precise information to design a transformation-cassette that would enable the nutrient-specific control of reporter activity. Thus, the choice of a core promoter to confer specific transgene expression, represent the major challenge we have to face in order to develop the next generation of bioindicators.

A typical plant promoter consists of CAAT and TATA boxes for recognition of DNA-dependent RNA polymerase, several-tens of bp upstream of the transcription initiation site (Yoshida and Shinmyo 2000). Specific DNA sequences, called *cis*-elements, generally upstream of the core promoter, drive the cell- or organ-specific expression of the downstream gene under certain environmental conditions. Specific factors, called *trans*-factors (or transcription factors), bind to the *cis*-elements affecting RNA polymerase activity. Generally, multiple-*cis*-elements and *trans*-factors work together to induce the full regulation of gene expression, since gene expression is generally under the control of several factors (Yoshida and Shinmyo 2000; Venter 2007).

In recent years, a wide range of different promoters have been characterised and extensively used for regulating the expression of transgenes in plant cells (Venter 2007). In several cases, the *cis*-elements that are necessary for transcriptional regulation and the *trans*-factors that interact with these elements have been identified. From these studies has emerged a complex picture in which DNA sequence *cis*-elements that are important for regulation are scattered over thousands of base pairs, and these elements interact with *trans*-factors that can be either ubiquitous or highly restricted in their distribution. In this way diverse expression patterns may be achieved through combinations of a limited number of regulatory elements and *trans*-acting factors. The knowledge of these combinatorial mechanisms should allow the generation different transcription patterns by ‘cut and pasting’ the components in different ways.

Analysis of the cauliflower mosaic virus (CaMV) 35S promoter has contributed to the understanding of transcriptional regulatory mechanisms and has allowed the design of

inducible transgene expression cassettes. The -343 to -46 upstream region relative to the site of initiation of transcription (+1) of the promoter is responsible for the strength of transcription. Two regions, -343 to -208 and -208 to -90, are responsible for transcriptional activation, and the -90 to -46 region plays an accessory role by further increasing the transcriptional activity (Odell et al. 1985; Fang et al. 1989). Artificial promoters are generally constructed by a combinatorial design of different promoter elements, with the minimal core DNA fragment (-46 to +8 bp) of the CaMV 35S promoter as the main component (Annex. 4). The core-promoter region contains a TATA-box necessary for recruiting RNA polymerase II and the orchestrated assembly of general transcription factors to form the pre-initiation complex (Novina and Roy 1996). The CaMV 35S core-promoter is ideal for transcription initiation and has been used in several synthetic plant promoters in which combinatorial engineering of *cis*-element have been introduced upstream of the core-promoter sequence.

The use of synthetic promoters allowing for targeted inducibility of a reporter gene is of considerable interest to develop engineering strategies aimed at creating plant bioindicators for real-time monitoring of nutritional status. For these purposes promoter sequence domains or *cis*-elements conferring nutrient- and organ-specificity should be combined in order to target the reporter expression in organs (shoot and leaves) in which signals should be easily detectable.

Many different plant promoters have been described as able to restrict gene expression to particular cells, tissues or organs. The *GaMYB2* promoter is cotton fibre- and *Arabidopsis* trichome-specific, and can drive gene expression specifically in glandular cells (head cells) of glandular trichomes in transgenic tobacco (Shangguan et al. 2008). Some *cis*-elements regulating tissue-specific gene expression have also been identified. For instance, mesophyll expression module 1 (*Mem1*), a 41 bp fragment of the *ppcA1* promoter, directs mesophyll-specific expression. The tetranucleotide sequence, CACT has been identified as a key component of *Mem1* by evolutionary and functional studies (Gowik et al. 2004). More recently, Ye et al. (2012) identified a rice green tissue-specific expression gene, *DX1*, and described two novel tissue-specific *cis*-elements (GSE1 and GSE2) within the *DX1* promoter. In particular, GSE1 acted as a positive regulator in all green tissues, whereas GSE2 acted as a positive regulator only in sheath and stem tissues.

Obviously, nutrient-specific *cis*-elements are equally as important for reporter expression as tissue-specific *cis*-elements. Nutrient-inducible plant promoters contain multiple *cis*-acting

elements, only some of which may specifically contribute to nutrient inducibility. A number of potential nutrient responsive *cis*-elements have recently been identified in the promoter of several nutrient responsive genes and have been indicated as key regulatory factors of gene expression under different nutritional conditions.

Sulfur-responsive elements (SUREs) have been identified in the promoter regions of the *Arabidopsis NIT3* nitrilase and β-subunit β-conglycinin gene from soybean (Awazuhara et al. 2002; Kutz et al. 2002), although no consensus sequences have been shown yet. However, an interesting study on *Arabidopsis* sulfate transporter SULTR1;1 promoter demonstrates that a 5 bp sequence is essential to promote sulfur response of SULTR1;1 (Maruyama-Nakashita et al. 2005). Such a sequence also appears in the promoter regions of many sulfur-responsive genes, suggesting its involvement in the transcriptional control of a gene set required for adaptation to sulfur-limiting conditions. Deletion analysis of the barley *IDS2* (iron deficiency-specific clone no. 2) gene promoter allowed the identification of two *cis*-acting elements, iron-deficiency-responsive element 1 and 2 (IDE1 and IDE2), which synergistically induced iron-specific expression in tobacco roots. Finally, comparative analyses of several nitrite reductase gene promoters from various higher plants have recently allowed identification of a conserved sequence motif as nitrate-responsive *cis*-element (Konishi and Yanagisawa 2010).

What do biondicators sense? A key problem

Modification of promoter architecture necessary for manipulating gene reporter activity requires accurate studies of the regulatory network involved in controlling gene expression under different nutritional conditions. Unfortunately, for the most part, these aspects are still largely unknown preventing the optimal design of a synthetic nutrient-inducible promoter, particularly in cases where a mineral nutrient undergoes complex assimilatory metabolisms (*i.e.* nitrate or sulfate) or interacts with other nutrients. In all these cases the specific question to be answered is: what do synthetic nutrient-specific promoters sense?

For example, the transcriptional regulatory mechanisms involved in sulfate uptake and assimilation reasonably result from direct sensing of the plant nutritional status rather than from the composition of the external soil solution (Lappartient and Touraine 1997; Lappartient et al. 1999). This control involves an inter-organ signaling mechanism in which key intermediates of the sulfate assimilatory pathway may act as negative or positive signals

in modulating the expression of the sulfur-responsive genes. Adequate levels of sulfur compounds would repress gene expression through a negative feedback loop preventing excessive sulfate uptake and reduction; *vice versa* a contraction of the intermediates along the assimilatory pathway would unrepress gene transcription allowing sulfate to enter the pathway. A second regulatory loop, involving OAS as a key intermediate, would act in promoting gene unrepression when nitrogen and carbon supply exceeds sulfur availability within the cells (Hawkesford 2000). In this context the need to dissect the molecular mechanisms involved in the nutritional signal perception and transduction is evident since, in several cases, the relationships existing between gene expression and the levels of the signal-intermediates are not always clear. Further research is needed to associate single gene expressions to a specific nutritional signal or sulfur-nutritional status.

Genome-wide expression analyses have revealed that nitrate supply induces changes in the expression of several genes, not only those involved in nitrate reduction and assimilation. Such behaviour is likely both due to the direct effects of nitrate itself and indirect effects caused by changes in nitrogen metabolite content or nitrogen nutritional status. In fact, nitrate is thought to act as a signal molecule influencing the expression of a number of genes, since their expression is rapidly induced by nitrate even in mutants severely compromised for nitrate reductase activity (Wang et al. 2004). In addition, it has been shown that nitrate-inducible expression NADH/nitrate reductase mRNA in maize roots, scutella and leaves also occurs in the presence of inhibitors of protein synthesis, suggesting that the signal transduction system mediating this response is constitutively expressed in plant cells, independently of the presence or the absence of nitrate in the growing medium (Price et al. 2004). Results of these studies clearly shows that dissection analyses of the signal transduction pathways controlling gene expression under different nitrogen supply should provide important information to define smart plants able to sense the cellular level of nitrite or the general nitrogen nutritional status of a crop system.

FUNCTION OF SULFUR IN PLANTS

Sulfur is an essential macronutrient required for the growth and physiology of plants. Sulfur is required for the synthesis of S-amino acids like cysteine (Cys) and methionine (Met), oligopeptides like glutathione (GSH) and phytochelatins (PC), vitamins and cofactors (thiamine, biotin, coenzyme A, S-adenosyl-Met), protein iron-sulfur clusters, in membrane

sulfolipids and in some secondary metabolites like glucosinolates, allyl cysteine, alliins and choline-*O*-sulfate (Maruyama-Nakashita et al. 2003; Saito 2004; Davidian and Kopriva 2010; Takahashi et al. 2011).

During the different developmental stages and under particular environmental conditions the sulfur demands can change (Yoshimoto et al. 2003; Buchner et al. 2004a) and plants response to this by tightly modulating sulfur uptake, assimilation and distribution through the plant.

SULFATE UPTAKE AND TRANSPORT

Sulfate (SO_4^{2-}) is the main form of sulfur present in the soil solution and the major source of sulfur for plants. In addition to the inorganic sulfate uptake from soil, plants are also able to absorb, by the foliage through the stomata, atmospheric sulfur gases (sulfur dioxide and hydrogen sulfide). In air polluted regions this way represents a significant source of the total amount of sulfur that plant needs (Van Der Kooij et al. 1997; Leustek et al. 2000; Buchner et al. 2004b).

Uptake from the soil, cellular compartmentalization and long distance translocation of the anion are mediated by several transmembrane transport systems. Once taken up by root, sulfate is distributed by xylem to mature leaves and in turn by the phloem to the younger ones. Inside the cells, sulfate moves toward the plastids, the major sites of its assimilatory reductive pathway, or is compartmentalized into the vacuole (Hawkesford 2003; Saito 2004; Hawkesford and De Kok 2006).

From the soil, sulfate is taken up into the root cells through specific transporters located on the plasma membrane of the roots cells allowing the entrance of the anion in the symplastic system. The influx of sulfate is a thermodynamically actively transport. Indeed, it take place against its electrochemical potential gradient existing across the plasma membrane by the activity of a proton/sulfate cotransport system adopting a $3H^+:1SO_4^{2-}$ stoichiometry in turn energized by the H^+ transmembrane electrochemical gradient generated by the activity of the pasmalemma H^+-ATPase (Hawkesford 2003; Saito 2004; Takahashi et al. 2011).

In different tissues, several membranes transporters are involved in the systemic sulfate distribution throughout the plant body. On the basis of the sequence similarity and function, five functional subgroups of sulfate transporters have been described in *A. thaliana*,

as well as in other plant species (Smith at al. 1997; Hawkesford 2003; Buchner at al. 2004a; Saito 2004; Kopriva 2006).

In Arabidopsis the first group include the high-affinity sulfate transporters *AtSULTR1;1* and *AtSULTR1;2* genes. These elements are predominantly expressed at root level and are responsible for the primary uptake of the anion from the soil solution. Both transporters are expressed in root hairs other than root epidermal and cortex cells. Moreover, *AtSULTR1;1* and *AtSULTR1;2* are specifically expressed also in leaf hydathodes and *AtSULTR1;2* also in the stomata guard cells suggesting their additional functions within the plant (Shibagaki et al. 2002; Hawkesford 2003). Both *AtSULTR1;1* and *AtSULTR1;2* are transcriptionally regulated in a promoter dependent manner in response to availability of sulfate in the growth medium (Hawkesford 2003; Hawkesford and De Kok. 2006) allowing the uptake of the anion into roots especially under sulfur-limiting conditions (Yoshimoto et al. 2007; Barberon et al. 2008). Moreover, the transcription of both *AtSULTR1;1* and *AtSULTR1;2* by sulfur limitation is dependent on the supply of carbon and nitrogen (Maruyama-Nakashita et al. 2004) and is modulated by the cellular concentration of metabolites belonging to the sulfur assimilatory pathways (Rouached et al. 2008). A third member in this first group of sulfate transporters is AtSULTR1;3, a phloem-localized sulfate transporter which mediate the control of long-distance transport of the anion between source and sink organs (Yoshimoto et al. 2003).

The elements belonging to the subgroup 2 of the Arabidopsis sulfate transporter family AtSULTR2;1 and AtSULTR2;2, are characterized by a low affinity for the sulfate (Takahashi et al. 2000) and are expressed in the vascular tissues of roots (*AtSULTR2;1* and *AtSULTR2;2*) and leaves (*AtSULTR2;1*), in siliques (Hawkesford 2003; Awazahara et al. 2005) where mediates the movement of sulfate into developing seeds. Therefore, it is suggested that the elements of the group 2 are mainly involved in the systemic allocation of the nutrient in the plant. Similarly to the sulfate high affinity transporters, the expression of *SULTR2;1* resulted to be transcriptionally regulated by the cellular availability of sulfur; in detail, it results induced under sulfur starved condition

The group 3 belong leaf tissues-localized transporters, including isoforms of unknown function. This large amount of the elements of this group would suggest some redundancy or indicate a great variety of expression pattern related to different sulfur availability during plant growth (Hawkesford 2003; Kopriva 2006). Elements of group 3 may participate in heterodimer association. In particular, it has been suggested that SULTR3;5 can function as a

heterodimers with SULTR2;1, as indicated facilitating the apoplastic transport of sulfate to the xylem parenchyma cells, especially under sulfur limiting conditions, increasing the rate of sulfate translocation from root to shoot by xylem (Kataoka et al. 2004a). Recently, by AtSULT3;1-GFP localization it has been demonstrated that AtSULTR3;1 is localized in the chloroplast membrane mediating the supply of sulfate to its reductive assimilation pathways (Cao et al. 2013). Interesting in plant under drought stress a correlation between the expression of this gene, the synthesis of cysteine and ABA has been reported (Cao et al. 2014).

The group 4 of sulfate transporter family mainly includes, tonoplast localized transporters (AtSULTR4;1 and AtSULTR4;1) mediating the efflux of the anion from the vacuole into the cytosol. The expression of both *AtSULTR4;1* and *AtSULTR4;1* is responsive to sulfate starvation, although the effect is more evident for the former than the latter (Kataoka et al. 2004b; Davidian and Kopriva 2010). Both transporters play a key role in ameliorating internal distribution of sulfate in the cell and in increasing the sulfate transported to the xylem (Kataoka et al. 2004b). Interestingly, the expression of both vacuolar *AtSULTR4;1* and *AtSULTR4;2* genes is significantly enhanced in leaves by drought and salt stress (Gallardo et al. 2014). Moreover, both the genes fall in QTL regions for tolerance to both stresses (Juenger et al. 2005; Mckay et al. 2008).

Finally, the group 5 sulfate transporter contains short sequences, with unknown function, presenting a low level of similarity with the rest of sulfate transporters members and with missing portions at the N and C terminal ends of the amino-acid (Hawkesford 2003; Kopriva 2006; Hawkesford and De Kok. 2006). Some years ago, SULTR5;2 was suggested also to function as molybdate transporter and since the absence of sulfate transport activity, it is renamed MOT1 (molybdenum transporter 1) (Tomatsu et al. 2007; Baxter et al. 2008).

SULFATE ASSIMILATION IN PLANTS

Plant sulfate assimilation, the metabolic pathways by which the inorganic sulfur form is converted by successive enzymatic steps in organic sulfur-containing compounds, (Saito 2004; Kopriva 2006) play an important function in the environmental sulfur cycle.

The cellular sulfate is assimilated by two metabolic pathways. The former is the reductive assimilation where once taken up from the soil is incorporated into adenosine-5′-phosphosulfate (APS), then reduced to sulfite (SO_3^{2-}) and sulfide (S^{2-}) in order to be

incorporated in the carbon skeletons of amino acid producing cysteine or homocysteine (Kopriva 2006).

For the assimilation in cysteine (Annex 5a) sulfate need to be transported into the plastids and activated through the adenylation reaction catalyzed by the enzyme ATP sulfurylase (ATPS) producing adenosine 5'-phosphosulfate (Saito 2004; Kopriva and Koprivova 2004; Kopriva 2006; Takahashi et al. 2011). Adenosine 5'-phosphosulfate is reduced by sulfite (SO_3^{2-}) by the enzyme APS-reductase using electrons derived from GSH. In turn, SO_3^{2-} is reduced to sulfide (S^{2-}) in a reaction catalyzed by the enzyme sulfite reductase (SiR) using six electrons derived from the reduced form of ferredoxin. Sulfide, a toxic molecule for the cells, is promptly incorporated by the activity of the enzyme *O*-acetylserine (thiol) lyase (OASTL) in the amino acid skeleton of *O*-acetyl serine (OAS) synthetizing cysteine. In turn OAS derives from the acetylation of serine with acetyl-Coenzyme A by the catalysis of the enzyme serine acetyltransferase (SAT).

Although cysteine (Cys) is the key metabolite in the synthesis of sulfur-containing compounds in plants, the major pool of non protein sulfur is the Cys-containing peptide GSH (Hell and Wirtz 2011). GSH plays a crucial role in plants such as cellular defense, redox status, signal transduction and detoxification (Noctor et al. 2012). Moreover, GSH forms conjugates, by the activity of GSH S-transferases, with electrophilic compounds such as heavy metal ions, secondary metabolites or xenobiotics via sulfhydryl group (Edwards and Dixon 2005; Cummins et al. 2011). The biosynthetic pathway of the tripeptide γ-glutamylcysteinylglycine (reduced form) GSH from cysteine involves two ATP-dependent reactions (Annex. 5a) catalyzed by two different enzymes. Firstly, the dipeptide γ-glutamylcysteine (γ-EC; γ-Glu-Cys) is synthetized by the enzyme γ-glutamylcysteine synthetase (γ-ECS) forming of a peptide bond between the amino group of cysteine and the glutamate. Secondly, the activity of the enzyme named glutathione synthetase (GS) allows the addition of a glycine at the C-terminal end of the γ-EC to produce GSH takes place. Both the enzymes have been found in the chloroplasts and cytosol (Noctor and Foyer 1998; Saito 2004; Takahashi et al. 2011).

The non reductive sulfate assimilation (Annex. 5b) take place in the cytosol. After that SO_4^{2-} is adenylated by the activity of the cytosolic ATP sulfuryalase (ATPS), APS is phosphorylated by the enzyme APS kinase (APK) to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS). This latter metabolite is used for sulfation reactions catalyzed by sulfotransferases (SOT) enzymes producing a variety of sulfonate organic compounds as

glucosinolates, flavonoids and jasmonates (Saito 2004; Kopriva and Koprivova 2004; Mugford et al. 2009; Takahashi et al. 2011; Ravilious and Jez 2012).

REGULATION OF SULFATE ASSIMILATION IN ARABIDOPSIS

Plant demand of reduced sulfur-containing compounds (i.e., Cys, γ-EC, and GSH) permits a high regulation of sulfate uptake and assimilation pathway (Leustek et al. 2000; Lappartient and Touraine 1996; Kopriva and Rennenberg 2004; Kopriva 2006; Takahashi et al. 2011).

The activities of the high affinity SULTR proteins and the enzyme APR are key points in controlling the sulfate reductive assimilation pathway in *Arabidopsis*. Both SULTRs and APR are regulated at transcriptional level as a function of availability of sulfate in the growth medium as well as the entity of the flux of the anion in the reductive pathway (Vauclare et al. 2002; Kopriva and Rennenberg 2004; Yochimoto et al. 2007; Scheerer et al. 2009).

Moreover, sulfate metabolism is also controlled at the level of the sulfite reductase (SiR) and of the mitochondrial SAT3 enzymes. The former enzyme controls the entire flow along the pathway. The latter provides adequate amount of amino acid carbon skeleton in order to efficiently incorporate the sulfide produced by SiR. The defective activity of both these enzymes causes severe growth and development limitation to the plant (Haas et al. 2008; Khan et al. 2010).

The transcriptional regulatory mechanisms modulating the uptake of sulfate and its assimilation is reasonably triggered by direct sensing of the sulfur nutritional status of the plant rather than from the concentration itself of sulfate in the external soil solution (Lappartient and Touraine 1997; Lappartient et al. 1999). The control involves an inter-organ signalling mechanism in which key intermediates of the sulfate assimilatory pathway may act as negative or positive signals in modulating the expression of sulfur-responsive promoters. Adequate levels of sulphur compounds would repress gene transcription of these genes through a negative feedback loop preventing excessive sulfate uptake and reduction. *Vice versa* a contraction in the cellular concentration of these intermediates of the assimilatory pathway would de-repress the transcription of the genes. The drawn picture defines a demand-driven model for the regulation of sulfate uptake and metabolism in plants (Lappartient and Touraine 1996).

Sulfate assimilation is regulated by Cys itself or some its metabolites upstream and downstream the pathways (Vidmar et al. 2000; Vauclare et al. 2002; Hopkins et al. 2005; Rouached et al. 2008; Takahashi et al. 2011). Firstly, it is largely reported that in Arabidopsis GSH acts as a negative modulator of the expression of the genes codifying for SULTR1:1, APR and γ-ECS. (Lappartient et al. 1999; Leustek et al. 2000; Vauclare et al. 2002; Hothorn et al. 2006) (Annex. 6). Secondly, the amino acid *O*-acetylserine (OAS) is considered as a positive modulator of the sulfate assimilatory pathway (Annex. 6). The existence of this regulation is suggested by analyzing the effects of exogenous OAS supply to the plant. Indeed, in the presence of sulfate when plant tissue are fed with OAS, the induction of numerous genes is observed; in particular SULTRs but also enzymes of sulfate reduction such as ATP sulfurylase and APR and many other including gene encoding enzymes of the breakdown of glucosinolate (Hell and Wirtz 2011 and therein references). OAS would act in promoting gene de-repression when nitrogen and carbon supply exceeds sulfur availability within the cells (Hawkesford 2000).

SULFUR METABOLISM UNDER SULFUR DEFICIENCY

As above described, sulfate transport and assimilation are regulated by mechanisms that involve specific or general intrinsic signals. The identification of the regulatory components of sulfate transport and assimilation is in progress (Schachtman and Shin 2007; Takahashi et al. 2011).

In 5'-promoter region of the gene *AtSULTR1;1* that in Arabidopsis codifies for a high affinity sulfate tranporter, a 16-bp sulfur-responsive element (SURE) containing a 5-bp core sequence (GAGAC) was identified (Maruyama-Nakashita et al. 2005). SURE was not present in the promoter region of the *AtSULTR1;2* gene. The responsive element is present also in the promoter region of the sulfur-responsive genes *SULTR2;1*, *SULTR4;2*, *APR3* and *NADPH* oxidoreductase (Maruyama-Nakashita et al. 2005).

Recently, the protein SLIM1 was identified as a transcriptional regulator of sulfate uptake, sulfur assimilatory metabolism and glucosinolates degradation in response to sulfate limitation. SLIM1 belongs to a group of ethylene insensitive3-like /EIL family transcription factor (Annex. 6). The function of SLIM1, differently from the other elements of the group, seems to be specific to sulfur response (Maruyama-Nakashita et al. 2006). Moreover, it has

been demonstrated that under sulfur starvation, SLIM1 controls the up-regulation of *SULTR1;2* but not of *SULTR1;1* suggesting the independence between this transcriptional regulator SLIM1 and the SURE *cis*-element (Davidian and Kopriva 2010).

Other components of the sulfur sensing and signaling system in plant are transcription factors belonging to the R2R3-type MYB family. In particular, MYB28, MYB29 and MYB76 induced the synthesis of aliphatic methionine-derived glucosinolates (Gigolashvili et al. 2007a and Hirai et al. 2007), whereas MYB34, MYB51 and MYB122 induced the synthesis of indole tryptophan-derived glucosinolate (Celenza et al. 2005 and Gigolashvili et al. 2007b). MYB34 and its downstream enzymes were negatively controlled by SLIM1 in response to sulfur limitation (Maruyama-Nakashita, et al. 2006). In other words, it is suggested that under sulfur starvation the activation of SLIM1 down regulates the MYB expression thus reducing the synthesis of glucosinolates favoring its reductive assimilation (Takahashi et al. 2011; Annex 6). This hypothesis is still under investigation since the effect of SLIM1 on MYB28 and MYB29 is unclear (Hirai et al. 2007). The R2R3-MYB transcription factors are also able to control genes of primary sulfate assimilation enzymes (ATPS, APK and APR genes) (Annex. 6) (Yatusevich et al. 2010).

MicroRNAs (miRNAs) are involved in the regulation of S metabolism (Mallory and Vaucheret 2006). In particular, recent results suggest the involvement of miRNAs in the responses to nutrient deprivation (Schachtman and Shin 2007). In sulfur limitation conditions, SLIM1 induces an increase miR395 of the level of miR395 in both shoots and roots of *A. thaliana* plants (Kawashima et al. 2009). In phloem companion cells, miR395 targets *SULTR2;1* (Annex. 6; Jones-Rhoades and Bartel 2004; Kawashima et al. 2009) leading to posttranscriptional degradations of its transcript. The suppression of *SULTR2;1* in shoot contribute to limit the distribution of sulfur from older to younger leaves (Liang et al. 2010).

Cysteine is a negative modulator of the activity of the enzyme serine acetyltransferase (SAT) (Annex. 6). Moreover, cysteine synthesis is post-translationally regulated by a protein-protein interaction at the level of the cysteine synthase complex (CSC). In plastid, cytosol and mitochondria the complex is stable if the concentration of sulfide (S^{2-}) maintain the SAT subunities in their active conformation. Under sulfate limitation, the cellular level of sulfide (S^{2-}) decline and OAS accumulates triggering the CSC to dissociate and in turn reducing the SAT activity to avoid OAS production. Therefore, the plant CSC functions as a determinant of cysteine biosynthesis, sensing the availability of sulfide. In turn, the accumulation of OAS due to CSC inactivation leds to the de-repression of genes encoding enzymes for sulfate uptake and reduction (Hawkesford and De Kok 2006; Writz and Hell 2006, 2007).

Several studies identified the plant hormones cytokinin, auxin, and jasmonate as signaling components in response to sulfur deficiency (Schachtman and Shin 2007). When *A. thaliana* plants are grown under sulfur deficiency, the expression of the gene *APR1* (APS reductase 1) is upregulated also by exogenous cytokinin supply (Ohkama et al. 2002). Maruyama-Nakashita and coworkers (2004) demonstrated that the exogenous application of cytokinin downregulates the expression of the high-affinity transporter SULTR1;2 which is upregulated by sulfur deprivation. The effect of cytokinin take place through the cytokinin response receptor (CRE1) (Schachtman and Shin 2007).

Several auxin responsive genes are upregulated by sulfur starvation (Hirai et al. 2003; Nikiforova et al. 2003). The enzyme nitrilase catalyzes the conversion of indole-3-acetonitrile in indole-3-acetic acid (IAA). The expression of the gene codifying for this enzyme (*NIT3* nitrilase) is markedly increased in sulfur deficient plants (Kutz et al. 2002; Maruyama-Nakashita et al. 2004). In *A. thaliana*, it has been suggested that the increased IAA production stimulates the differentiation of later roots thus improving plant capacity to absorb sulfate from the media (López-Bucio et al. 2003).

Finally, jasmonate (JA) is also suggested as possible signaling component of plant response to sulfur deficiency. Indeed, under low sulfate conditions, genes of the JA biosynthesis pathway are upregulated by jasmonate (Hirai et al. 2003, Jost R et al. 2005). Moreover, both JA and methyl-JA application positively affect the activity of some sulfur assimilation enzymes (Jost R et al. 2005).

OBJECTIVES

The general aim of this thesis was to develop plant bioindicators, exploiting the gene fusion concept, in which the expression of a reporter gene is under the control of a promoter sensing the sulfur nutritional status of the plants.

The rational of the work envisages:

a) The identification, by a gene-trap approach in *Arabidopsis*, and the characterization of sulfate responsive promoters;
b) The identification of sulfur responsive and regulative elements (*cis-acting elements*) within the putative promoters;
c) The exploitation of the regulative elements in generating recombinant promoters able to specifically control the expression of reporter genes as a quantitative function of the sulfur nutritional status of the plant.

MATERIAL AND METHODS

PLANT MATERIAL

All Arabidopsis (*Arabidopsis thaliana*) plants used in this study were in the Landsberg erecta (Ler) or Wassilewskija (Ws) backgrounds. The ecotype Landsberg erecta (Ler) wild-type or belonging to the collection EXOTIC (generated by Exon Trapping Insert Consortium) mutant gene traps and the ecotype Wassilewskija (Ws) wild-type or T-DNA insertion mutant line obtained from Versailles's T-DNA collection (National Institute for Agricultural Research (INRA) Versaille, France) were used for the experiments.

PLANT GROWTH CONDITIONS

Sterilization of seeds

The seeds were firstly surface sterilized by Tween 20 (0.01%) for 20 min, washed with 70% (v/v) ethanol for 2 min and rinsed with distilled water followed by immersion in an equal volume of sodium hypochlorite solution as sterilizing agent for 10 min. At the end of the treatment, seeds were then washed four times with sterile distilled water to be finally resuspended in 1 ml of sterile distilled water.

Sowing, culture and treatments

The sowing took place in three different mediums:

- The first, plants were grown on sterile plates containing agar medium.
  The experimental plan used for the different tests is summarized below:
  - Analysis of sulfur deficiency effect on the expression of the genes flanking the intergenic region
    Seeds of FLAG line and wild-type (ecotype Ws) were planted on 0.8% (w/v) agar medium containing 1% (w/v) sucrose at two concentrations of sulfate, +S (1500 µM) and -S (0 µM), for 14 days. Concentrations of sulfate were adjusted by replacing this ion with equal moles of chloride to maintain the same concentration of magnesium (Annex. 7).

- Selection and propagation of transformant lines

  Seeds of transgenic plant lines (ecotype Ler) were sown directly on suitable medium: 25 ml of 0.8% (w/v) agar medium containing 1% (w/v) sucrose and half-strength MS salts (Murashige and Skoog 1962) supplemented with 50 μg ml^{-1} kanamycin.

  Transformants T_1 kanamycin resistant seedlings were transferred into plastic pots (5 cm diameter) with soil (Tercom plus) and were grown to maturity producing the T_2 seeds. The vernalised T_2 seeds of the transformed T_1 lines were sown on soil wet with water in array of 42 individual pots placed on solid plastic trays in the growth chamber. Upon inflorescence development, plants were covered to prevent the cross-fertilization and in each pot was placed a plastic container for the collection of seeds and plastic tube inside this container to prevent touching neighboring plants (www.arasystem.com). At maturation, the progeny families (T_3 seeds), from individual selfed T_2 plants, were collected and chronologically coded according to the plant of origin.

- Culture on agar medium with different sulfur sources:

  Seeds were planted on 1.25% (w/v) agar medium containing 1% (w/v) sucrose with two different concentrations of sulfate (+S: 1500 μM and -S: 0 μM). For glutathione and cysteine treatments, -S agar medium solution was supplemented with 1.5 mM GSH and 1.5 mM Cys, and plants left grow vertically for 20 days. Concentrations of sulfate in culture solutions were adjusted by replacing this ion with equal moles of chloride to maintain the same concentration of magnesium (Annex. 7).

- Culture on agar medium in the absence or presence of sucrose.

  Seeds were planted on 1.25% (w/v) agar medium containing or not 1% (w/v) of sucrose at two different concentrations of sulfate (+S: 1500 μM and –S: 0 μM) and plants left grow vertically for 20 days. Concentrations of sulfate in culture solutions were adjusted by replacing this ion with equal moles of chloride to maintain the same concentration of magnesium (Annex. 7).

- Culture on agar medium with the presence of cadmium (Cd):

  The seeds of the transgenic lines were sown on complete 1.25% (w/v) agar media plates containing 1% (w/v) sucrose supplemented with cadmium ($CdCl_2$) and the plates were placed vertically for 20 days.

- Gradient plates

  To have increasing concentrations of sulfate in the medium, we used agar plates with gradient of sulfate concentrations. SO_4^{2-}-gradient plate termed SGAP (0–150 μM)

was prepared using 224 ml SO_4^{2-}-plus medium and 277.8 ml SO_4^{2-}-free medium. The agar medium was prepared using the basic nutrient solution (Annex. 7), 1% (w/v) sucrose, 1.25% agar to prevent roots penetrating the surface of the medium. For plates with Cd, it was in the form $CdCl_2$. SO_4^{2-}-plus medium contains $MgSO_4^{2-}$ at a concentration of 150 µM. In the presence of Cd, SO_4^{2-}-plus and free medium contained $CdCl_2$ at a concentration of 50 µM. For the preparation of SGAP (0-150 µM), a square Petri dish (22.4 cm × 22.4 cm) was placed in an inclined position on a glass bar (Annex. 8a). At the left end of the dish, a SO_4^{2-}-free zone was about 2.4 cm which represent the limit of the melted SO_4^{2-}-plus medium poured into the bottom of the dish. After solidified, the dish was placed in a horizontal position then melted SO_4^{2-}-free medium was poured into the dish until it covered the solidified SO_4^{2-}-plus medium. Solidified SGAPs were used directly in experiment for sowing seeds (Watanabe et al. 2010).

Arabidopsis FLAG seeds were sown on a SGAP in horizontal line (11.2 cm distant from the top of the plate) across the gradient (Annex. 8b) from the left to the right side of the plate respectively from the lowest to the highest sulfate concentration. Plates then were transferred to growth chambers and Arabidopsis seedlings were grown vertically for 14 days.

- The second, in soil (Tercom plus) to grow plants for floral dip transformation or to produce seeds.

  For transformation, 10 plants (ecotype Ler) were grown in 7 cm square pots in soil (Tercom plus) until the flowering stage in the growth chamber. Plants were dipped when most inflorescences were about 1–10 cm tall (Clough and Bent 1998).

- The third, *Arabidopsis thaliana* plants (ecotype Ler and Ws) were grown hydroponically under non sterile conditions.

  Firstly, seeds were germinated directly on top of modified plastic pipette tips filled with prewetted Grodan. The tips were then placed on plastic tray with direct contact with tap water. After 7 days, seedlings in plastic tips were positioned on floating rafts and transferred to 3-L plastic tanks (40 seedlings per tank) containing ½ Hoagland nutrient solutions (Annex. 9). Concentrations of sulfate in culture solutions were adjusted by replacing this ion with equal moles of chloride to maintain the same concentration of magnesium (Annex. 9). Nutrient solutions were renewed weekly and plants were grown for 14 d before the experiments.

In all cases, the seeds were vernalized at 4°C for 4 days to break dormancy, sowed, and then transferred in a growth chamber. Seedlings were grown under a 12-h photoperiod at 23±2 °C and 70% relative humidity. At the level of the plants, light intensity was 100 µmol m^{-2} s^{-1}.

DETERMINATION OF NON-PROTEIN THIOLS (NPT)

Total non protein thiol (NPT) were extracted and determined by this methodology. Immediately after harvesting, shoot were frozen and then pulverized using mortar and pestle in liquid N_2. NPT were extracted by grinding 200 mg of shoot powders in 600 µl of a mixture containing 1 M NaOH and 1 mg ml^{-1} $NaBH_4$, and the homogenate was centrifuged for 5 min at 13000 *g* and 4°C. The last centrifugation was repeated with the resultant supernatant. The collected 400 µl of supernatant were neutralized by adding 66 µl of 37% HCl and then centrifuged again for 10 min at 13000 *g* and 4°C. Then, volumes of 200 µl of the supernatant were collected and mixed with 800 µl of 1M K-Pi buffer (pH 7.5) containing or not 0.6 mM Ellman's reagent [(5,5'-dithiobis(2-nitrobenzoic acid); DTNB)] which react with thiols to form TNB quantified spectrophotometrically by measuring the samples' absorbances at 412 nm (Nocito et al. 2011). Thiol concentration was then determined by interpolating the obtained values in a calibration curve previously constructed by determining the level of total GSH. All results were expressed as nanomoles of GSH equivalents.

DETERMINATION OF GLUTATHIONE (GSH)

The total glutathione of a tissue is mainly represented by the reduced form (GSH), while the oxidized glutathione disulfide (GSSG) represents a minimum portion. Total GSH was measured according to Anderson (1985) by using an enzymatic assay that evaluates the rate of oxidation of GSH through the coupling of two separate reactions, a non-enzymatic and enzymatic. In the first GSH is oxidized by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to give GSSG with formation of 5-thio-2-nitrobenzoic acid (TNB); in the second reaction, catalyzed by the enzyme glutathione reductase, the GSSG is reduced to GSH with consumption of NADPH. The rate of oxidation of GSH was followed by evaluating the rate of formation of TNB at 412 nm. The assay was prepared in a stock buffer (pH = 7.5) containing 143 mM potassium phosphate buffer (KPi) and Na_2-EDTA 6.3 mM, a daily buffer, consisting of the

stock buffer to which was added NADPH (0.248 mg ml^{-1}), DTNB 6 mM in stock buffer, and glutathione reductase (50 U ml^{-1}) in stock buffer.

Briefly, shoot samples of about 150 mg were homogenized in a mortar at 4°C in 400 µl of 5-sulfosalicylic acid 5% (w/v) to remove the proteins. The homogenate was centrifuged at 14000 *g* for 30 min, and then recuperate 400 µl of supernatant for each sample. The reaction mixtures were prepared by adding 700 µl of daily buffer, 50 µl or 100 µl of sample, 100 µl of DTNB and distilled H_2O to reach a final volume of 1 ml and then kept at 30°C for 2-3 min. The reaction was started by adding 10 µl of glutathione reductase.

The formation of TNB was followed spectrophotometrically in a time interval of 3 min. The concentration of total glutathione in the tested samples is determined from obtained calibration curve previously constructed using the method described with predetermined amounts of GSH.

ANALYSIS OF *GUS* EXPRESSION

The *GUS* reporter gene coding for the protein β-glucuronidase which catalyzes the cleavage of a great variety of β-glucuronic acid derivatives, which are commercially available as substrates for spectrophotometric, fluorometric and histochemical assays (Jefferson 1987).
Histochemical assay was performed and β-glucuronidase activity was detected through staining with the acid 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). The enzyme β-glucuronidase is able to cleave this molecule releasing a molecule of glucuronic acid and one molecule of 5-bromo-4-chloro indigo, that following an oxidative dimerization reaction form an insoluble and blue precipitate (5'-dibromo-4, 4'-dichloro-indigo).
For the histochemical localization of *GUS* activity, at the end of the growth period the plants were incubated with 3 ml of GUS staining solution composed as follows: 50 mM sodium phosphate (NaPi) at pH 7.0, 0.5 mM fericyanate stock, 0.1% (v/v) Triton X100, X-Gluc (0.5 mg ml^{-1}) conveyed in dimethyl sulfoxide (DMSO). The plants were incubated overnight at 37°C and in the absence of light. At the end of the incubation period, the observation of the organs under a binocular microscopy was preceded by discoloration of the tissues with 100% ethanol (v/v) to remove chlorophyll.

TOTAL RNA EXTRACTION AND QUANTIFICATION

The total RNA extraction was conducted in TRIzol® reagent (LifeTechnologies), in accordance with the protocol reported by the manufacturer.

Frozen samples were pulverized in liquid N_2 within pre-cooled mortars. The powders (200 mg) were transferred into sterile centrifuge tubes, added with 1 ml of TRIZOL®, mixed and kept at room temperature for 5 min. The samples were then added with 200 µl of chloroform and centrifuged at 12000 *g* for 15 min at 4°C. The upper aqueous phase (400 µl) was taken and mixed with 400 µl of 70% ethanol (v/v) to precipitate the total RNA. 700 µl of the sample were transferred to a Spin Catridge with a Collection Tube and centrifuged at 12000 *g* for 15 s at room temperature. Then, the flow-through was discarded, 700 µl of Wash Buffer I was added to the Spin Catridge to be centrifuged at 12000 *g* for 15 s at room temperature. After transferring the Spin Cartidge to a new Collection Tube, 500 µl of Wash Buffer II with ethanol was added and centrifugation at 12000 *g* for 15 s at room temperature was done (step to repeat once). Centrifuge at 12000 *g* for 1 min at room temperature to dry the membrane then transfer the Spin Cartidge into a Recovery Tube where 30 µl of DEPC H_2O was added. Finally, after incubation at room temperature for 1 min, centrifuge at 12000 *g* for 2 min at room temperature and stored at -20°C. The total RNA was quantified by using the Thermo Scientific NanoDrop 1000 Spectrophotometer measuring the concentration in ng/µl based on absorbance at 260 nm of 1µl nucleic acid sample. In order to verify the quality of the RNA, the same spectrophotometer measure the ratio of sample absorbance at 260 and 280 nm used to assess the purity of RNA (ratio 260/280 of ≈2.0 accepted as pure RNA) and the ratio of sample absorbance at 260 and 230 nm used as secondary measure of the purity of RNA (ratio 260/280 in the range of 1.8-2.2 accepted as pure RNA).

ISOLATION OF cDNA ENCODING GENES *AT1G12030, GUS* AND *S16,* AND EVALUATION OF GENE EXPRESSION

Through the technique of RT-PCR (Reverse Transcriptase-PCR), cDNA coding for the genes flanking the intergenic region in the FLAG line, for *GUS* and *S16* gene were isolated. This procedure combines the reverse transcriptase reaction with a normal technique of DNA amplification, to synthesize specific cDNA molecules, using RNA as a template. To obtain the first-strand cDNA, it was used the protocol of SuperScript III first-strand synthesis system for RT-PCR (LifeTechnologies) according to the manufacturer's instructions.

Before the application of the mentioned protocol and in order to remove contaminating DNA from RNA preparation, it was used the protocol of Amplification grade DNase I (LifeTechnologies) according to the manufacturer's instructions. It was prepared a mixture containing 8 µl of RNA, 1 µl 10× DNase I buffer and 1 µl DNase I Amplification grade. After incubation for 15 min at room temperature, 1 µl of 25 mM EDTA solution was added to inactivate DNase I. Then, it was added a mixture containing 1 µl of 10 mM dNTPs mix and 1 µl of Oligo $dT_{(20)}$, which was subsequently incubated at 65° C for 5 min for heat inactivation and kept on ice for at least 1 min.

The RNA sample is now ready to be used directly in a reverse transcription reaction. The samples were then added 10 µl of cDNA synthesis mix containing 2 µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT and 1 µl of RNaseOUT (Recombinant RNase Inhibitor). Subsequently was added to each sample 1 µl of SuperScript III RT (reverse transcriptase) to begin the reverse transcription reaction that occurred at a temperature of 50°C in a time of 50 min before being terminated by bringing the samples to 85°C for 5 min in order to denature the enzyme. The mixture was cooled in ice for 2 min and centrifuged briefly; added then 1 µl of RNase H and then incubates at 37°C for 20 min.

The subsequent cDNA amplification reactions were carried out by PCR on 2 µl aliquots of the diluted mixture with distilled H_2O.

The primer pairs used to amplify the sequences of *AT1G12030* gene, *GUS* gene and of the gene *S16* in the FLAG line were designed and are listed in the Annex 10a. Preparing reaction mixtures containing cDNA, the pair of primers specific for the sequence, each at a concentration of 10 µM, 2,5 mM dNTPs, 5 u/µl of GoTaq® DNA polymerase (Promega), the corresponding buffer 5x (GoTaq® Flexi Buffer), 2 mM $MgCl_2$, 25 mM dNTPs and water to achieve a final reaction volume of 17 µl (Annex. 10b). To each sample were added two drops of mineral oil to prevent evaporation during the PCR cycles. The thermal profile used for each amplification reaction is given in Annex 10c. The PCR products were then visualized by electrophoresis on agarose gel. The primers, the reaction mixtures and the thermal profiles described above were used for the assessment of gene expression by semiquantitative RT-PCR.

AMPLIFICATION OF FRAGMENTS OF INTEREST

The fragments of interest were amplified by PCR from MIDI 718 vector containing the intergenic region 1331-bp fragment using primers designed on the fragments of interest (Annex. 11a).

In the PCR reaction, 2 µl of DNA from a 1:1000 dilution of MIDI 718 vector was amplified using various primer pairs: *1331 for*, *440 rev* for the amplification of 440-bp with the 5' sequence of each primer was extended 10-bp to generate an Xba I site (underlined) (Annex. 11b.1). For the amplification of 1331-bp, we used *1331 for* and *1331 rev* with the 5' sequence of each primer was extended 10-bp to generate a Hind III site (underlined) (Annex. 11b.2). Each sample was amplified in quintuplicate. The sequences of these primers, the reaction mixture and the thermal profile of the reaction are shown in Annex 11b, c and d.

The products of the amplifications were loaded on agarose gel 1% (w/v) in adjacent lanes. The gel was prepared by dissolving a suitable amount of agarose in 1x TBE buffer [89 mM Tris, 89 mM H_3BO_3, 2 mM Na_2-EDTA (pH = 8.2)] supplemented with ethidium bromide (5 mg ml^{-1}).

The results of electrophoresis were visualized on a UV transilluminator. Image analysis allowed identifying products of interest which were eluted and then cloned into a vector.

CLONING OF AMPLIFIED PRODUCTS

The resulting fragments of interest were extracted and purified according to the protocol of DNA and gel band purification kit (Promega) by centrifugation from agarose gels in Tris borate (TBE) (Wizard® SV Gel and PCR Clean-Up System). We proceeded to DNA purification procedure by centrifugation. Starting with dissolving the gel slice in a 1.5 ml microcentrifuge tube by Adding 10 µl Membrane Binding Solution per 10 mg of gel slice, mixed with vortex and incubated at 50–65°C for 5 min. Then the dissolved gel mixture was transferred the Minicolumn assembly, incubated at room temperature for 1 minute and centrifuged at 16000 *g* for 1 min to bind the DNA which was then washed with 700 µl Membrane Wash Solution (ethanol added), centrifuged at 16000 *g* for 1 min and followed by another wash by adding 500 µl Membrane Wash Solution and a centrifugation at 16000 *g* for 5 min. To allow the evaporation of any residual ethanol, a recentirifugation for 1 min was done to reach the elution by adding 20 µl of PCR water to the Minicolumn present in clean 1.5 ml microcentrifuge tube and then Incubated at room temperature for 1 min and a final

centrifuge at 16000 *g* for 1 min. The purified fragments were ligated, according to the protocol Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen), into the plasmid vector pCR® Blunt II-TOPO®, which allows a high-efficiency ligation of the insert.

The ligation reactions were conducted for 25 min at room temperature in a mixture consisting of 2 µl purified amplicon, 0,5 µl of the linearized pCR® Blunt II-TOPO® vector and 0,5 µl of salt solution (1.2 M NaCl, 0,06 M $MgCl_2$).

The TOPO® Cloning Reaction is transformed into chemically competent cells. The transformation reactions were conducted by adding 2 µl of TOPO® Cloning Reaction to 25 µl of One shot® TOP 10 chemically competent E. coli cells, which were then kept on ice for a period of 15 min. Then followed with heat-shock at 42°C for 30 seconds, and after the cells were immediately placed in ice. After adding 250 µl of LB medium, cells were then incubated at 37°C for 1 hour with continuous shaking. An aliquot of the processed (130 µl) transformation was finally transferred on plates of LB agar medium supplemented with kanamycin (50 µg ml^{-1}), which were incubated at 37°C for 12 hours. pCR® Blunt II-TOPO® permit direct selection of recombinants via disruption of the expression of the lethal gene *ccd*B of E. coli, fused to the LacZα fragment and since the plasmid has the gene for resistance to the antibiotic kanamycin; so the only cells able to grow on medium with added antibiotic are, therefore, the transformed one and containing the blunt-end PCR product (insert).

Positive colonies were picked and used to inoculate LB media with agar and were put in LB media containing 50 µg ml^{-1} kanamycin to incubate overnight at 37°C and then the liquid cultures were used for the extraction of the recombinant plasmid.

The isolation of plasmid DNA from transformed cells was conducted according to the protocol of PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). From 5 ml overnight liquid cultures were taken 2 ml which were subsequently centrifuged at 5000 *g* for 3 min to allow the precipitation of the bacterial cells. The pellets were then resuspended in 250 µl of resuspension buffer and added with 250 µl of lysis buffer, and then the cells were maintained at room temperature for a maximum time of 5 min. They were then added to 350 µl of a precipitation buffer and, after mixing, centrifugation at 12000 *g* for 10 min. The supernatant was loaded onto a Spin column. We proceeded to purification procedure using centrifugation and a wash tube containing the spin column was centrifuged at 12000 *g* for 1 min, a wash buffer was added followed by a centrifugation at 12000 *g* for 1 min. The plasmids were finally eluted by washing the column with 75 µl of preheated H_2O and centrifuged at 12000 *g* for 2 min after incubation for 1 min at room temperature.

PLANT EXPRESSION VECTOR CONSTRUCTION

To generate a binary vector containing the *GUS* gene fused to both constructs, two different fragments (444 and 1331-bp) were inserted into pBI101.3 (Annex. 12), an empty plant transformation cloning vector resistant to kanamycin (www.arabidopsis.org) containing *GUS* gene, leading to pBI101.3-440 and pBI101.3-1331.

Plasmid construction with 35Smp

To generate a reporter construct containing both fragments fused to the 35S minimal promoter, 35Smp sequence was first inserted upstream of the *uidA* reporter gene in pBI101.3 vector. To obtain the desired sequence of 35Smp, we synthesized an oligonucleotides corresponding to its sequence with the 5'sequence of each oligonucleotide was extended 5-bp and the 3'sequence 1-bp to generate an Xba I and BamHI site (underlined) in Oligo1 and Oligo2. The sequences of these oligonucleotides are shown in Annex 13a. The annealing reaction mixture and the thermal profile of the reaction are shown in Annex 13b and c.

Firstly, the plasmid pBI101.3 was digested with the two enzymes Xba I and BamHI, to introduce the 35Smp sequence. The restriction reactions were performed at 37°C for 240 min and then to 65°C for 15 min in mixtures containing 14 µl of plasmid DNA, 10 U of Xba I and BamHI (1 µl), 2 µl 10x buffer E, 2 µl BSA (1:10) and H_2O up to a final volume of 20 µl. After the digestion, we proceeded to the ligation of 35Smp. The reactions of ligation were conducted at 22°C for 180 min in mixtures containing 3 µl of the digested plasmid DNA, 1 µl of the insert (1:20), 1 µl 10x ligase buffer, 3U T4ligase and H_2O up to a final volume of 10 µl.

The resultant plasmid pBI101.3-35Smp was used to transform *E. coli* competent cells; XL1-Blue strain as a host for propagation of the plasmid. The transformation reactions were conducted by adding 10 µl of the resultant plasmid to 200 µl of the competent *E. coli* cells, which were then kept on ice for a period of 30 min. Then followed with heat-shock at 42°C for 60 seconds, and after the cells were immediately placed in ice for 1 min. After adding 1 ml of LB medium and then cells were incubated at 37°C for 1 hour with continuous shaking. An aliquot of the processed (300 µl) transformation was finally transferred on plates of LB agar medium supplemented with kanamycin (50 µg ml^{-1}), which were incubated at 37°C for 12 hours. From some *E. coli* colonies found, colony PCR permitted to screen for plasmid inserts

(35Smp) if it is present or not. The sequences of the primers used, the reaction mixture and the thermal profile of the reaction are shown in Annex 14.

Positive colonies were picked and used to inoculate LB with agar and were put in LB medium containing 50 μg ml^{-1} Kanamycin to incubate overnight at 37°C and then the liquid cultures were used for the extraction of the recombinant plasmid.

The isolation of plasmid DNA from transformed cells was conducted according to the protocol of PureYield™ Plasmid Midiprep System (Promega). We proceeded to DNA purification procedure by centrifugation and 50 ml overnight liquid bacteria cultures were firstly centrifuged at 3500 *g* for 15 min to allow the precipitation of the bacterial cells. The pellets were then resuspended with 3 ml Cell Resuspension Solution and subsequently added with 3 ml of Cell Lysis Solution followed by a gentle mix and incubation for 2-3 min at room tempreature. They were then added 5 ml Neutralization Solution followed also by a gentle mix and incubation in upright position for 3 min. After that, the lysate was loaded onto a Clearing column present into a new 50 ml disposable plastic tube, incubated for 2 min to collect cellular debris and centrifugation at 1500 *g* for 5 min. Then, the filtered lysate was poured onto a Binding column present into a new 50 ml disposable plastic tube which was centrifuged at 1500 *g* for 5 min. For wash step, Endotoxin Removal Wash solution was added followed by a centrifugation at 1500 g for 3 min. After discarding the flowthrowgh, 20 ml of Column Wash solution was added and followed by a centrifugation at 1500 *g* for 5 min. An additional centrifugation at 1500 *g* for 10 min to ensure the removal of ethanol present in Column Wash solution. The plasmids were finally eluted by washing the binding column with 600 μl of preheated H_2O and centrifugation at 2000 *g* for 5 min. The clone that showed an insert of the expected size was then sequenced.

Plasmid construction with 440-bp

To introduce the 440-bp fragment of interest in the pBI101.3-35Smp, a separated digestion of obtained plasmids with the enzyme XbaI was done. Concerning the digestion of the plasmid TOPO-440 bp, the reactions of restriction were conducted at 37°C for 60 min (3 cycles) followed with a cycle of 65°C for 15 min in mixtures containing 20 μl of plasmid DNA, of 10 U XbaI , 3 μl 10x buffer D, 3 μl BSA (1:10) and H_2O up to a final volume of 30 μl. Concerning the digestion of the plasmid pBI101.3-35Smp, the reactions of restriction were conducted at 37°C for 60 min (16 cycles) followed with a cycle of 65°C for 15 min in mixtures containing 20 μl of plasmid DNA, 3 U of XbaI (0,3 μl), 3 μl 10x buffer D, 3 μl BSA

(1:10) and H_2O up to a final volume of 30 µl. The resultant digested vector was redigested with 20 U XbaI (2 µl) at 37°C for 75 min followed with a cycle of 65°C for 15 min. The restriction fragments were, then, displayed by electrophoresis on agarose gel. pBI 101.3-35Smp and 440-bp DNA bands was excised from gel and purified by centrifugation using Wizard® SV Gel and PCR Clean-Up System (Promega). We proceeded to DNA purification procedure by centrifugation. The volume of the eluted DNA was 15 µl and 20 µl PCR water respectively for 440-bp DNA fragment and for pBI 101.3-35Smp.

After the digestion**,** the vector DNA pBI 101.3-35Smp was then dephosphorylated. The reactions of dephosphorylation were conducted at 37°C for 15 min followed with 74°C for 15 min in mixtures containing 15 µl of plasmid DNA, 2 µl 10x buffer D, 2 µl BSA (1:10), 1 µl TSAP (Thermosensitive Alkaline Phosphatase) and H_2O up to a final volume of 20 µl. After the dephosphorylation, we proceeded to the ligation of 440-bp DNA fragment. The reactions of ligation were conducted at 4°C overnight in mixtures containing 5 µl of the digested/dephosphorylated plasmid DNA, 3 µl (3/1 molar ratio of insert:vector) and 1 µl (1/1 molar ratio of insert:vector) of the 440-bp DNA insert (1:10), 1 µl 10x ligase buffer, 3U T4 DNA ligase and H_2O up to a final volume of 10 µl.

The resultant plasmid pBI101.3-35Smp-440 bp was used to transform *E. coli* competent cells; XL1-Blue strain as a host for propagation of the plasmid. After incubation overnight, *E. coli* colonies found in the two molar ratios of vector: insert were picked and used to inoculate LB with agar and were put in LB medium containing 50 µg ml^{-1} kanamycin to incubate overnight at 37°C and then the liquid cultures were used for the extraction of the plasmid (pBI101.3-35Smp-440 bp).The isolation of plasmid DNA from transformed cells was conducted according to the protocol of PureLink™ Quick Plasmid Miniprep Kit (Invitrogen).

Plasmid construction with 1331-bp

To introduce the 1331-bp fragment of interest in the pBI101.3-35Smp, a separated digestion of obtained plasmids with the enzyme Hind III was done. Concerning the digestion of the plasmid TOPO-1331 bp, the reactions of restriction were conducted at 37°C for 60 min (16 cycles) followed with a cycle of 65°C for 15 min in mixtures containing 20 µl of plasmid DNA, of 10 U Hind III (1µl), 3 µl 10x buffer E, 3 µl BSA (1:10) and H_2O up to a final volume of 30 µl. The restriction fragments were, then, displayed by electrophoresis on agarose gel. 1331-bp DNA bands was excised from gel and purified by centrifugation using Wizard® SV Gel and PCR Clean-Up System (Promega). We proceeded to DNA purification

procedure by centrifugation and by adding 20 µl PCR water for the elution. Concerning the digestion of the plasmid pBI101.3-35Smp, the reactions of restriction were conducted at 37°C for 240 min followed with a cycle of 65°C for 15 min in mixtures containing 20 µl of plasmid DNA, 3 U of Hind III (1µl), 3 µl 10x buffer E, 3 µl BSA (1:10) and H_2O up to a final volume of 30 µl. After the digestion**,** the vector DNA pBI101.3-35Smp was then dephosphorylated. The reactions of dephosphorylation were conducted at 37°C for 15 min followed with 74°C for 15 min in mixtures containing the 30 µl mixture of plasmid DNA digestion with 2 µl TSAP and without extra buffer. The dephosphorylated vector was, then, displayed by electrophoresis on agarose gel. Plasmid DNA band was excised from gel and purified by centrifugation using Wizard® SV Gel and PCR Clean-Up System (Promega). We proceeded to DNA purification procedure by centrifugation and by adding 20 µl PCR water for the elution.

After the dephosphorylation, we proceeded to the ligation of 1331-bp DNA fragment. The reactions of ligation were conducted at 22°C for 4 hours and then 4°C overnight in mixtures containing 4 µl of the digested/dephosphorylated plasmid DNA, 4 µl (3/1 molar ratio of insert:vector) of the 1331-bp DNA insert (1:10), 1 µl 10x ligase buffer, 3U T4 DNA ligase (0.33 µl) and H_2O up to a final volume of 10 µl.

The resultant plasmid pBI101.3-35Smp-1331 bp was used to transform *E. coli* competent cells; XL1-Blue strain as a host for propagation of the plasmid. After incubation overnight, *E. coli* colonies found were picked and used to inoculate LB with agar and were put in LB medium containing 50 µg ml^{-1} Kanamycin to incubate overnight at 37°C and then the liquid cultures were used for the extraction of the plasmid (pBI101.3-35Smp-1331 bp). The isolation of plasmid DNA from transformed cells was conducted according to the protocol of PureLink™ Quick Plasmid Miniprep Kit (Invitrogen).

CONFIRMATION OF THE PRESENCE AND THE DIRECTION OF INSERTS

In order to verify the results relating to the presence of 440-bp insertion, this DNA fragment was then searches by digestion of the plasmids obtained with the enzyme XbaI. The digestion of the plasmid pBI101.3-35Smp-440 bp, the reactions of restriction were conducted at 37°C for 60 min (16 cycles) followed with a cycle of 65°C for 15 min in mixtures containing 10 µl of plasmid DNA, 3 U of XbaI (0.3µl), 2 µl 10x buffer D, 2 µl BSA (1:10) and H_2O up to a final volume of 20 µl. The restriction fragments were, then, displayed by electrophoresis on agarose gel. In order to verify the direction of the 440-bp insert in the

vector, amplification reactions were conducted using 2 µl of DNA template (1:1000) present in different clones founds. The primer pairs used were 3 primers (Annex. 15). *pBI for* and *440 rev* in the first reaction mixture searching for 440-bp (+) (Annex. 15a) and *pBI for* and *1331 for* searching for 440-bp (-) (Annex. 15b). The sequences of these primers are shown in Annex 11b.1 and Annex 14a, the reaction mixtures and the thermal profiles of the reaction are shown in Annex. 14b and 14c1.

In order to verify the direction of the 1331-bp insert in the vector, amplification reactions were conducted using 2 µl of DNA template (1:1000) present in different clones founds. The primer pairs used were 3 primers (Annex. 16). *pBI for* and *1331 rev* in the first reaction mixture searching for 1331 bp (+) (Annex. 16a) and *pBI for* and *1331 for* searching for 1331 bp (-) (Annex. 16b). The sequences of these primers are shown in Annex 11b.2 and Annex 14a, the reaction mixtures and the thermal profiles of the reaction are shown in Annex 14b and 14c2.

The amplified fragments were, then, displayed by electrophoresis on agarose gel and the clones that showed the presence of the insert (440-bp) with the expected size in the different orientations (+/-) were then subjected to sequencing.

TRANSFORMATION OF *ARABIDOPSIS THALIANA*

We proceeded stable transformation of *Arabidopsis thaliana* via *Agrobacterium tumenfaciens*-mediated transformation and transformed plants in the progeny were characterized.

Electroporation of *Agrobacterium tumefaciens*

In plant genetic engineering, *Agrobacterium*-mediated transformation is the most commonly used method. Electrocompetent *Agrobacterium tumefaciens* cells strain was used. On ice, these cells were thawed. For each DNA sample to be electroporated and present in a 1.5 ml microfuge tube on ice, was added 20 µl of electrocompetent *A.tumefaciens* cells and mixed gently. After setting the MicroPulser system for the electroporation of *A.tumefaciens*, transfer the DNA-cell sample to the bottom of 0.1 cm chilled electroporation cuvette which then placed in the shocking chamber electrodes providing optimum electrotransformation efficiency of the cells using a voltage 2.2 kV and just one pulse. Remove the cuvette from the

chamber and transfer the cells to 2 ml tube containing 1 ml of LB. Tubes with cells were incubated 1 h at 30°C and shaking at 250 r.p.m.

Then, plate the aliquots of the electroporated cells on LB agar plates carrying added kanamycin (50 µg ml^{-1}) and gentamicin (50 µg ml^{-1}) and incubate it for 48 h at 30°C. Each *A.tumefaciens* colonie found was put in 50 µl PCR water and conducted at 94°C for 4 min before making colony PCR to screen for inserts if it are present or not. The sequences of the primers used, the reaction mixture (Annex. 14a and b) and the thermal profile of the reaction are shown in Annex 14c1 (for 440-bp) and in Annex 14c2 (for 1331-bp).

Selecting transformed *Agrobacterium* using PCR colony

To screen for plasmid inserts directly from *Agrobacterium* colonies, amplification reactions were conducted using 2 µl of DNA template (1:1000) present in different clones founds. The primer pairs used were 3 primers (Annex. 15). *pBI for* and *440 rev* in the first reaction mixture searching for 440-bp (+) (Annex. 15a) and *pBI for* and *1331 for* searching for 440-bp (-) (Annex. 15b). The sequences of these primers are shown in Annex 11b.1 and Annex 14a, the reaction mixtures and the thermal profiles of the reaction are shown in Annex 14b and 14c1.

In order to verify the direction of the 1331-bp insert in the vector, amplification reactions were conducted using 2 µl of DNA template (1:1000) present in different clones founds. The primer pairs used were 3 primers (Annex. 16). *pBI for* and *1331 rev* in the first reaction mixture searching for 1331-bp (+) (Annex 16a) and *pBI for* and *1331 for* searching for 1331-bp (-) (Annex 16b). The sequences of these primers are shown in Annex 11b.2 and Annex 14a, the reaction mixtures and the thermal profiles of the reaction are shown in Annex 14b and 14c2.

The amplified fragments were, then, displayed by electrophoresis on agarose gel and the clones that showed the presence of the insert (440-bp and 1331-bp) with the expected size in the different orientations (+/-) were selected to transform Arabidopsis plant by floral dipping method.

Floral dip

A.tumefaciens cells strain carrying the binary plasmids with the different inserts (pBI101.3-35Smp, pBI101.3-35Smp-440 bp and pBI101.3-35Smp-1331 bp) was used. Bacteria were grown to stationary phase at 28-30°C, 300 r.p.m in liquid culture media containing sterilized LB carrying added kanamycin (50 μg ml^{-1}) and gentamycin (50 μg ml^{-1}). Bacterial cultures were started from a 1:100 dilution of smaller overnight cultures from 10, 50 and then 500 ml and grown for 18–24 h to a final OD_{600} of approximately 1 prior to use. Cells were harvested by centrifugation for 10 min at room temperature at 3500 *g* and then resuspended in infiltration medium. The floral dip inoculation medium contained 5% sucrose and 0.05% Silwet L-77 added just before inoculation (Clough and Bent 1998).

The inoculation medium containing *Agrobacterium* cell suspension was added to a beaker where watered plants (the night before) were inverted into this suspension such that all flower buds were submerged. After 3-5 min, plants were then removed from the beaker, placed in a plastic tray in horizontal position and covered with plastic wrap to maintain humidity. Then, were left overnight in a low light and returned to the growth chamber the next day. Plants were grown for a further 3-5 weeks keeping plants from each pot together and separated from neighboring pots until T_1 seeds were dry to be harvested and stored in microfuge tubes and kept at room temperature.

Selection of transformant plants using an antibiotic marker

The selection system used in *Agrobacterium*-mediated transformation is the antibiotic resistance since the presence in the T-DNA of the selectable marker "*nptII*" (neomycin phosphotransferase II) gene, which confers resistance to kanamycin antibiotic. Sterilized T_1 seeds were resuspended in sterile distilled water and plated on kanamycin selection plates at a density of approximately 2000 seeds per 150 mm plate, vernalised for 2 days, and then grown for 14-20 days in a growth chamber at 22°C under 12 h light. Selection plates contained ½ X MS medium, 0.8% agar and 50 μg ml^{-1} kanamycin. Transformants T_1 surviving on kanamycin, were characterized as kanamycin resistant seedlings that produced green leaves and well developed roots.

Screening of transgenic plants

We proceeded an important step for transgenic studies: the isolation of the homozygous T_3 transgenic plants having a single copy of the transformed promoter (single T-DNA insertion) identified genetically estimating the genotype of transgenic plant by segregation test of kanamycin resistance marker using the seeds progenies T_3 obtained. Within the transgenic lines analyzed is therefore possible to detect the presence of individuals wild-type and transformant individuals heterozygous and homozygous for the T-DNA insertion.

T_3 seeds are germinated on half-strength MS medium containing 50 μg ml^{-1} of kanamycin and scored for Kan^R and Kan^S seedlings 20 days after germination. Kan^R seedling developed normally on this medium and remains green. Kan^S individuals cannot form true leaves with bleaching of cotyledons, and the seedlings finally die.

QUANTITATIVE REAL-TIME RT-PCR FOR GRADIENT PLATES

Total RNA extraction and quantification

The total RNA extraction was conducted in TRIzol® reagent (LifeTechnologies), in accordance with the protocol reported by the manufacturer. Frozen shoot samples were pulverized in 1 ml of TRIZOL® within mortars for homogenization, were transferred into sterile centrifuge tubes, mixed and kept at room temperature for 5 min. The samples were then added with 200 μl of chloroform, shaked by hand for 15 s, incubated for 2-3 min at room temperature and then centrifuged at 12000 *g* for 15 min at 4°C. The upper aqueous phase (400 μl) was taken and mixed with 0.5 ml of 100% isopropanol (v/v) to precipitate the total RNA. After incubation 10 min at room temperature, samples were centrifuged at 12000 *g* for 15 s at 4°C. Then, the supernatant was discarded and 1 ml of 75% ethanol was added to the tube to wash the RNA-pellet. After brief vortex, the samples were centrifuged at 7500 *g* for 5 min at 4°C. Finally, the wash was discarded and the RNA-pellet was vacuum dried from the ethanol for 5-10 min to be resuspended with 30 μl of DEPC H_2O. Finally, after incubation at 60°C for 15 min, the total RNA was quantified by using the Thermo Scientific NanoDrop 1000 Spectrophotometer measuring the concentration in ng μl^{-1} based on absorbance at 260 nm of 1 μl nucleic acid sample. In order to verify the quality of the RNA, the same spectrophotometer measure the ratio of sample absorbance at 260 and 280 nm used to assess the purity of RNA.

Isolation of cDNA and evaluation of gene expression

To obtain the first-strand cDNA for use in Real-Time quantitative Reverse Transcription PCR (qRT-PCR), it was used the protocol of SuperScriptIII First-strand synthesis SuperMix for qRT-PCR (LifeTechnologies) according to the manufacturer's instructions.

Before the application of the mentioned protocol and in order to remove contaminating DNA from RNA preparation, it was used the protocol of Amplification grade DNase I (LifeTechnologies) according to the manufacturer's instructions. It was prepared a mixture containing 4 µl of RNA, 0.5 µl 10× DNase I buffer and 0.5 µl DNase I Amplification grade. After incubation for 15 min at room temperature, 0.5 µl of 25 mM EDTA solution was added to inactivate DNase I. Then, it was incubated at 65°C for 5 min for heat inactivation.

The RNA sample is now ready to be used directly in a reverse transcription reaction. 4 µl of samples were then added to a master mix containing 5 µl of 2X RT reaction mix (oligo $(dT)_{20}$, random hexamers, $MgCl_2$, dNTPs) and 1 µl of RT enzyme mix (SuperScript III RT and RNaseOUT). Subsequently each sample was gently mixed and the reverse transcription reaction was occurred at 25°C for 10 min, then at a 50°C in a time of 50 min before being terminated by bringing the samples to 85°C for 5 min in order to denature the enzyme. The mixture was cooled in ice; added then 0.5 µl of RNase H and then incubates at 37°C for 20 min.

The subsequent cDNA amplification reactions were carried out by quantitative PCR (qPCR) using GoTaq qPCR Master mix reagent system (Promega).

The primer pairs used to amplify the sequences of genes *S16*, *GUS* and *AT1G12030* in the FLAG line were designed. The primer pairs used are listed in the Annex 17a. Analysis of transcript levels of each gene was achieved by quantitative PCR using an Applied Biosystems 7300 Real-Time PCR System. Quadruplicate reactions were performed for each gene and sample using well-plates consisting of reaction mixtures containing 5 µl aliquots of the diluted cDNA template (20% of the reaction volume), the pair of primers specific for the sequence, each at a concentration 10 µM, GoTaq® qPCR Master mix (Promega), the CXR reference dye, and water to achieve a final reaction volume of 20 µl (Annex. 17b). A control reaction was done for each sample to confirm that there was no contaminating DNA in the original sample. The reaction plate was finally centrifuged at low speed for 1 min to keep the components together and remove bubbles. The quantitative PCR reaction conditions were given in Annex

17c. The cycle threshold values were determined for each sample during the quantitative PCR cycling reaction. Transcript levels of each gene were normalized to the *S16* control gene.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SigmaPlot for Windows version 12.0 (Systat Software, Inc.). Quantitative values are presented as mean ± standard error of the mean (SE). Significance values were adjusted for multiple comparisons using the Bonferroni correction. Statistical significance was at $P < 0.05$.

RESULTS AND DISCUSSION

ARABIDOPSIS GENE TRAP LINES

Gene trapping is a powerful strategy for gene discovery and functional genomics in plants by exploiting the insertion of the gene trap construct into an expressed gene. The detection of reporter gene expression allows the determination of the expression patterns of different genes (Springer 2000).

In the framework of the EXOTIC (EXON Trapping Insert Consortium) project funded by the UE Fifth Program for Plant Biotechnology, *Arabidopsis thaliana* (ecotype Landsberg erecta) gene trap lines from the EXOTIC collection were generated by insertional mutagenesis exploiting the *Zea mays* Ac/Ds transposable elements system (Sundaresan et al. 1995; Chin et al. 1999). In detail, the EXOTIC lines carry a Dissociation (*Ds*) transposable element (Fig. 1) appropriately modified (*DsG*) containing a promoterless β-glucuronidase (*GUS*) reporter gene flanked by a triple splice acceptor and an intron, other than the Neomycin phosphotransferase (*NPTII*) gene, which confers resistance to kanamycin. The presence of the insertional *DsG* element in a gene interrupts its sequence, but contemporaneously allows its promoter, if activated, to control the expression of the *GUS* gene. This strategy allows the identification of genes and/or promoters responsive to the selective factor(s) adopted in the screening.

In our lab, the EXOTIC lines were previously screened for *GUS* expression after having been grown in the absence or in the presence of sulfate, the main S source for plants, in the medium (Lancilli et al. 2008). This screening identified the EXOTIC line 718 that showed GUS expression in root apices and in shoots only when grown under sulfate starvation (Fig. 2). In this line insertion the *DsG* element is located in chromosome 1, in the intergenic region between the *AT1G12030* and the *AT1G12040* genes. The *DsG* element is inserted at 1331-bp and at 2509-bp from the start of the *AT1G12030* and *AT1G12040* genes, respectively (Fig. 3A).

AT1G12030 is a gene with unknown function, encoding a putative protein probably expressed in the mitochondria, while *AT1G12040* encodes the LRX1 (Leucine-Rich Repeat/EXTENSIN1) protein, expressed in the roots, where it is involved in root hair morphogenesis and elongation (Baumberger et al. 2001).

The 1331-bp region resulted able to simultaneously control transcription in opposite directions of the *GUS* reporter and of the *AT1G12030* genes under sulfur-deficiency conditions.

In order to identify, in the 1331-bp, the minimal region able to activate the transcription of the reporter gene under S starvation, we looked for the existence of other *Arabidopsis* mutant lines with insertions in this intergenic region. Among the different available *Arabidopsis* gene trap systems based on T-DNA transfer, a FLAG line (Versailles's T-DNA collection) belonging to the Wassilevskija ecotype resulted interesting (Bechtold et al. 1993; Bouchez et al. 1993). In the FLAG line, the T-DNA insertion contains the promoterless *GUS* reporter gene, the *NPTII* gene and a *bar* gene conferring resistance to the herbicide bialaphos (Basta). This insertion is present in chromosome 1 and in the intergenic region between the *AT1G12030* and the *AT1G12040* genes, at 440-bp and at 3400-bp from the start of the *AT1G12030* and *AT1G12040* genes, respectively (Fig. 3B).

Expression of the *GUS* reporter gene in the FLAG line

When plants of the FLAG line were grown for 14 days in the presence of 0 μM sulfate in the nutrient solution (sulfur starvation condition: -S), expression of the *GUS* reporter gene was observed whereas the gene was not expressed when plant were grown in the presence of 1500 μM sulfate (control: +S). The *GUS* positive signals were detectable in both roots and shoots of the S-starved plants. No coloration due to *GUS* activity was detectable in the tissues of sulfur-sufficient plants (+S) (Fig. 4).

Effects of sulfur deficiency on the expression of the genes flanking the intergenic region in the FLAG line

In order to understand the effects of sulfur starvation on the expression of the genes (*AT1G12030* and *GUS*) flanking the intergenic region in the FLAG line, wild-type and mutant plants were grown in -S and +S condition for 14 days; at the end of this time period, total mRNAs were extracted from plants and the levels of the two mRNAs of interest were evaluated by semi-quantitative RT-PCR. As shown in Fig. 5, the mRNA of the *GUS* reporter gene was detectable at very low levels in plants of the FLAG line grown under adequate S availability (+S). Consistent with the histochemical observation described above (Fig. 4), the –S condition induced a marked accumulation of *GUS* mRNA in plant tissues.

Different than reported for the Landsberg genetic background (Lancilli et al. 2008), the *AT1G12030* gene resulted constitutively expressed in plants of the Wassilevskija ecotype

grown in control condition (+S). However, also in this ecotype, in plants experiencing S starvation a significant increase in the *AT1G12030* mRNA level was observed in comparison to that detectable in control plants. The T-DNA insertion in the FLAG line negatively affected the expression of the *AT1G12030* gene. Nevertheless, in these plants the S starvation condition markedly increased the expression of this gene.

Densitometry analysis of each band (using ImageJ programme) indicated that the levels of *AT1G12030* transcripts significantly increased compared to the controls (by about 250% and 1600%) when wild-type and FLAG plants, respectively, were exposed to S starvation (Fig. 6). The same analysis showed that the *GUS* transcript levels in plants grown under S starvation were by about 12-fold increased with respect to control plants.

Taken as a whole, these results suggest that 440-bp fragment might be sensitive to sulfur starvation and responsible for regulating the expression of the *AT1G12030* and *GUS* genes. Since these two adjacent genes are located in a divergent orientation on the chromosome 1 of the FLAG *Arabidopsis* genome, it is possible to suppose that the 440-bp intergenic region might share a putative bidirectional promoter containing sulfur responsive *cis*-regulatory elements.

Moreover, it is interesting to underlie that this group of experiments led to the identification of an *A. thaliana* line presenting differential *GUS* expression in S-deficient conditions in the aerial portions. In our opinion, this is a particularly interesting starting point for the development of a bioindicator of the plant sulfur nutritional status.

Expression of *GUS* and *AT1G12030* genes as a function of the plant sulfur nutritional status

In order to establish whether the FLAG line might be considered as a bioindicator able to signal the actual plant sulfur nutritional status, activation of the *GUS* gene in these plants was related to their sulfate critical concentration (n_c), i.e., the minimum concentration of sulfate necessary to achieve 95% of maximum biomass (Fageria 2014). The value of n_c was evaluated by measuring the growth of both roots and shoots of plants grown vertically in agar plates (Fig. 7) under a continuous gradient (0-150 μM) of sulfate concentrations (SO_4^{2-}-gradient agar plates: SGAPs).

The stability of the continuous gradient of sulfate concentrations in the agarized medium was checked daily by measuring for 14 days the concentrations of the anion in progressive points of the agar medium in plates without plants. Figure 8A and 9A shows the

behaviors of plant shoot fresh weight (FW) and root length (Lr) increases, respectively, as a function of sulfate concentration in the medium. As expected, both functions are represented by a hyperbolic curve described by the following Michaelis-Menten equations:

$$FW = \frac{FW_{max}\ [SO_4^{2-}]}{K_{½} + \ [SO_4^{2-}]}$$

$$Lr = \frac{Lr_{max}\ [SO_4^{2-}]}{K_{½} + \ [SO_4^{2-}]}$$

where FW_{max} and Lr_{max} are the function asymptotes and $K_{½}$ is the concentration of sulfate in the medium corresponding to the half of the value of either FW_{max} or Lr_{max}. The double-reciprocal plots of both functions describe linear functions (Fig. 8B and 9B) with R^2 values of 0.99 and 0.97 for shoot and root, respectively. The values of function asymptotes (FW_{max} and Lr_{max}), $K_{½}$ and n_c for both shoot and root growth are reported in Table 1. Considering a value equal to 95% of FW_{max} and Lr_{max}, the corresponding extrapolated value of critical sulfate concentration n_c, resulted equal to 130.4 μM and 89.4 μM for shoot and root growth, respectively (Fig. 8A, 9A and Table 1).

GSH was considered as the main cellular thiol whose concentration is tightly related to the internal plant sulfur status (Lappartient et al. 1999). We evaluated also, in the shoot of the FLAG line plants, the dependence of GSH concentration on sulfur availability in the SGAPs growth medium (Fig. 14). Although the experimental curve did not fit with a Michaelis-Menten hyperbolic curve, it reached a maximum GSH value at about 120 μM external sulfate concentration, quite close to the n_c value (130.4 μM) calculated considering the dependence of shoot biomass increase on sulfate availability (Table 1).

The steady-state levels of *GUS* mRNA were evaluated by real-time PCR in shoots of the FLAG line plants obtained from SGAP experiments. The levels of *GUS* transcripts decreased progressively as the sulfate concentration in the medium increased (Fig. 12); the behavior of the function is well described by an exponential decay curve. From data of Figure 12 it is also possible to establish that the lowest concentration of sulfate where the *GUS* reporter gene was not expressed was about 130 μM, very close to the n_c value calculated for the shoot biomass increase.

A very similar behavior was obtained by analyzing, by means of SGAP experiments, the steady-state levels of *AT1G12030* mRNA as a function of sulfate concentration in the medium (Fig. 13). Also in this case, the levels of *AT1G12030* transcripts progressively decreased, according to an exponential decay function, as the concentration of sulfate in the medium increased. Similarly to what observed for the *GUS* gene, transcripts of the *AT1G12030* gene resulted detectable only when the concentration of sulfate in the external medium was higher than 130 μM.

This group of results suggests that the FLAG line plants are able to signal, also in quantitative terms, their sulfur nutritional status through the expression of both *GUS* and *AT1G12030* genes. This is an important prerequisite in order to consider the FLAG line as a sulfur nutritional status biondicator, as well as the FLAG intergenic region (440-bp) as an interesting element in order to constitute a sulfur-sensitive pilot transgenic bioindicator plant.

Nevertheless, it should be stressed out that the quantitative relationship between the plant nutritional status and the levels of *GUS* transcripts resulted detectable only by real-time PCR technique and not by the simpler histochemical assay. This implies that *GUS* is not the best reporter gene for easily and promptly disclosing the amount of sulfur that a plant needs. However, other reporter genes whose expression can be monitored and quantitatively evaluated with more ease and sensitiveness than the *GUS* gene are known. The possibility to substitute *GUS* into the FLAG line is presently under investigation.

The 440-bp intergenic region conserves the same characteristics of sulfur sensitiveness previously described for the 1331-bp intergenic region of the 718 EXOTIC line (Lancilli et al. 2008). Therefore, it seems probable that within this region regulative element/s, able to drive in a bidirectional way the expression of flanking genes when the plant sulfur nutritional status is below its optimum, is/are present.

Cadmium exposure and additional sulfur needs

Biotic and abiotic stresses increase the levels of cysteine-derived metabolic compounds inducing activation of the sulfate reductive assimilatory pathway (Rausch and Wachter 2005). This is particularly evident when plants are exposed to cadmium (Cd). Indeed, when it happens plants activate adaptive responses that ultimately lead to an increase in the demands of sulfate, sulfur containing compounds, and carbon skeletons (Lee and Luestek 1999; Nocito et al. 2002, 2007, 2008). In particular, under Cd stress are induced both

the activities that lead to the synthesis of the tripeptide glutathione (GSH) and those that consume it (Nocito et al. 2007). Indeed, GSH not only acts as an antioxidant in mitigating Cd-induced oxidative stress, but also represents the key intermediate for the synthesis of phytochelatins (PCs) which are a class of cysteine-rich heavy metal-binding peptides involved in buffering cytosolic metal-ion concentration (Noctor et al. 2012). The large amount of PCs produced by Cd-stressed plants thus represents an additional sink for reduced sulfur that, by increasing the metabolic request for GSH, generates a typical demand-driven coordinated transcriptional regulation of genes involved in sulfate uptake, sulfate assimilation and GSH biosynthesis (Lancilli et al. 2014).

On the basis of the above described considerations, one can assume that Cd exposure represents a suitable experimental tool to induce in the plant a higher internal requirement of sulfur. In other words, in the presence of Cd the optimal sulfur nutritional status is expected to be furnished to the plant by higher sulfate availability in the growth medium. With the aim to verify this hypothesis, the FLAG line plants were grown in a SGAP experiment (0-150 μM sulfate) in the presence of a constant concentration (50 μM) of Cd (as chloride salt) (Fig. 7B).

The presence of 50 μM Cd induced a marked delay in plant growth (Figures 7A and 7B), as well as evident shoot chlorosis. Detailed analysis of the effect of 50 μM Cd on shoot biomass showed that very similar growth values were observed at higher sulfate availability compared to experiments without Cd (Fig. 10A). Least square fittings (Fig. 10A) revealed that also in the presence of Cd the dependence of shoot biomass increase as a function of sulfate concentration in the medium and can be properly described by the hyperbolic Michaelis-Menten equation. Double-reciprocal plot (Fig. 10B) allowed extrapolating the $K_{½}$ for sulfate and FW_{max} values. As reported in Table 2, the values of both parameters were higher than those obtained in the Cd-free SAGP experiments. The value of sulfate n_c in FLAG line plants grown in the presence of Cd resulted more than two-fold (268 μM) with respect to that of the same plants grown in the absence of Cd, whereas the length of primary roots was not significantly affected by the presence of the Cd. The dependence of Lr_{max} on sulfate concentration in the medium (Fig. 11A) can also be described according to a hyperbolic Michaelis-Menten function whose equation constants values (Lr_{max} and $K_{½}$) were not significantly different than those calculated in the Cd-free growth condition (Table 1 and 2). Therefore, the value of sulfate n_c (89.4 μM vs 94.55 μM) was not affected.

Analysis of GSH concentration in shoots of the FLAG line plants grown in the presence of Cd (Fig. 14) indicated that even at the highest sulfate availability in the medium

the GSH concentration did not reach a maximum. This could explain the evident chlorosis observed (Fig. 7B), indicative of oxidative stress condition experienced by plants. This negative effect of Cd on GSH levels may be explained by the increased metabolic request for GSH in order to produce PCs (Nussbaum et al. 1988; Ruegsegger and Brunold 1992; Nocito et al. 2002) for metal detoxification. Under Cd stress the value of sulfate n_c dramatically increased.

In the presence of cadmium (Fig. 15), FLAG gene trap line respond to the sulfur nutritional status by activating *GUS* reporter gene and the presence of signal had a complementary pattern to the expression level in the aerial portion of plants throughout the gradient whereas it exhibited a gradual decreased root *GUS* activity until a concentration of ± 90 μM which belong the optimum range in root above which lesser GUS activity was clearly induced just in shoot.

In conclusion, the results described above indicate that the pilot lines are capable to correctly indicate the critical concentration of sulfate in the external medium also in the presence of interfering metal ions (such as cadmium) able to increase the plant metabolic demand for sulfur.

Effects of cysteine or GSH on the response of *Arabidopsis* gene trap lines

Whether the cellular contents of sulfate, cysteine and GSH are considered the primary signal for controlling sulfate uptake and metabolism or rather they act indirectly is not completely clear (Buchner et al. 2004a; Davidian and Kopriva 2010). This because very little is known about the molecular mechanisms involved in the perception and transduction of nutritional signals (Maruyama-Nakashita et al. 2005, 2006; Takahashi et al. 2011).

Since, other than sulfate, cysteine and GSH are also absorbed by plant roots (Lappartient and Touraine 1996; Bolchi et al. 1999; Lappartient et al. 1999; Vauclare et al. 2002; Rouached et al. 2008), the internal sulfur status of tissues and the consequent molecular and metabolic responses can be modified and thus studied by growing plants in the presence of these two metabolites as sole sulfur source. Indeed, it has been reported that cysteine and GSH induce down regulation of sulfur-responsive genes (Smith et al. 1997; Lappartient et al. 1999; Vauclare et al. 2002; Maruyama-Nakashita et al. 2004).

With the aim to better characterize the signal(s) capable to trigger the sulfur starvation responses in both the 718 EXOTIC and the FLAG lines, plants were grown vertically for 14 days on agarized S-starved medium (-S; 0 μM sulfate) or, alternatively, in the presence of 1.5 mM cysteine (Cys) or 1.5 mM GSH as sole sulfur sources. The results obtained by staining the shoots with X-Gluc substrate showed that the presence of cysteine as sole sulfur source in the agar medium was capable to completely revert the induction of *GUS* expression in both lines. The effect was evident in both roots and shoots. In the presence of GSH in the medium, the *GUS* expression activity was completely absent in roots and was markedly reduced (although a weak signal could still be observed) in shoots, (Fig. 16).

The results suggest that the internal sulfur status as represented by the thiol contents in both the 718 EXOTIC and the FLAG gene trap lines might control, through the activity of sulfur-sensitive elements present in both the 1331-bp and the 440-bp region, the expression of the *GUS* flanking gene. In other words, both the *Arabidopsis* lines appear capable to signal, through the expression of the *GUS* gene, the metabolic effects produced by sulfur starvation and not the presence/absence of sulfate in the growing medium (Fig. 16).

The difference shown by cysteine and GSH treatments might be due to different causes, such as different root- to-shoot transport ability and/or metabolization of the two compounds, defects in long-distance root-to-shoot signalling of sulfur status, or defects in adjusting the shoot metabolism to the conditions of GSH as sole sulfur source encountered by the roots.

In this framework, to understand the mechanism/s by which cysteine and GSH differently affect the response of gene trap bioindicators is the challenge for future investigations.

Gene trap sulfur status bioindicators in controlled hydroponic culture.

In order to exclude that the observed responses of the *Arabidopsis* 718 EXOTIC and FLAG lines to sulfur starvation do not depend on the low solute diffusion rate in the agarized growth substrate, plants were grown in hydroponic cultures with different (1-100 μM) sulfate concentrations. After 14 days, shoot growth, non-protein thiol (NPT) concentrations, and GUS activity were determined (Fig. 17).

The shoot fresh weight of both the 718 and FLAG lines increased with the increase in sulfate concentration in the medium. The total biomass produced by the FLAG line (Wassilevskija ecotype) was markedly higher than that produced by the 718 line (Landsberg ecotype) (Fig. 17A). At the same sulfate availability in the medium, the concentrations of non-protein thiols (NPT) in the 718 line were significantly higher than in the FLAG line,

resulted probably for a dilution effect due to different plant sizes. Within the same line, NPT concentrations increased with sulfate concentration (up to 10 μM external sulfate). In both lines, when external sulfate concentrations increased from 10 μM to 100 μM, the NTP concentrations remained unchanged (Fig.17B).

Table 3 shows the results of X-Gluc coloration assay performed on both the 718 and FLAG lines grown in the presence of different sulfate concentrations. Relevant *GUS* expression activity was detectable only in the FLAG line for sulfate concentrations ≤5 μM, when the NPT concentration was lower than the maximum reached at the two highest sulfate concentrations (10 and 100 μM). Unexpectedly, no *GUS* expression activity was detectable for the 718 line even at the lowest sulfate concentration tested (1 μM), corresponding to dramatically low NPT concentrations.

It has been shown that presence of sucrose in the growth medium can significantly affect the expression of some sulfur-responsive genes. Expression of *APS reductase*, encoding the key enzyme of sulfate reduction, as well as of genes encoding the sulfate transporters SULTR1;1 and SULTR1;2, is stimulated by feeding sucrose and glucose to plants (Kopriva et al. 1999, 2002; Hesse et al. 2003; Lejay et al. 2003; Maruyama-Nakashita et al. 2004). Therefore, in this set of experiments conducted on plants grown in hydroponic culture, the lack of *GUS* expression in the 718 line, even when the tissue levels of NTPs were very low, might be due to the absence of sucrose (present in the agarized medium) in the hydroponic growth medium.

In order to verify the possible role of sucrose in regulating *GUS* gene expression, plants of both the 718 and the FLAG lines were grown for 20 days in agarized medium, in the presence (+S: 1500 μM) or in the absence (-S: 0 μM) of sulfur and in the presence or in the absence of sucrose (1% w/v).

In conditions of sulfur deprivation (–S), absence of sucrose in the medium remarkably attenuated the expression of the *GUS* reporter gene in plants of the 718 line, whereas plants of the FLAG line showed evident expression of the *GUS* gene (Fig. 18). These results seem to suggest that *GUS* expression activity in the 718 line is regulated by a combined effect of sulfur deficiency and sucrose availability to the plant. This combined effect was absent or less evident in FLAG line plants.

Under nitrogen and carbon skeletons excess, *O*-acetylserine (OAS), the substrate of cysteine synthase, is accumulated de-repressing the transcription of genes encoding sulfur assimilatory proteins (Hawkesford 2000; Hirai et al. 2003). On this basis, addition of sucrose

to the growth medium (either agarized medium or hydroponic solution), should activate these de-repressive mechanism acting on the expression of sulfur-sensitive genes.

Gene transcription is positively (activation) or negatively (repression) regulated by transcriptional activators or repressors (Hanna-Rose and Hansen 1996; Wray et al. 2003). Therefore, the different behavior observed in the two 718 and FLAG mutant lines with higher expression of the reporter *GUS* gene under sulfur starvation in the case of the shorter fragment (440-bp of the FLAG line) than of the longer fragment (1331-bp of the 718 line), might be explained by assuming the presence of a repressor capable to bind to a DNA regulative sequence (*cis*-element) located in the 718 line between -441 and -1331 bp with reference to the *AT1G12030* gene. This transcriptional repressor might prevent the transcription of sulfur assimilative genes in conditions of limited carbon skeleton availability, preventing the hazardous accumulation of toxic free sulfide (S^{2-}). In the FLAG line, the repressor-binding domain should be absent, allowing expression of the *GUS* reporter gene even in the absence of the excess of carbon skeletons due to sucrose supply. Clarification of the mechanism underlying the regulatory effects of sucrose needs further studies.

EVALUATION OF THE ACTIVITY OF PUTATIVE PROMOTERS IN GENE TRAP LINES

The results described above allow to conclude that in the intergenic regions located in both the 718 and FLAG lines (1331-bp and 440-bp, respectively) are present sulfur-responsive sequences capable to regulate the expression of flanking genes when plants are grown under sulfur deficiency. More thorough identification and characterization of such sequences might allow their exploitation as part of a synthetic promoter for constitution of transgenic bioindicators of the plant sulfur nutritional status.

Sequences analysis of the FLAG line intergenic region (440-bp)

Analysis of the 440-bp region of the FLAG gene trap line (using BioEdit programme), revealed the presence of the 5-bp core sequence GAGAC of the sulfur-responsive element (SURE) in position -101 referred to the 5'end of the *AT1G12030* gene. The SURE element first identified in the promoter of the *Arabidopsis* gene *SULTR1:1* (Fig. 19A) and encoding a high-affinity sulfate transporter, is sufficient to drive the expression of the gene in sulfur-deprived condition (Maruyama-Nakashita et al. 2005).

The SURE core element is characteristic of many sulfur-responsive genes playing a key role in their induction (Maruyama-Nakashita et al. 2005). Nevertheless, it is not present in all genes that respond to sulfur deficiency, as well as it has been found in the promoter of genes not regulated by sulfur availability (Wawrzynska et al. 2010). For these reasons, the observed response in the gene trap lines could require other additional regulatory sequences.

Sequence analysis of the 718 line intergenic region (1331-bp)

The whole sequence of the 1331-bp intergenic region between *AT1G12030* and the transposon insertion in the 718 EXOTIC gene trap line was analyzed. Other than the sulfur-responsive *cis*-elements SURE, that also in this line was located at position -101 referred to the 5' end of *AT1G12030* gene, a conserved 20-bp UPE box motif was detected in the intergenic region between -683 bp and -703 bp with reference to the 5'end of the *AT1G12030* gene (Fig. 19B). This motif has been described as sufficient for the binding of the transcriptional factor SLIM1 (Sulfur LIMitation 1) that regulates the sulfur responses of some genes (Maruyama-Nakashita et al. 2006; Wawrzynska et al. 2010) and is reported to be present only in the promoter of some sulfur deficiency responsive genes (Wawrzynska et al. 2010).

***ARABIDOPSIS* TRANSFORMATION**

In eukaryotic organisms promoters are not always located in the proximity of the gene transcription start sites (TSSs), but can be located in a position distant from the 5' upstream regions, or at the 3' downstream regions of genes (Xu et al. 2004; Kapranov et al. 2007). Thus the identified 1331- and 440-bp intergenic regions might not contain functional promoters of the *GUS* and *AT1G12030* genes. This promoter can be located far away from the TSSs.

Therefore, in order to verify whether the 1331- and 440-bp intergenic regions contain a putative sensitive promoter able to control and modulate the transcription of *GUS* gene in sulfur-deficiency conditions independent of their original position in the genome, *A. thaliana* plants (Landsberg ecotype) were engineered by transformation with a construct containing the intergenic regions upstream of the *GUS* gene. Moreover, using the same experimental approach, the actual capability of these intergenic regions to control in a bidirectional way the expression of divergent genes was tested. Two constructs for each fragment were generated.

The former construct contained the fragments in the orientation [1331 (+) and 440 (+)] found in the gene trap lines, whereas the other two constructs contained the same fragments in their inverse orientation [1331 (-) and 440 (-)]. In all constructs the *GUS* gene was fused to the 35S minimal promoter (35Smp) typically containing a TATA box and a transcription start site (Novina and Roy 1996). This minimal promoter is not capable to drive reporter gene expression when alone, but it can be activated by neighboring *cis*-acting regulatory elements (Springer 2000).

The constructs, named 1331(+)::35Smp:GUS, 1331(-)::35Smp:GUS, 440 (+)::35Smp:GUS and 440 (-)::35Smp:GUS, are schematically depicted in Figure 20. These constructs were stably introduced into *Arabidopsis thaliana* by floral deep technique. As a control, transgenic plants containing only the *GUS* gene under the control of the 35S minimal promoter were also generated.

Transgenic plants were screened looking for single locus homozygosis. Only T_3 transgenic both 440-bp (+) and 440 (-) homozygous lines were isolated and used for further analysis. Transgenic T_3 440-bp (+ or -) plants were grown 20 days in the absence (-S) or in the presence of 1500 μM sulfate (+S) in the agar medium.

As reported in Figure 21, growth in the absence of sulfate determined the expression of the *GUS* reporter gene in both 440 (+) and 440 (-) T_3 transgenic plants, indicating that the intergenic region is capable to support the activity of the 35S minimal promoter in both orientations. The activities of the intergenic regions were evident in both roots and shoots. In the same growth condition (absence of sulfate), transgenic plants containing only the 35S minimal promoter without the intergenic region did not show any *GUS* signal (Fig. 21).

The results obtained with transgenic plants indicates that: a) the putative 440-bp intergenic region functions as a bidirectional promoter which shares regulatory elements capable to regulate gene expression in opposite orientations under sulfur deficiency conditions; b) the putative 440-bp intergenic region acts as a sulfur-deficiency-induced promoter independent of its position in the genome.

The bidirectional control of the simultaneous expression of two genes has been described in different organisms (Beck and Warren 1988; Keddie et al. 1994; Trinklein et al. 2004) and also in *Arabidopsis thaliana* (Williams and Bowles 2004; Mitra et al. 2009; Wang et al. 2009; Kroumpetli et al. 2013; Banerjee et al. 2013). In the human genome, putative bidirectional promoters are defined as intergenic DNA sequences between the two transcription start sites (TSSs) of a bidirectional gene pair separated with not more than 1000 base pairs (Adachi and Lieber 2002; Trinklein et al. 2004). In plants, Mitra and coworkers

(2009) reported also the existence of bidirectional promoters longer than 1000 bp. We here describe shorter sequences characterized by bidirectional capability to modulate the expression of flanking genes. We conclude that the 440-bp intergenic region is probably an enhancer, i.e. a *cis*-acting DNA sequence that increases gene transcription in a manner that is independent of the orientation and distance relative to the RNA start site (Blackwood and Kadonaga 1998; Williams and Bowles 2004).

When 440-bp::35Smp:GUS transgenic plants were grown for 20 days in a complete agar medium (1500 µM sulfate) in the presence of 50 µM Cd, signals of GUS activity were found independent of the orientation of the 440-bp intergenic region (Fig. 22). Interestingly, the signals were exclusively located in the vascular tissue of the leaves and in root tips. This result confirms that the 440-bp intergenic region is capable to activate in a bidirectional way the expression of the gene flanking it in response to the sulfur nutritional status of the plant independently of its position in the plant genome.

CONCLUSION AND PERSPECTIVES

In the present study, we carried out the initial characterization of two *Arabidopsis* bioindicators of sulfur nutritional status carrying *GUS* reporter gene under the control of sulfur responsive promoters.

We identified, by gene-trap approach, two intergenic regions (1331-bp and 440-bp) modulating in a bidirectional way the expression of the flanking genes in response to S limitation.

Plants in which the reporter gene *GUS* is under the control of these intergenic regions signal their sulfur nutritional status. Indeed:

a) The addition of cysteine or GSH to plants growing under sulfate deprivation inhibits or downregulates the expression of the *GUS* gene;
b) The exposure of plants to Cd, that generates an additional sink for reduced sulfur in the cells, induce the expression of the *GUS* gene, although in the external medium an adequate amount of sulfate exists.

Transgenic *Arabidopsis* lines carrying the 440-bp DNA fragment in two orientations have been characterized in response to sulfur limitation conditions and have shown *GUS* activity in shoots and roots. These results confirm:

a) The sufficiency and the ability of 440-bp fragment to drive the expression of *GUS* reporter gene in sulfur deficient conditions;
b) That 440-bp fragment is able to modulate in bidirectional way the expression of *GUS* reporter gene under sulfur deficiency;
c) That 440-bp fragment functions independently from his position in the genome.

The 440-bp intergenic region is suggested as possible promoter to develop specific bioindicators useful to monitor plant sulfur nutritional status.

The identified putative promoters are suitable for developing “sentinel plants” that will serve as sensors of sulfur deficiency to optimize agronomic practices allowing efficient temporal, spatial applications of S fertilizer and further the development of decision-making systems for precision farming to realize the growth potential of crop plants. It could be particularly important not only for areas of intensive agriculture but also for agriculture in developing countries where accessibility to fertilizers could be a problem.

In addition to its role in monitoring plant S status, the transgenic plants generated in this study have the ability to serve as “smart plants” and be used as a powerful research laboratory tool to:

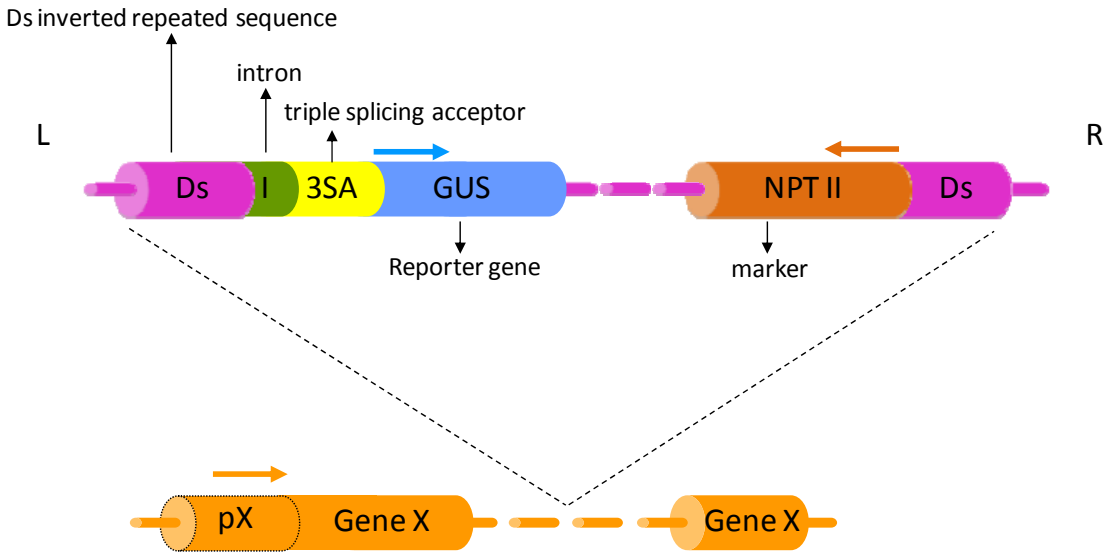
a) Sense the Fe-S clusters which play a fundamental role in the electron transport chains of chloroplasts and mitochondria;
b) Identify the impact of elevated CO_2, sulfur gases such as H_2S and SO_2 on the response to sulfur deficiency;
c) Identify plants with enhanced sulfate use efficiency;
d) Support computer model predictions for the application of S fertilizers.

Additionally, the fact that bidirectional promoters activate the expression of multiple genes might be an important tool for plant biotechnology offering an important approach for:

a) The construction of selection expression vectors co-expressing two genes giving different traits on either side of the control region (Beck and Warren 1988);
b) Generating protein products from two adjacent genes (Banerjee et al. 2013) as using two different reporter genes (*GFP* and *GUS*);
c) Optimizing plant genetic engineering by reducing the number of introduced promoters to minimize gene silencing (Mitra et al. 2009).

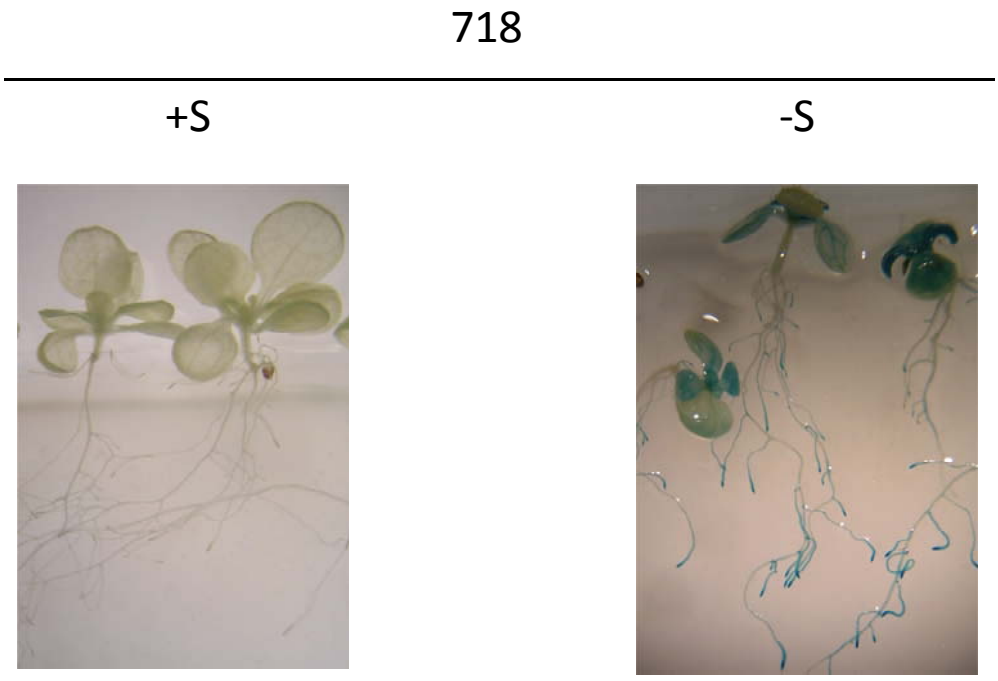
FIGURES AND TABLES

Figure 1. Schematic representation of the interruption of the gene X by the *DsG* element.



DsG construct carrying the *GUS* reporter without promoter; Gene *X* whose expression is driven by *pX* promoter. The images are not representative of the real size between the elements of the constructs.

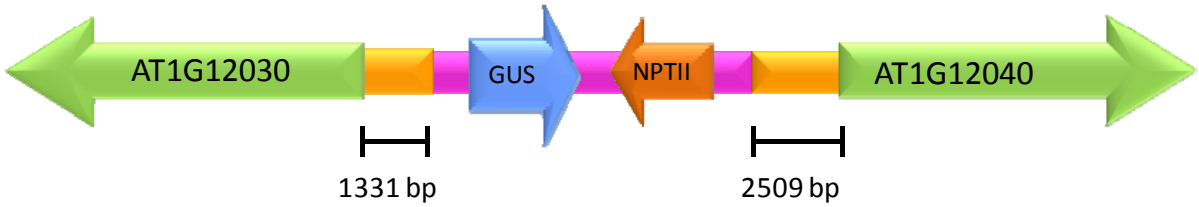
Figure 2. *GUS* reporter gene expression pattern in 718 line (from Lancilli et al., 2008).



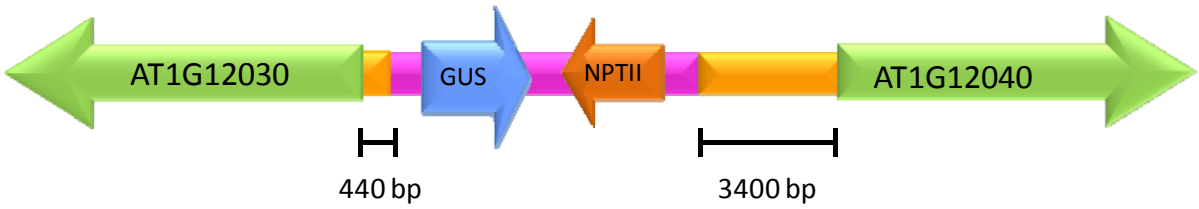
Plants were grown for 2 weeks in complete (+S: 1500 µM) or sulfate deprived (-S: 0 µM) agar media. *GUS* expression was detected by histochemical assay. The images are representatives of one typical experiment repeated 10 times with similar results.

Figure 3. Schematic diagrams of the insertion element in 718 and FLAG lines.

3A. 718 line: insertion in the intergenic region between the *AT1G12030* and the *AT1G12040* genes.



3B. FLAG line: insertion in the intergenic region between the *AT1G12030* and the *AT1G12040* gene.



Insertion element

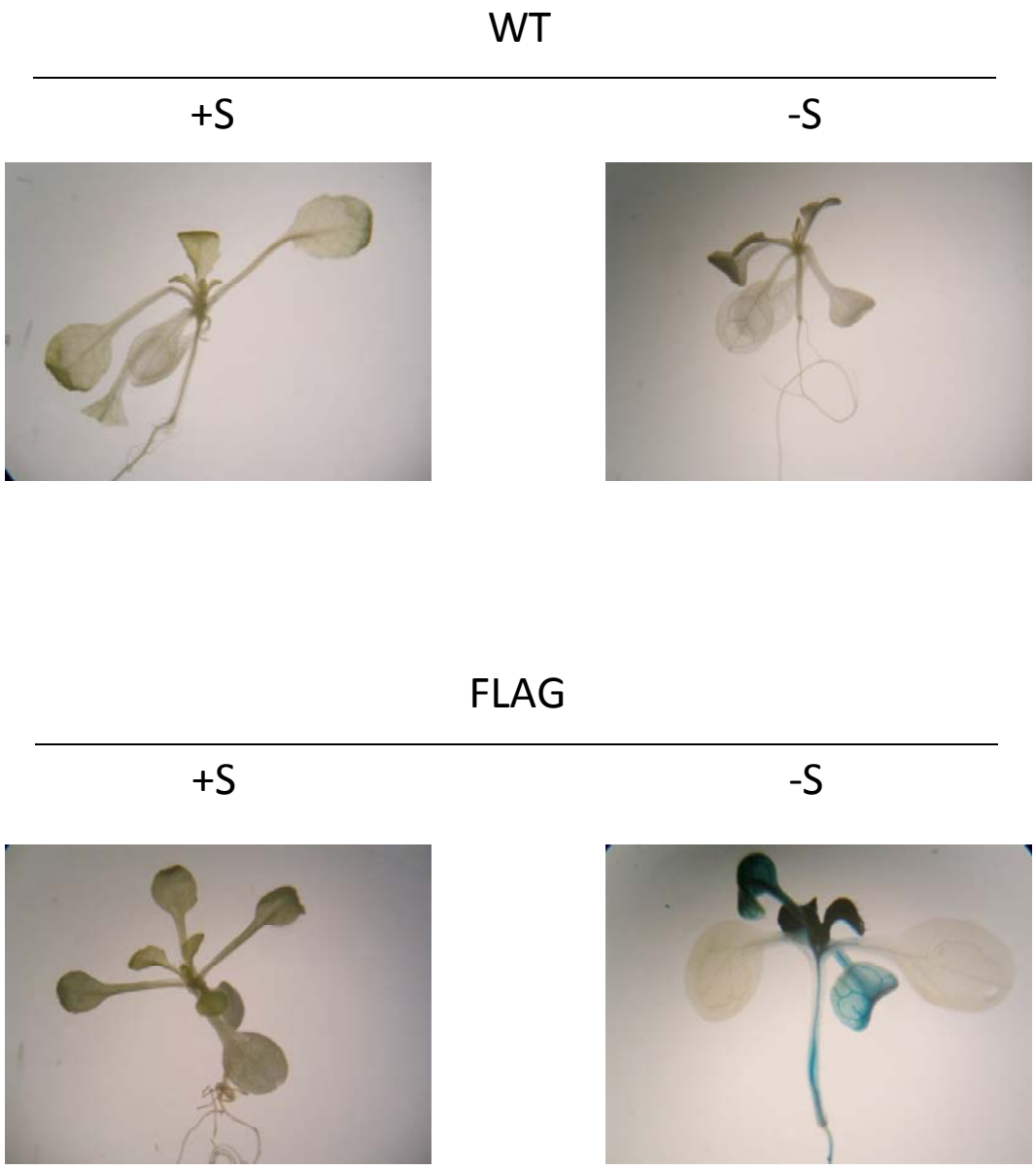
β-glucuronidase (GUS) gene

Neomycin phosphotransferase (NPTII) gene

Intergenic region

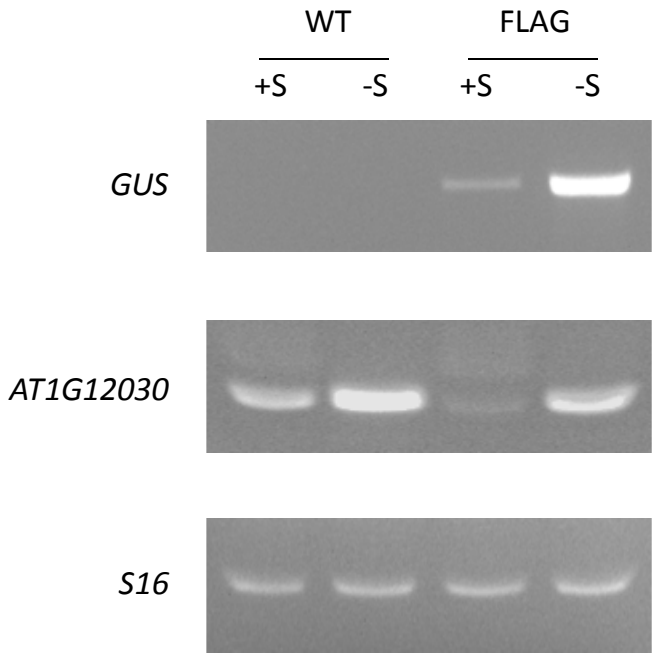
The images are not representative of the real size between the elements of the constructs.

Figure 4. *GUS* reporter gene expression pattern in wild-type and in FLAG line plants.



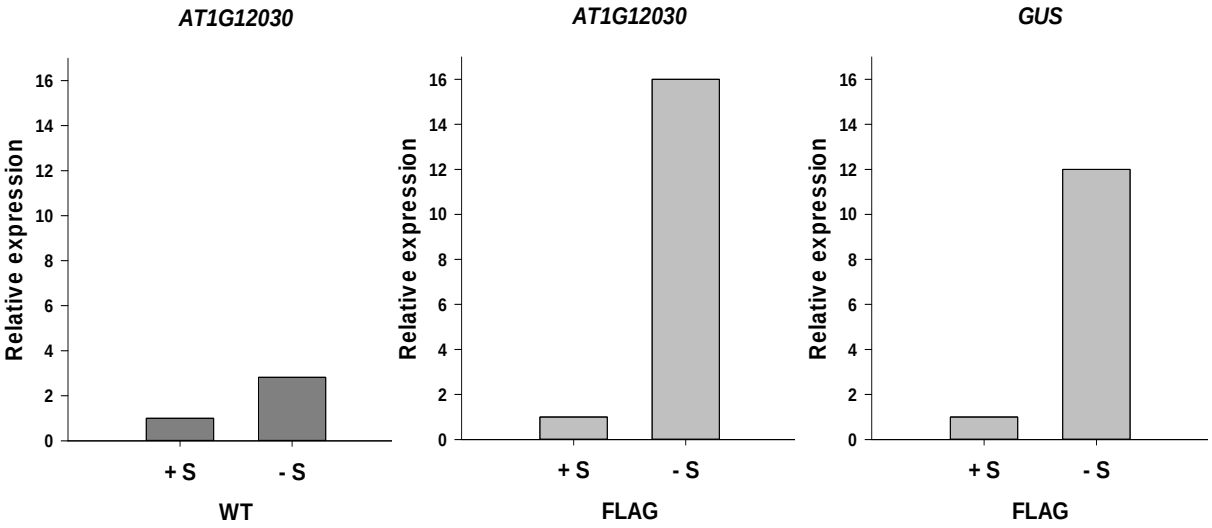
The plants were grown in complete (+S: 1500 µM) or without sulfur (-S: 0 µM) media. After 14 days, plants were vacuum infiltrated with X-Gluc staining solution and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol. Plants were finally photographed under a binocular microscope. The images are representatives of one typical experiment repeated 10 times with similar results.

Figure 5. Sulfur deficiency effect on the expression of the genes flanking the intergenic region in the FLAG line.



The photo shows the semi-quantitative RT-PCR analysis of the expression pattern of the *GUS*, *AT1G12030* and of the reference gene *S16* in wild-type plants (Wassilevskija) and FLAG mutant grown in the absence (-S: 0 µM) or presence (+S: 1500 µM) of sulfate for 14 days. The amplification products were visualized under UV light after electrophoretic separation on agarose gel 1% (w/v) in 1x TBE buffer supplemented with Ethidiumbromide 5 mg ml^{-1}.

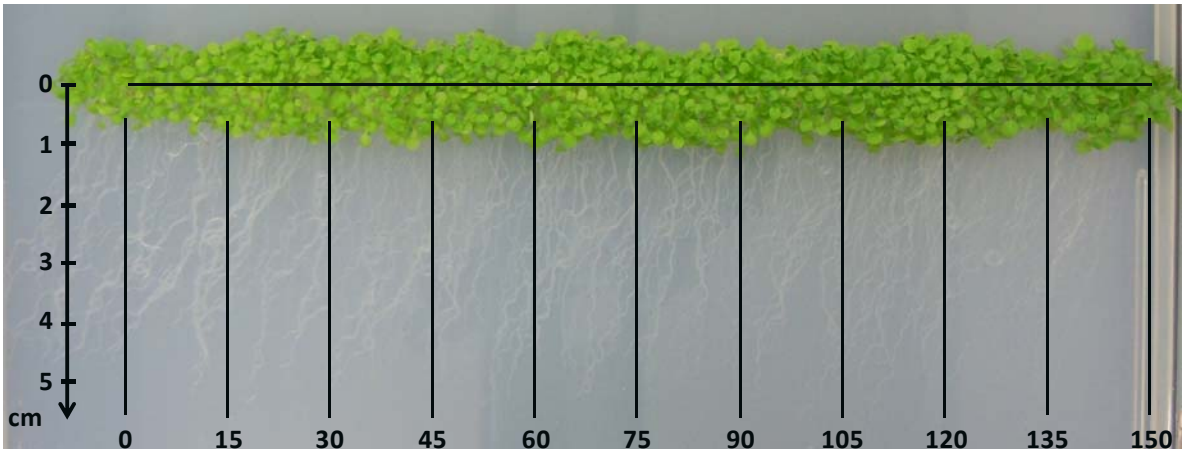
Figure 6. Relative expression of the *GUS*, *AT1G12030* genes in *A. thaliana* wild-type and FLAG mutant grown for 14 d in the presence (+S) or in the absence (-S) of 1500 µM sulfate in the medium.



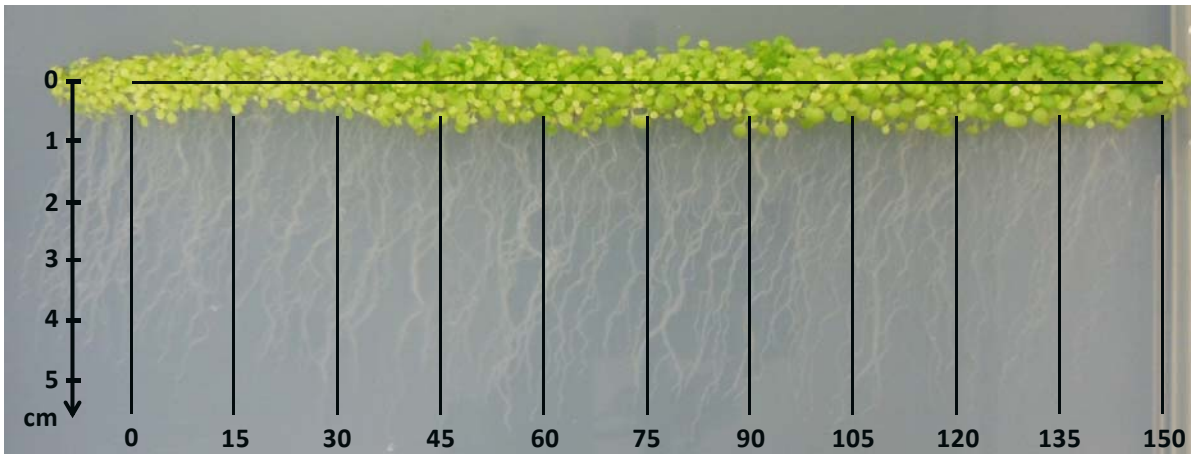
Relative expression levels of each gene were referred to a calibrator set to the value 1, which was represented by the treatment with the lowest expression.

Figure 7. Gradient plates.

7A. SO_4^{2-}-gradient agar plates (SGAPs: 0-150 µM)

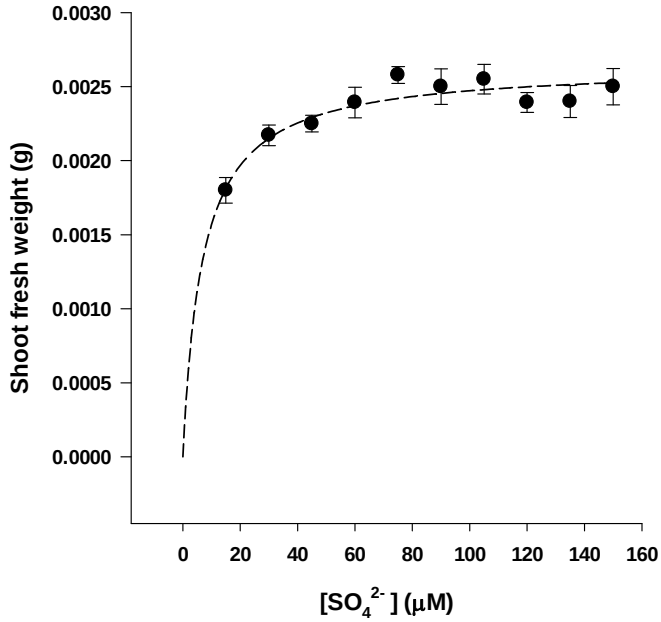


7B. SO_4^{2-}-gradient agar plates (SGAPs: 0-150 µM) added with 50 µM Cd.



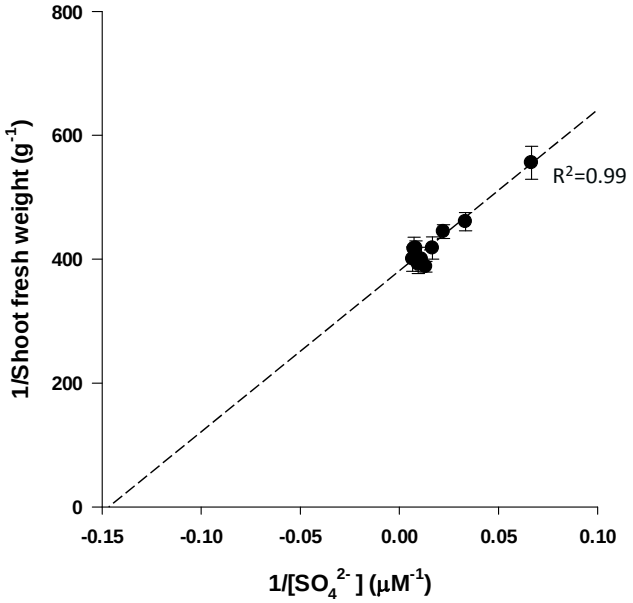
FLAG line seeds were planted in a line as shown. The picture shows the seedlings 14 days after germination. Four SGAPs were prepared for each experiment. The images are representatives of one SGAP from one typical experiment repeated two times with similar results.

Figure 8A. Relationship between sulfate concentrations and shoot fresh weight.



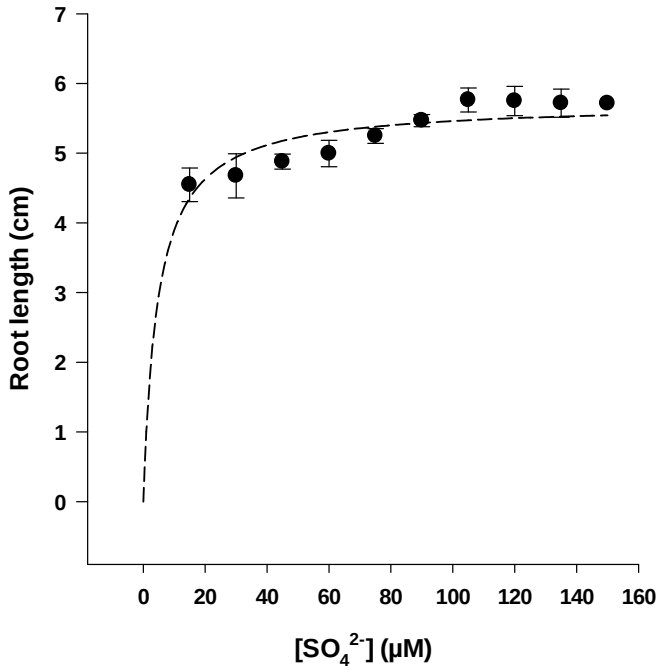
At each point, the shoot fresh weight was measured in 14 representative plants from each plate. The data represent means ± SE of two experiments.

Figure 8B. A Double-Reciprocal or Lineweaver-Burk Plot.



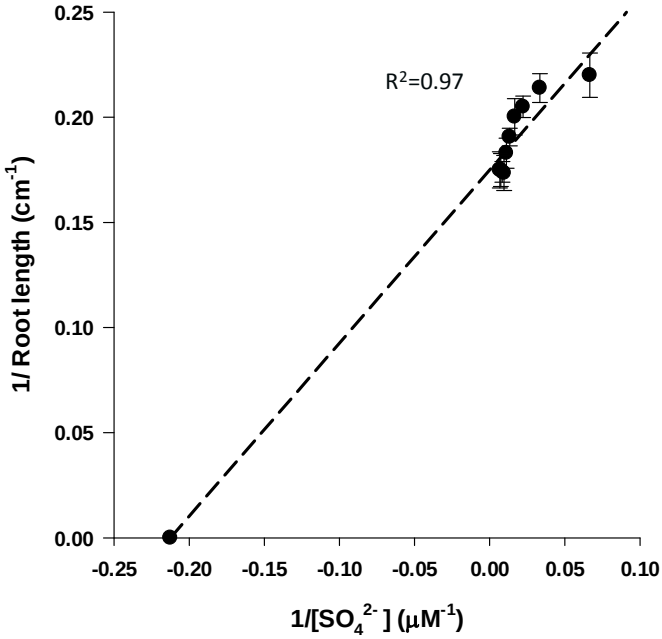
Slope is the value of the $\mathbf{K_{1/2}/FW_{max}}$ ratio; x-intercept represents the value of $-1/\mathbf{K_{1/2}}$.

Figure 9A. Relationship between sulfate concentration and root length.



At each point, the root length was measured in 6 representative plants from each plate. The data represent means ± SE of two experiments.

Figure 9B. A Double-Reciprocal or Lineweaver-Burk Plot.



Slope is the value of the $\mathbf{K_{1/2}/FW_{max}}$ ratio; x-intercept represents the value of $-1/\mathbf{K_{1/2}}$.

Table 1. Constants describing the relationship between sulfate concentration in the medium and shoot fresh weight or root length.

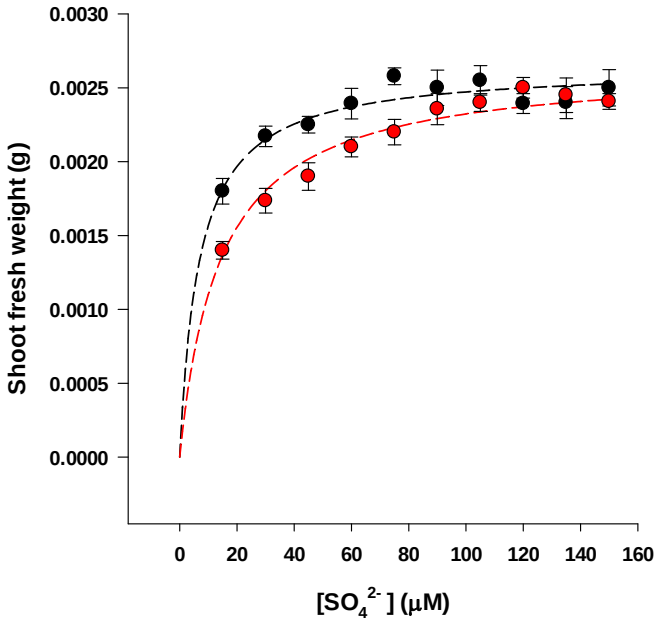
Parameters	Shoot FW	Root length
$\mathbf{FW_{max}}$	0.0026415 g	-
$\mathbf{Lr_{max}}$	-	5.71 cm
$\mathbf{K_{1/2}}$	6.86 µM	4.70 µM
$\boldsymbol{n_c}$	130.40 µM	89.41 µM

$\mathbf{FW_{max}}$ is the asymptote of the function $FW = \frac{FW_{max}\ [SO_4^{2-}]}{K_{½} + \ [SO_4^{2-}]}$

$\mathbf{Lr_{max}}$ is the asymptote of the function $Lr = \frac{Lr_{max}\ [SO_4^{2-}]}{K_{½} + \ [SO_4^{2-}]}$

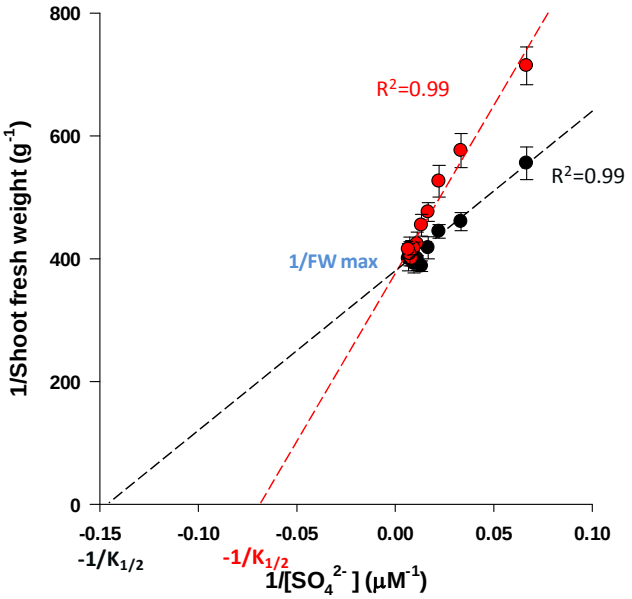
$\mathbf{K_{½}}$ represent the concentration of sulfate in the medium at which correspond the half value of either $\mathbf{FW_{max}}$ or $\mathbf{Lr_{max}}$.
$\mathbf{FW_{max}}$, $\mathbf{Lr_{max}}$ and $\mathbf{K_{½}}$ were evaluated by the Lineweaver-Burk Plot.

Figure 10A. Relationship between sulfate concentration and shoot fresh weight in the absence or in the presence of 50 µM Cd.



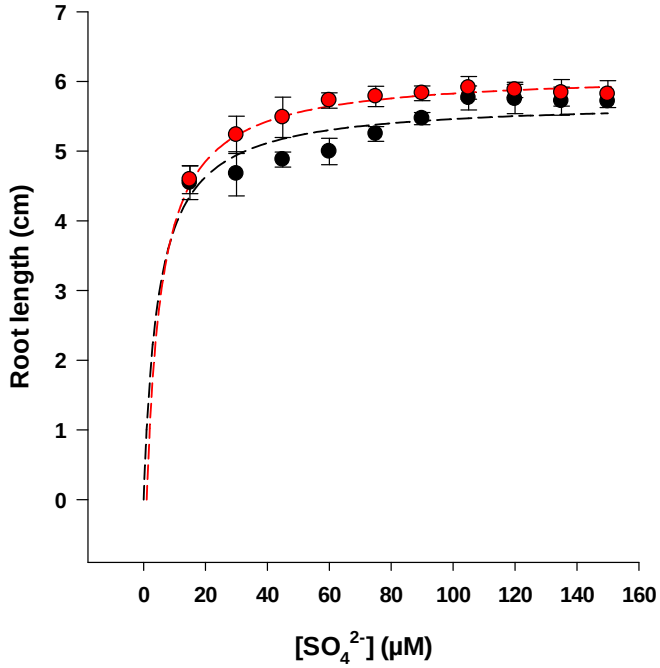
SO_4^{2-}-gradient agar plates (black curve) and SO_4^{2-}-gradient agar plates added with 50 µM $CdCl_2$ (red curve). At each point, the shoot fresh weight was measured in 14 representative plants from each plate. The data represent means ± SE of two experiments.

Figure 10B. A Double-Reciprocal or Line weaver-Burk Plot.



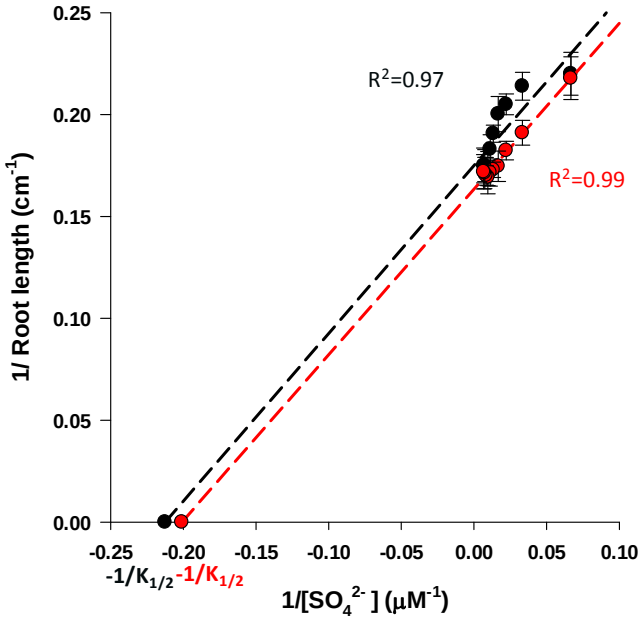
Slope is the value of the $\mathbf{K_{1/2}/FW_{max}}$ ratio; x-intercept represents the value of $-1/\mathbf{K_{1/2}}$.

Figure 11A. Relationship between sulfate concentration and root length in the absence or in the presence of 50 μM Cd.



SO_4^{2-}-gradient agar plates (black curve) and SO_4^{2-}-gradient agar plates added with 50 μM $CdCl_2$ (red curve). At each point, the root length was measured in 6 representative plants from each plate. The data represent means ± SE of two experiments.

Figure 11B. A Double-Reciprocal or Lineweaver-Burk Plot.



Slope is the value of the $\mathbf{K_{1/2}/FW_{max}}$ ratio; x-intercept represents the value of $-1/\mathbf{K_{1/2}}$.

Table 2. Constants describing the relationship between sulfate concentrations in the medium added with 50 µM Cd and shoot fresh weight or root length.

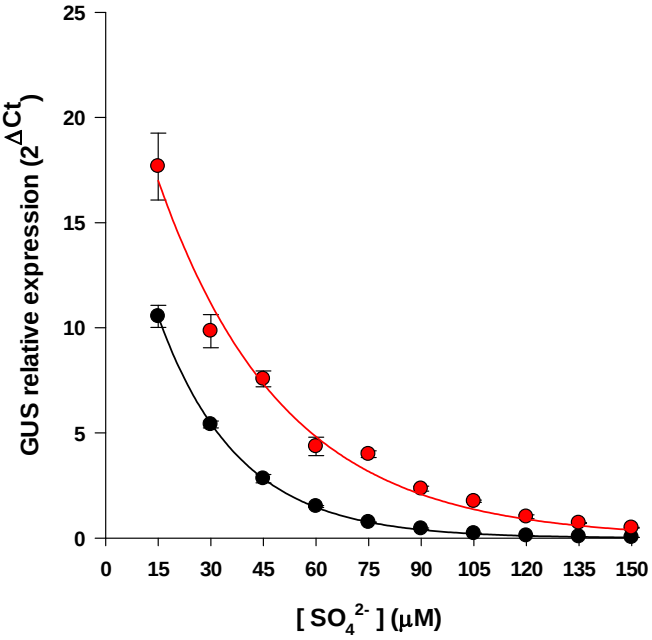
Parameters	Shoot FW	Root length
$\mathbf{FW_{max}}$	0.0026506 g	-
$\mathbf{Lr_{max}}$	-	6.11 cm
$\mathbf{K_{1/2}}$	14.10 µM	4.97 µM
n_c	267.99 µM	94.55 µM

FW_{max} is the asymptote of the function $FW = \frac{FW_{max}\ [SO_4^{2-}]}{K_{½} + \ [SO_4^{2-}]}$

Lr_{max} is the asymptote of the function $Lr = \frac{Lr_{max}\ [SO_4^{2-}]}{K_{½} + \ [SO_4^{2-}]}$

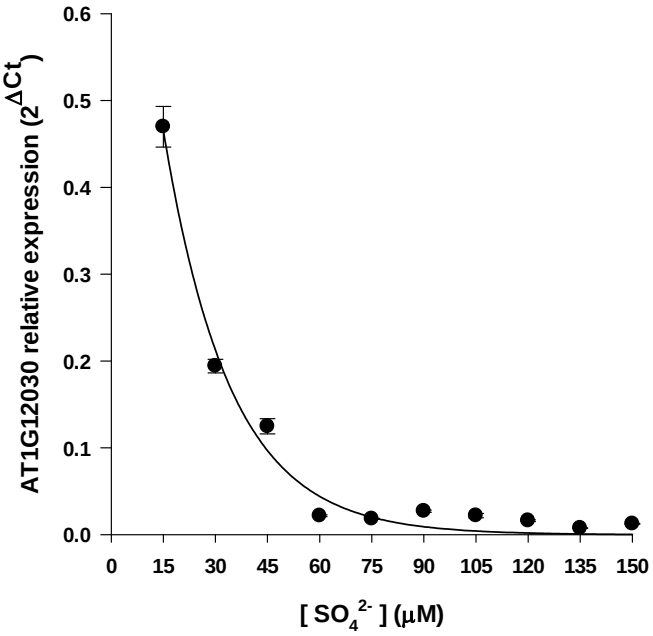
$K_{½}$ represent the concentration of sulfate in the medium at which correspond the half value of either **FW_{max}** or **Lr_{max}**.
FW_{max}, **Lr_{max}** and **$K_{½}$** were evaluated by the Lineweaver-Burk Plot.

Figure 12. Relative expression of *GUS* gene in shoot of plants grown at different sulfate concentrations in the presence or in the absence of 50 µM Cd.



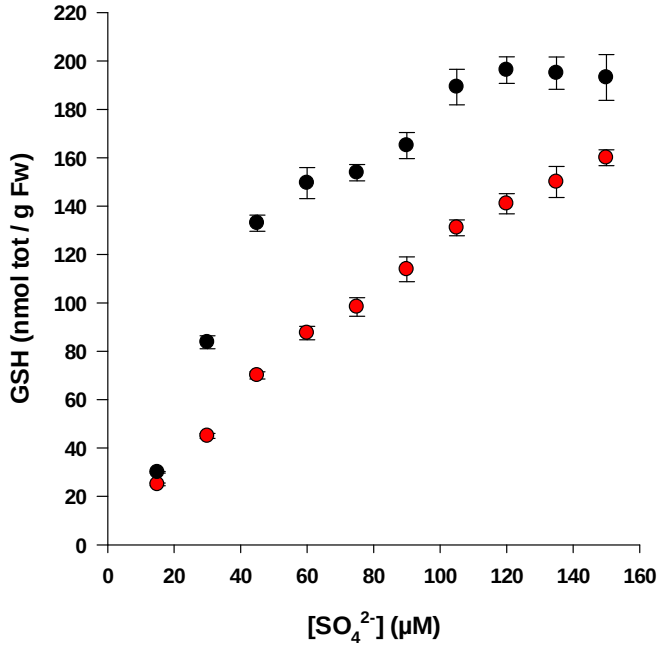
Total RNA was extracted from shoots and the gene expression was assessed by Real Time PCR. SO_4^{2-}-gradient agar plates (black curve) and SO_4^{2-}-gradient agar plates added with 50 µM $CdCl_2$ (red curve). The *GUS* levels were normalized using *S16* as an internal standard. The data represent means ± SE of two experiments run in quadruplicate (n = 8).

Figure 13. Relative expression of *ATG12030* gene in shoot of plants grown at different sulfate concentrations.



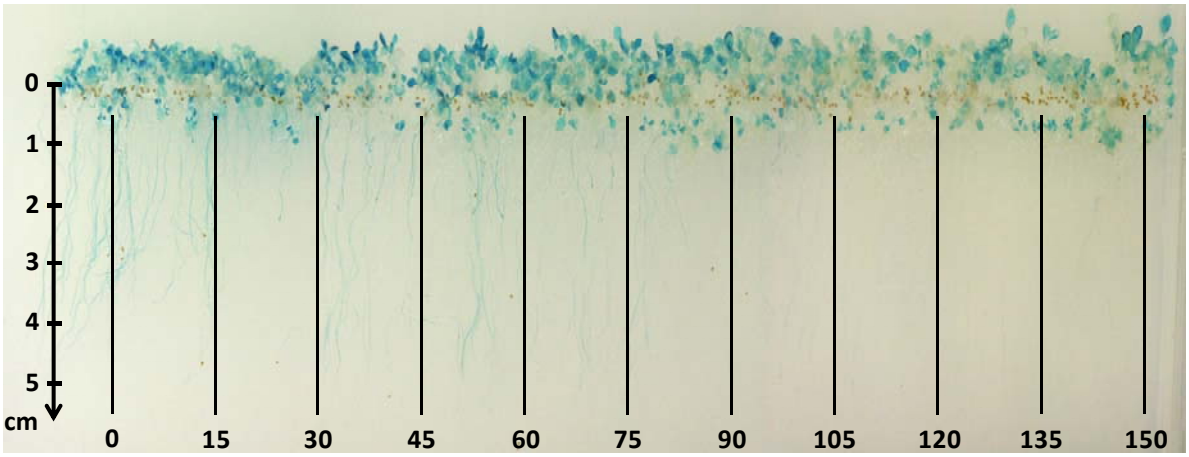
Total RNA was extracted from shoots and the gene expression was assessed by Real Time PCR. The *AT1G12030* levels were normalized using *S16* as an internal standard. The data represent means ± SE of two experiments run in quadruplicate (n = 8).

Figure 14. Relationship between sulfate concentration and the concentration of GSH in FLAG line shoots from plants grown in the presence or in the absence of 50 µM Cd.



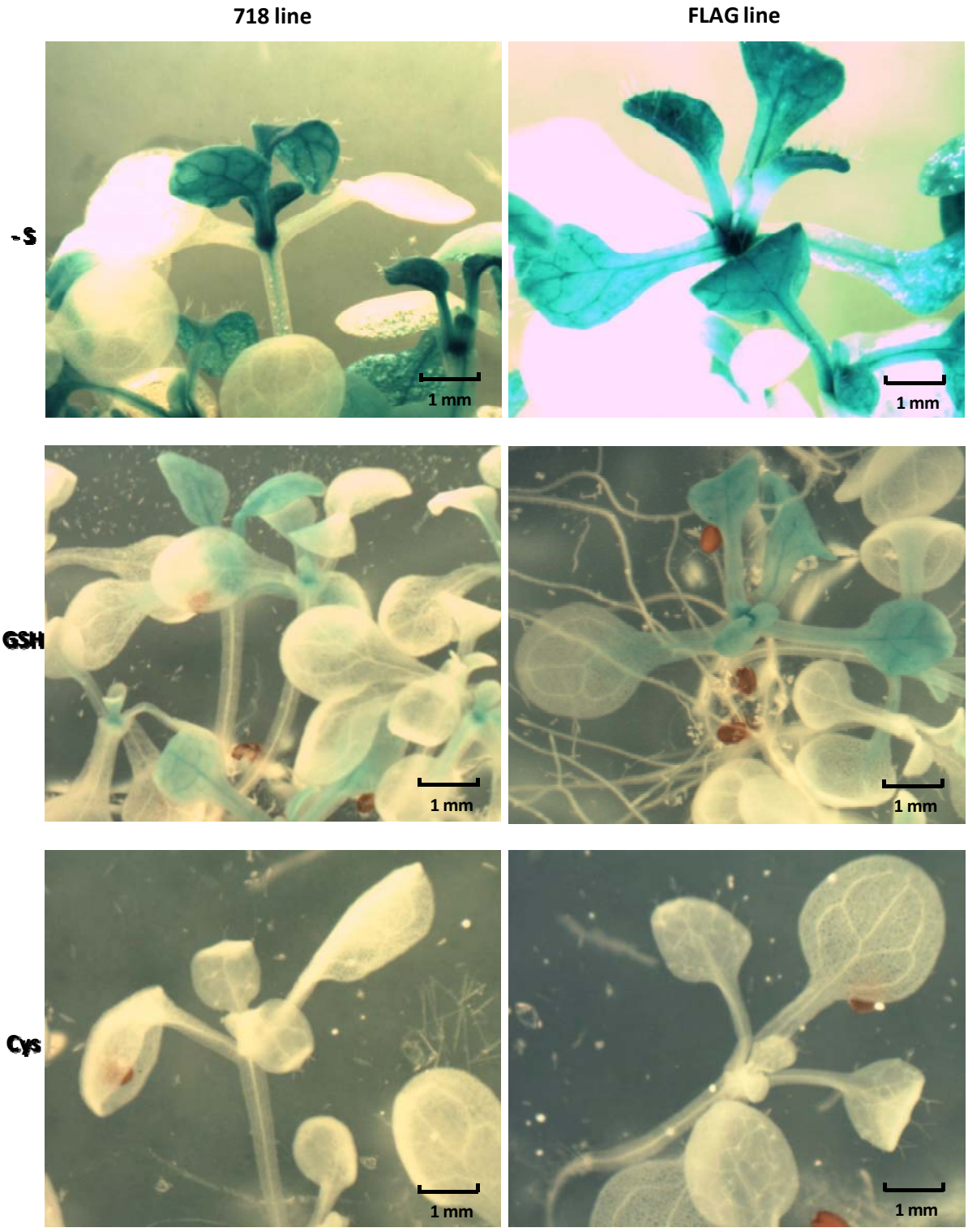
SO_4^{2-}-gradient agar plates (black circle) and SO_4^{2-}-gradient agar plates added with 50 µM $CdCl_2$ (red circle). The data represent means ± SE of two experiments run in duplicate (n = 4).

Figure 15. Activity of GUS in shoots and roots of FLAG line plants grown in the SO_4^{2-}-gradient agar plates in the presence of 50 µM Cd.



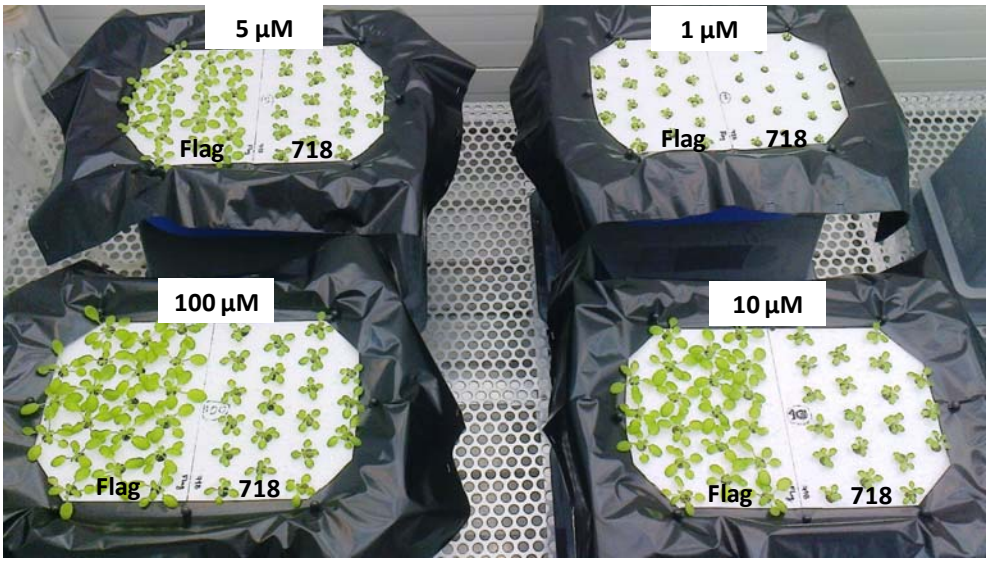
SAGPs 0-150 mM SO_4^{2-} added with 50 µM $CdCl_2$. After 14 days, plants were vacuum infiltrated with X-Gluc staining solution and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol. Plants were finally photographed. The image is representative of one typical experiment repeated two times with similar results.

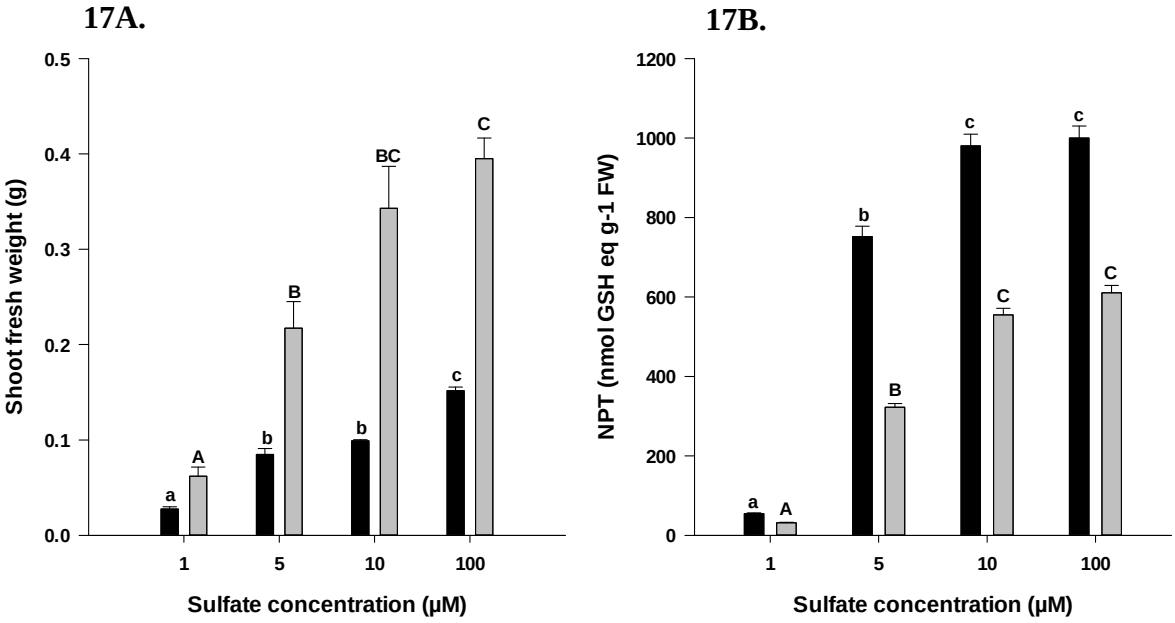
Figure 16. Effect of different sources of sulfur on the expression of the *GUS* reporter in gene 718 and FLAG line plants.



The plants were grown on agar medium in the presence of different sources of sulfur (**-S**: medium-S; **Cys**: medium-S supplemented with 1.5 mM; **GSH**: medium-S supplemented with 1.5 mM GSH; -S: medium-S). After 14 days plants were vacuum infiltrated with staining solution containing X-Gluc and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol and then photographed under a binocular microscope. The images are representatives of one typical experiment repeated two times with similar results.

Figure 17. Effect of different sulfate concentrations on shoot fresh weight **(A)** and on shoot NPT levels **(B)** in 718 and FLAG lines.





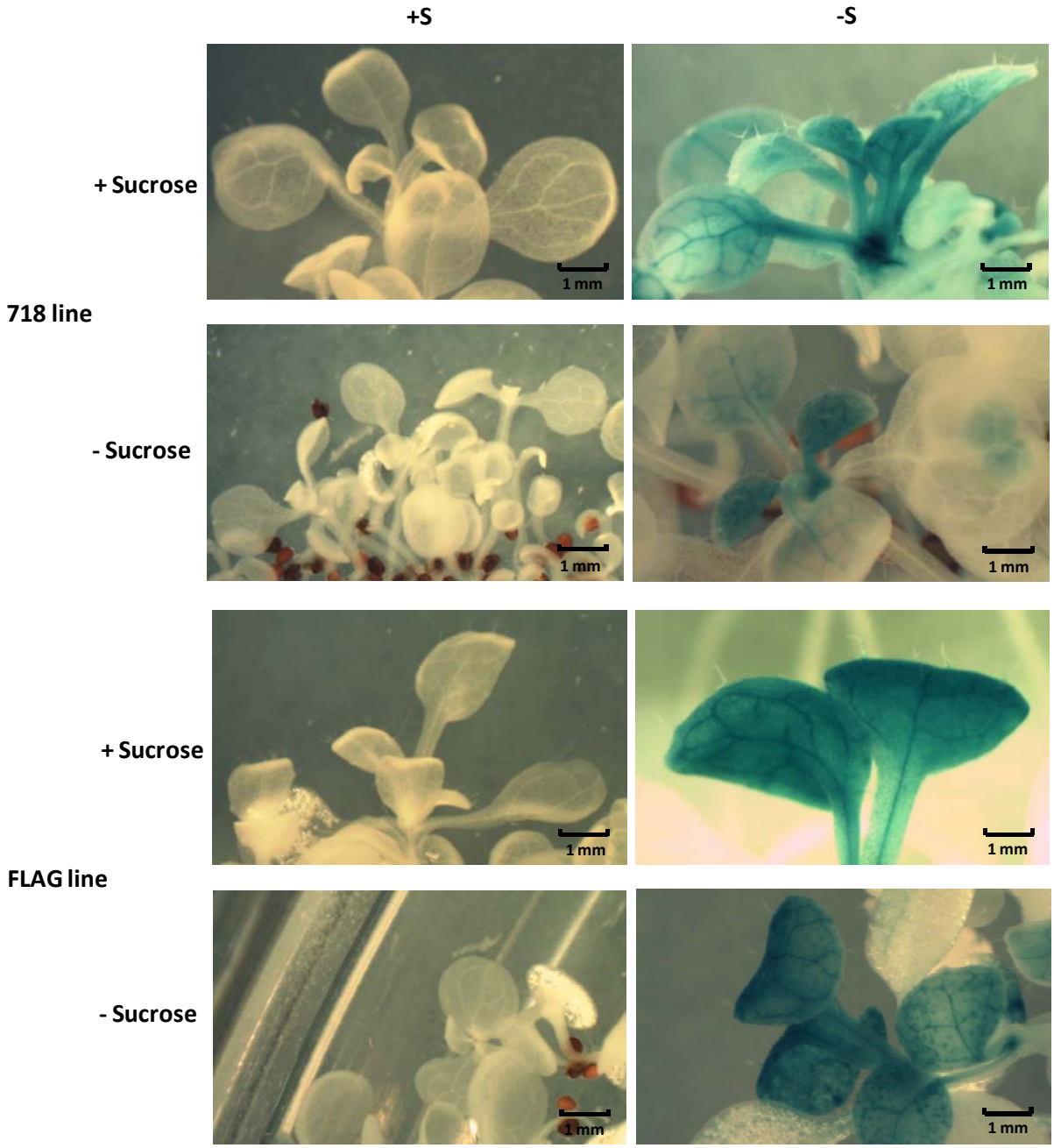
Plants were grown for 2 weeks in hydroponic solutions at 1, 5, 10 and 100 µM sulfate. 718 line (black bars) and FLAG line (grey bars). NPT concentrations were determined by spectrophotometric assays. The data shown in the graphs are mean values ± SE obtained in two experiment performed in triplicate (n = 6). Different letters indicate significant differences ($P < 0.05$).

Table 3. The activity of the *GUS* reporter gene in 718 and FLAG lines after histochemical staining.

	1 µM	**5 µM**	**10 µM**	**100 µM**
718 line	ND	ND	ND	ND
FLAG line	**X**	**X**	ND	ND

Plants were grown for 2 weeks in hydroponic solutions at 1, 5, 10 and 100 µM sulfate. After 14 days, plants were vacuum infiltrated with X-Gluc staining solution and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol. Plants were finally photographed under a binocular microscope. (**X**, *GUS* activity; ND, not detectable). The images are representatives of one typical experiment repeated two times with similar results.

Figure 18. Effect of sucrose on the activity of the *GUS* reporter gene in 718 and FLAG lines

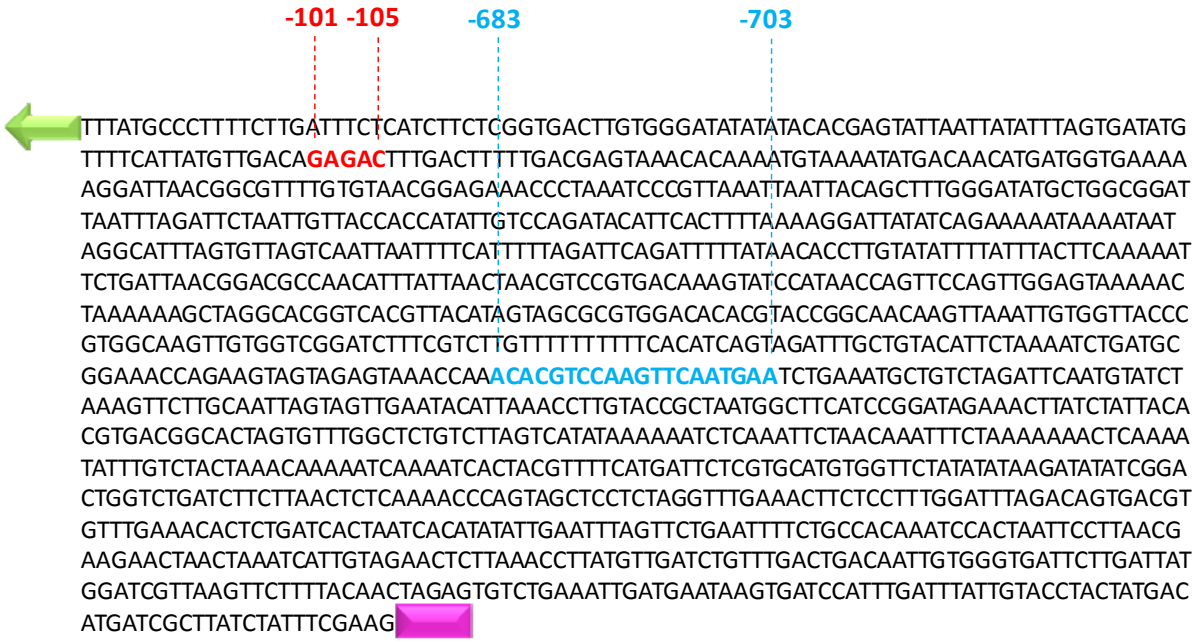


The plants were grown in complete (+S: 1500 µM) or without sulfur (-S: 0 µM) media in the presence or in the absence of sucrose (1% w/v). After 20 days, plants were vacuum infiltrated with X-Gluc staining solution and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol. Plants were finally photographed under a binocular microscope. The images are representatives of one typical experiment repeated two times with similar results.

Figure 19. SURE and UPE box within the sequence between the start codon of *AT1G12030* and the insertion site of the element *DSG* in the FLAG and 718 lines.

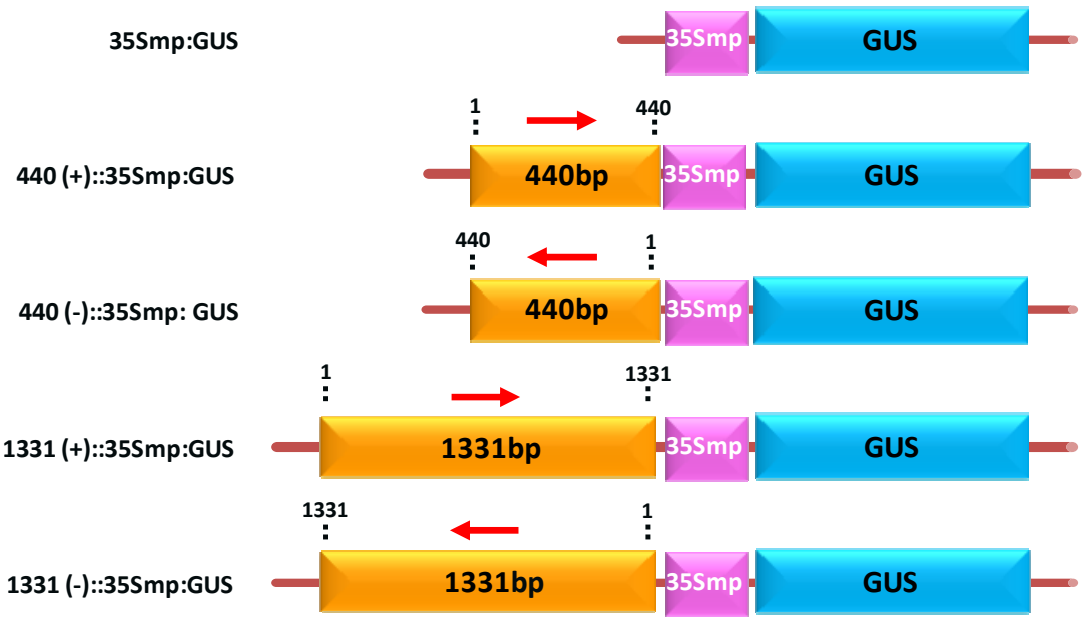
19A. FLAG line

19B. 718 line



The arrow indicates the gene *AT1G12030* (green) and the box (violet) indicate the *DsG* element. The SURE element is highlighted in red and the UPE box is highlighted in blue; the position of the SURE and UPE box are reported with respect to *AT1G12030* gene.

Figure 20. Schematic diagram of the different chimeric promoter constructs. 35Smp, 35S core promoter.



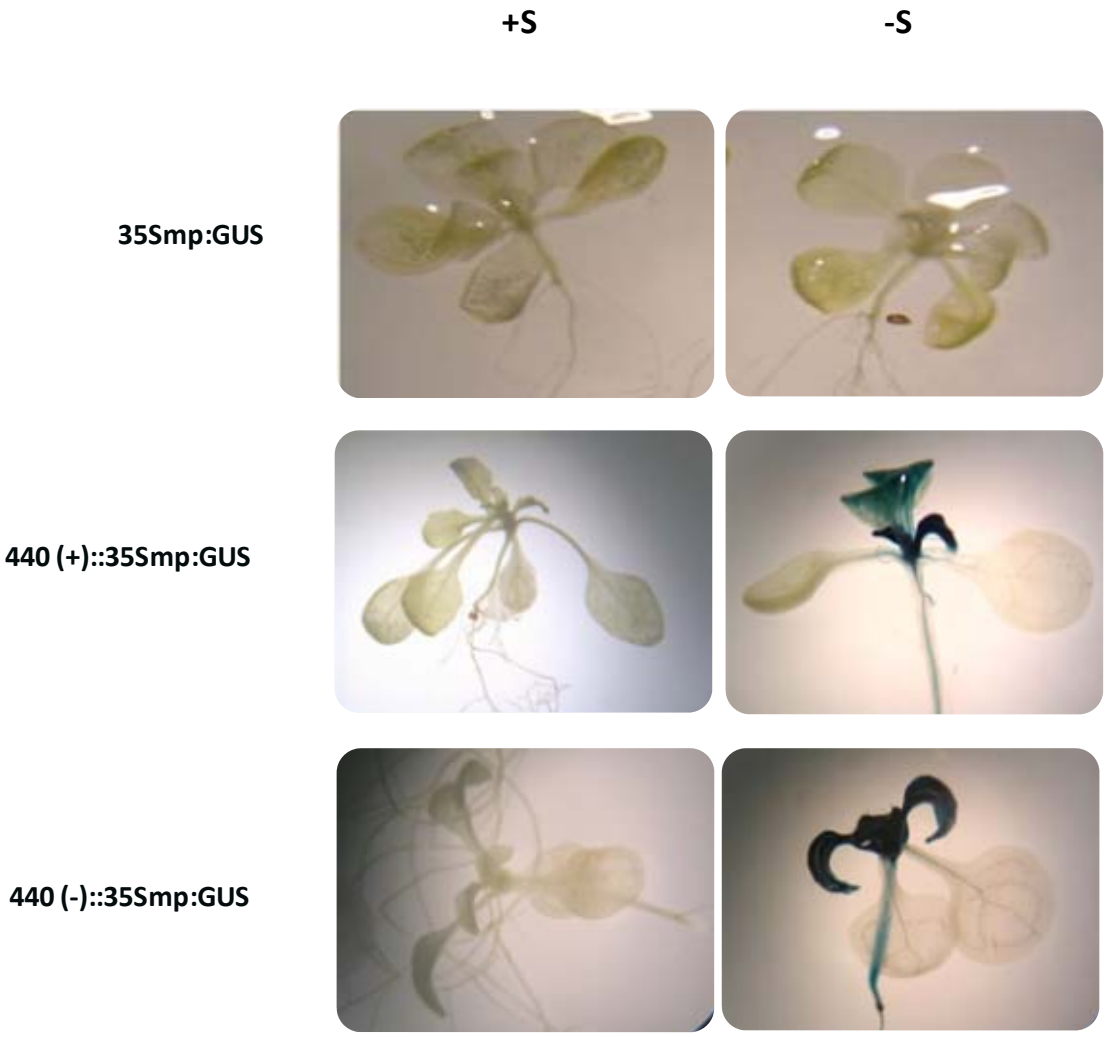
35Smp, 35S minimal promoter

β-glucuronidase (GUS) gene

Intergenic region

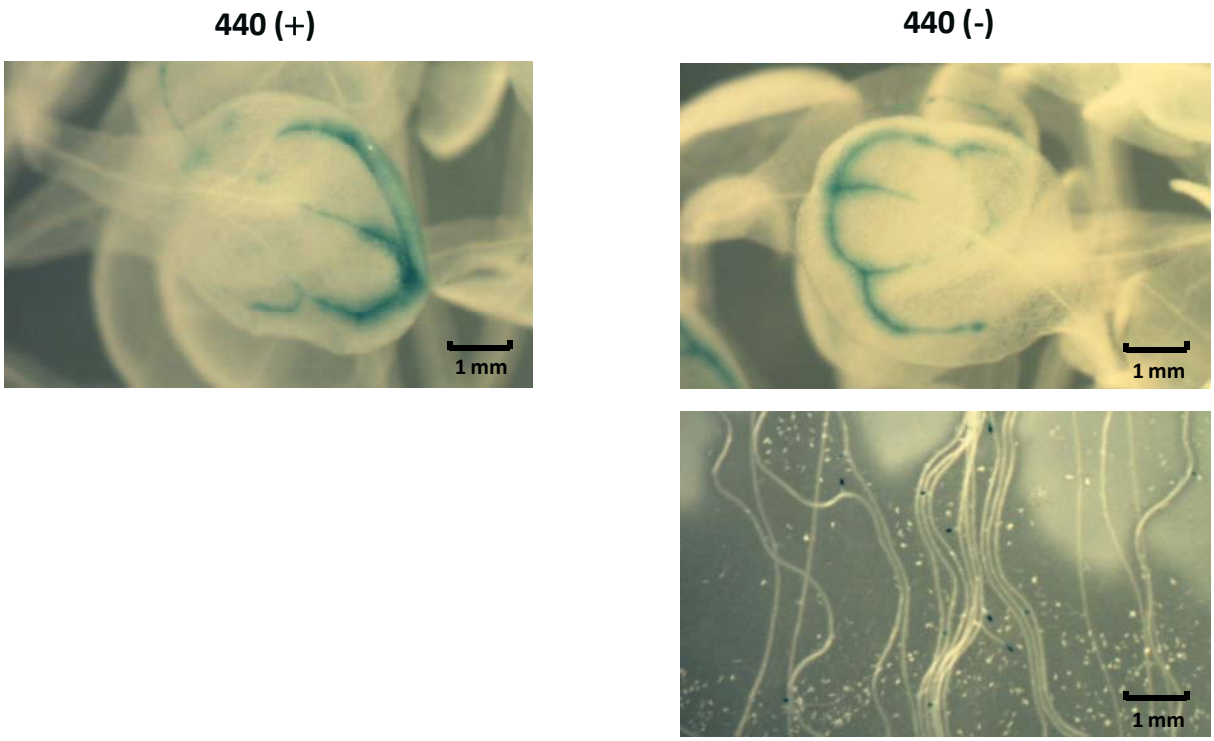
The images are not representative of the real size between the elements of the constructs.

Figure 21. Activity of the *GUS* reporter gene in the homozygous transgenic plants carrying 440 bp fragment.



Transgenic plants were grown in complete (+S: 1500 µM) or without sulfur (-S: 0 µM) media. After 20 days plants were vacuum infiltrated with X-Gluc staining solution and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol. Plants were finally photographed under a binocular microscope. The images are representatives of one typical experiment repeated two times with similar results.

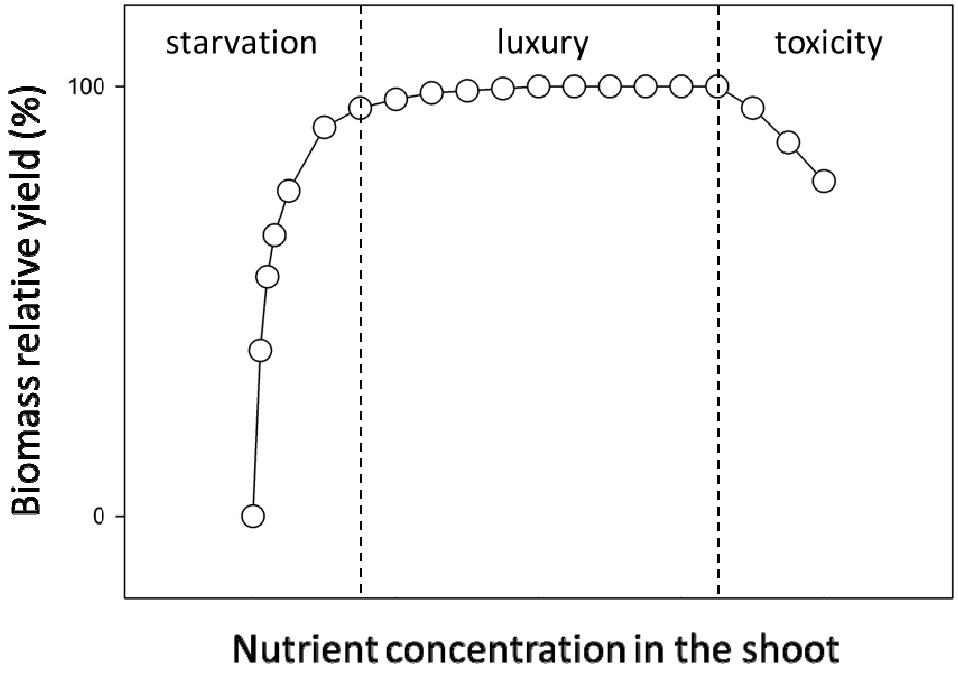
Figure 22. Activity of the *GUS* reporter gene in the homozygous transgenic plants carrying 440-bp fragment in the presence of 50 µM Cd.



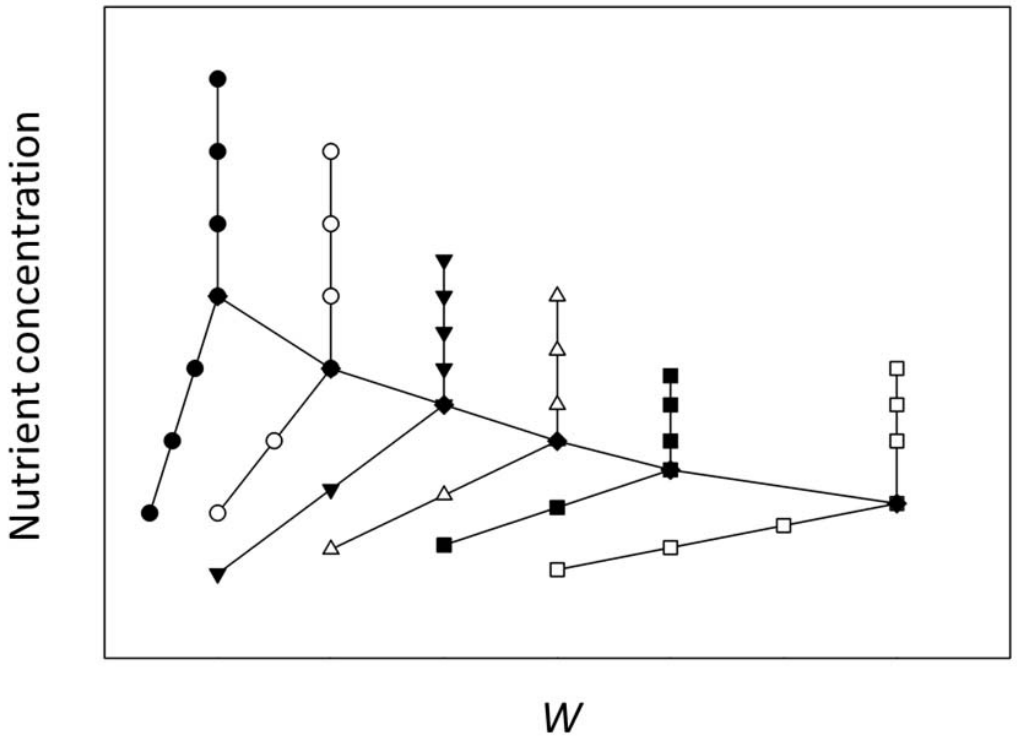
Transgenic plants were grown in complete medium in the presence of cadmium (Cd: 50 µM). After 20 days, plants were vacuum infiltrated with X-Gluc staining solution and incubated overnight at 37°C. In order to allow the visualization of the blue product, chlorophyll was removed from leaves by stepwise replacement of the staining solution with ethanol. Plants were finally photographed under a binocular microscope. The images are representatives of one typical experiment repeated two times with similar results.

ANNEX

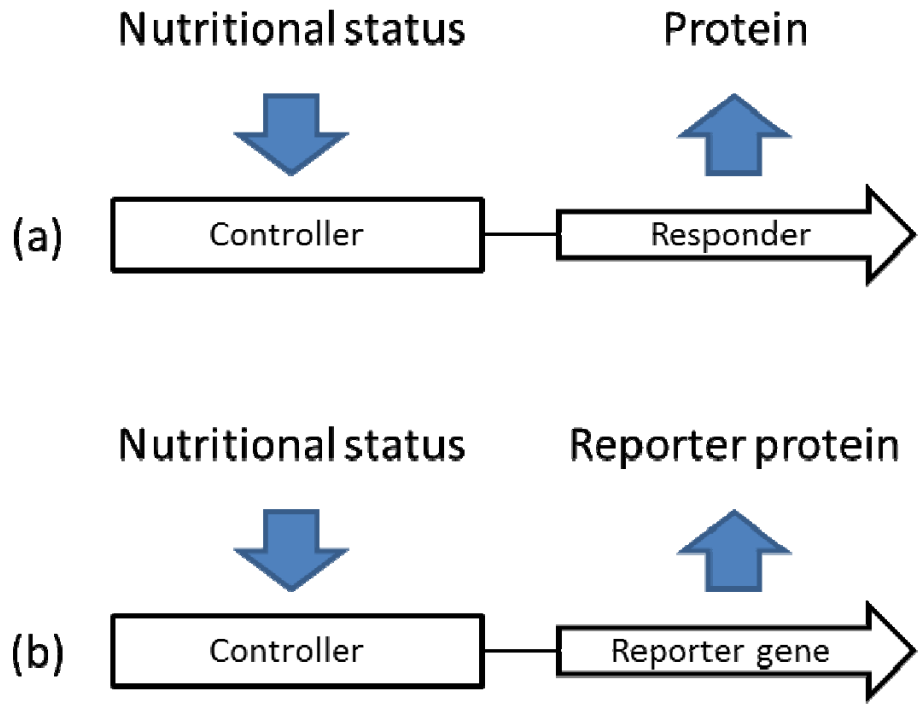
Annex 1. Relationship between the concentration of a nutrient in the shoot and the biomass relative yield.



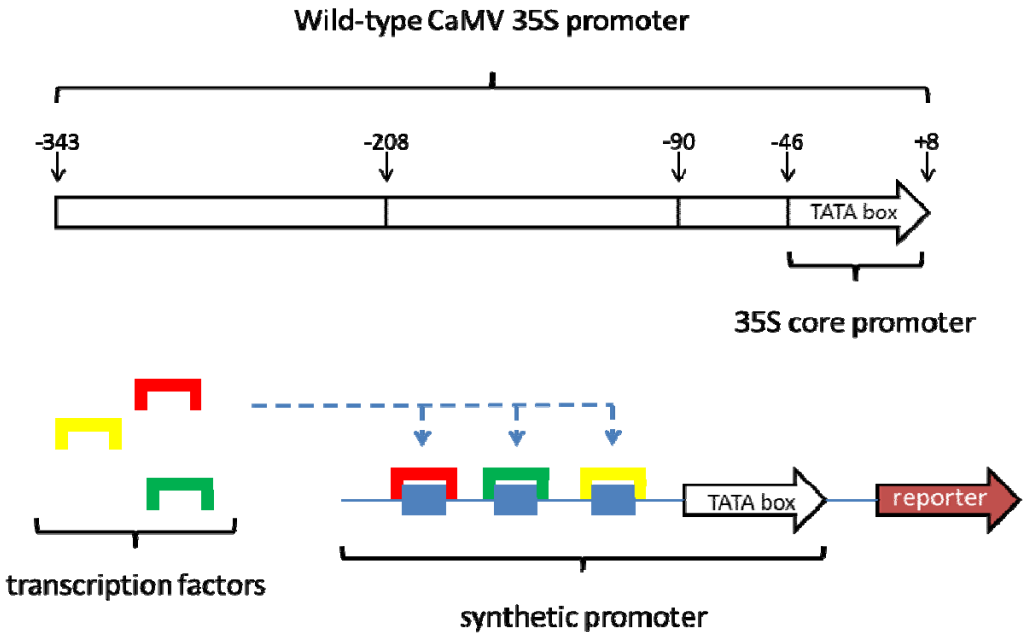
Annex 2. Dilution curve for a generic essential nutrient as defined by six n_c values determined for six plant developmental stages.



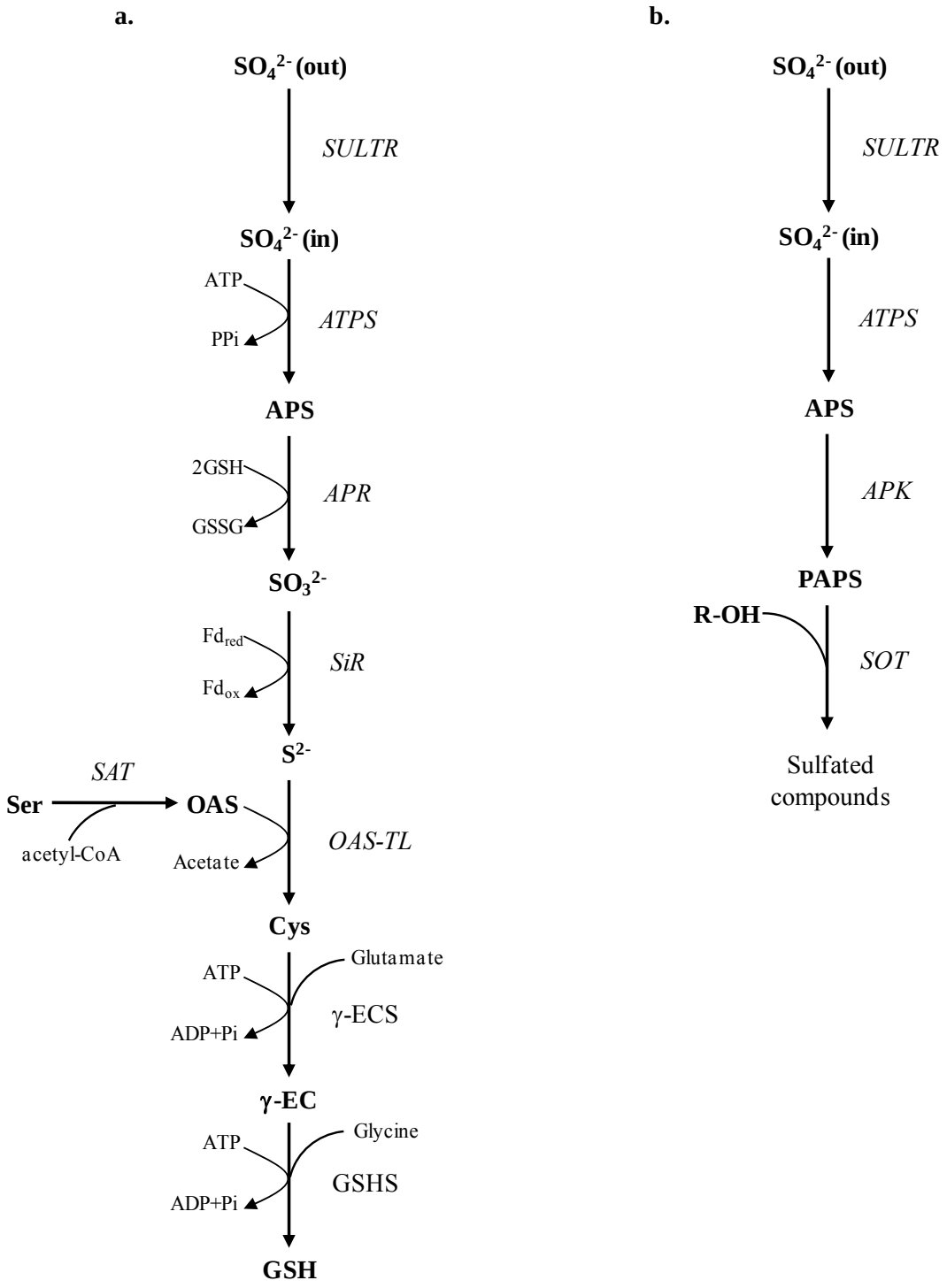
Annex 3. Gene fusion is a DNA construction in which the coding sequence from one gene (reporter) is transcribed and/or translated under the direction of the controlling sequence of another gene (controller). (a) Gene structure. (b) Gene fusion.



Annex 4. Schematic representation of a synthetic promoter useful for bioindication purposes. The core region of the CaMV 35S promoter is fused with a combinatorial engineering of cis-elements (blue boxes) which, following the interaction with specific transcription factors, drives the reporter expression under particular conditions.

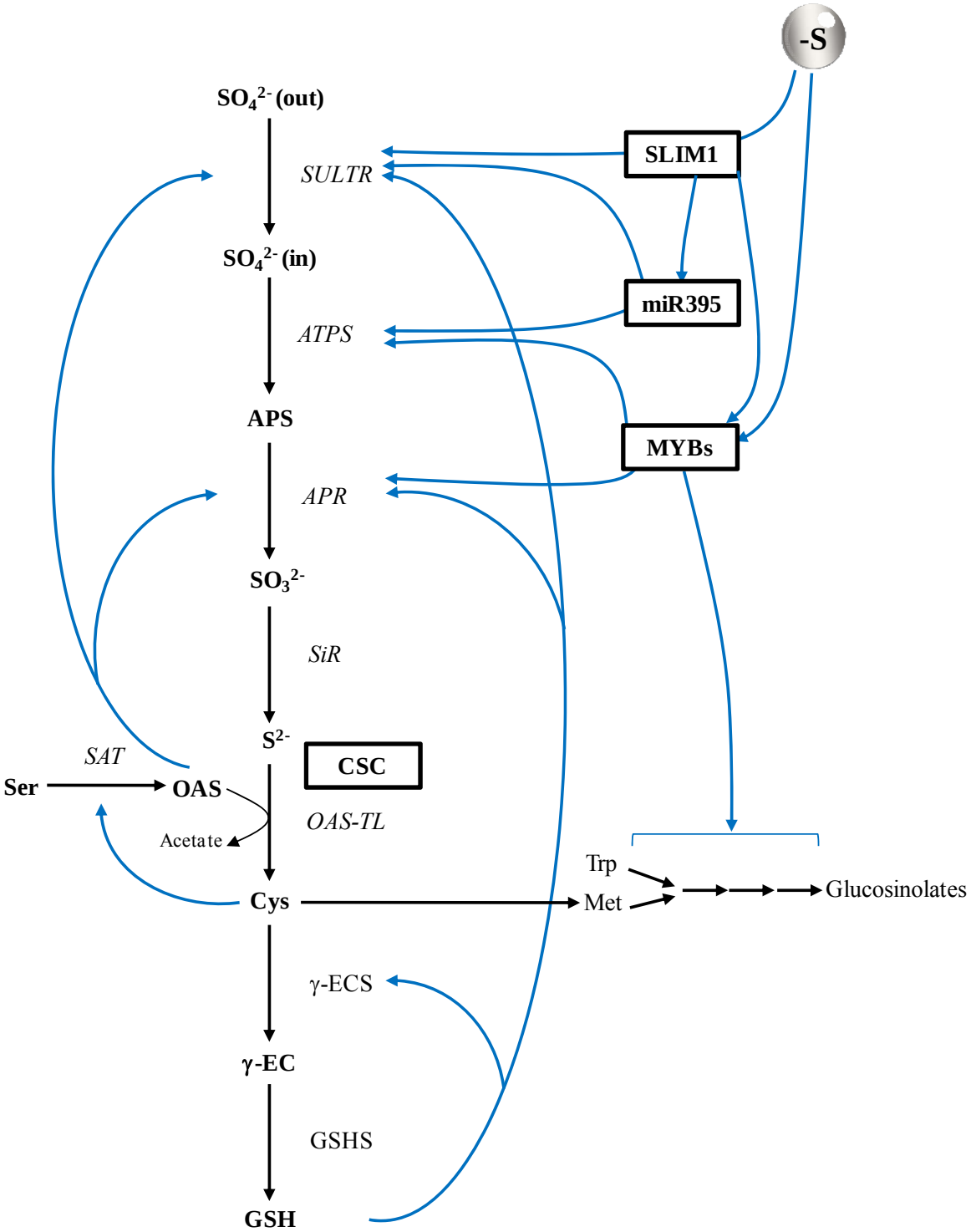


Annex 5. Reductive assimilatory sulfate pathway in plastid (a) and cytosol (b) of higher plants.



APS, adenosine 5-phosphosulfate; Cys, cysteine; γ-GluCys, γ-glutamylcysteine; GSH, glutathione; OAS, *O*-acetylserine; SO_3^{2-}, sulfite; S^{2-}, sulfide; PAPS, 3-phosphoadenosine 5-phosphosulfate; R-OH, hydroxylated precursor. *APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; γ -ECS, γ-glutamylcysteine synthetase*; *GSHS, glutathione synthetase; OAS-TL, OAS(thiol)lyase; SAT, serine acetyltransferase; SiR, sulfite reductase; SOT, sulfotransferase; SULTR, sulfate transporter.*

Annex 6. Regulatory pathways and components controlling sulfate transport and metabolism.



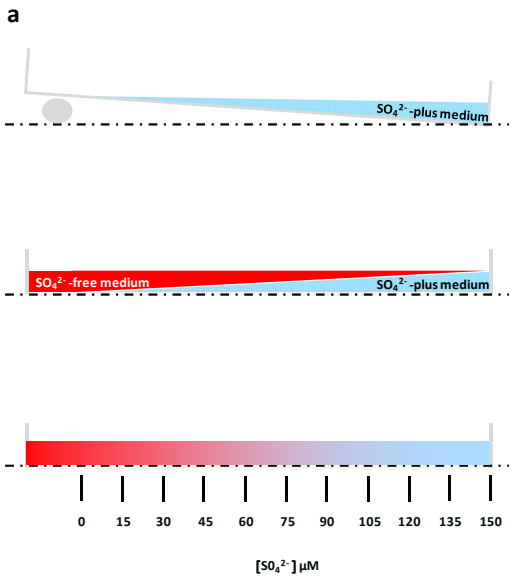
Annex 7. Composition of the basic nutrient agar solution.

Base medium	
component	**concentration**
NH_4NO_3 (mM)	20.61
$CaCl_2$ (mM)	2.99
KNO_3 (mM)	18.79
KH_2PO_4 (mM)	1.25
H_3BO_3 (μM)	100
Na_2-EDTA (μM)	100
$MnCl_2$ (μM)	100
$ZnCl_2$ (μM)	29.91
$Fe_2(CH_4O_6)_3$ (μM)	25
$CoCl_2$ (μM)	0.11
$CuCl_2$ (μM)	0.1
$(NH_4)_6Mo_7O_{24}$ (μM)	1.03
KI (μM)	5
Sucrose (g l^{-1})	10
Agar (g l^{-1})	8

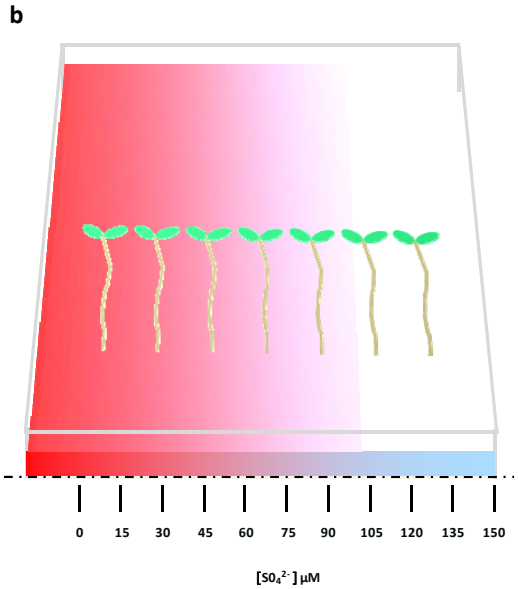
At the basic nutrient solution, $MgSO_4$ and $MgCl_2$ were added in different concentrations to obtain complete media (+S), deprived media (-S) according to the following scheme:

Medium	**$MgSO_4$** (μM)	**$MgCl_2$** (μM)
+S	1500	-
-S	-	1500

Annex 8. SO_4^{2-}-gradient agar plates (SGAPs). Sulfate concentration gradient at the medium surface of SGAPs (0–150 µM).



8a. Preparation of SGAPs. A square plastic dish is inclined on a glass bar, and SO_4^{2-}-plus medium is added. When the medium has solidified, the dish is placed horizontally. Then, SO_4^{2-}-free medium is poured into the dish from the left end, also covering the SO_4^{2-}-plus medium. A gradient of sulfate concentration is thus formed at the surface of the medium. The SGAPs contain 1.25% (w/v) agar to prevent the Arabidopsis roots penetrating the surface of the medium.



8b. Growing plants using SGAPs. SGAPs are set vertically and Arabidopsis FLAG line seeds are planted in a horizontal line from the left to the right side of the plate respectively from the lowest to the highest sulfate concentration. Root growth and shoot biomass are monitored for 14 days after germination.

Annex 9. Composition of the basic nutrient solution (Hoagland ½) used in the hydroponic culture at different concentrations of sulfate.

Base medium	
component	**concentration**
$NH_4H_2PO_4$ (mM)	0.5
Ca $(NO_3)_2$ (mM)	2
KNO_3 (mM)	3
H_3BO_3 (μM)	46
$MnCl_2$ (μM)	9
$ZnSO_4$ (μM)	0.8
Fe-EDTA (μM)	25
$CuSO_4$ (μM)	0.3
$(NH_4)_6$ Mo_7O_{24} (μM)	0.1

At the basic nutrient solution, $MgSO_4$ and $MgCl_2$ were added in different concentrations to obtain different concentrations of sulfate, according to the following scheme:

$MgSO_4$ (μM)	**$MgCl_2$** (μM)
100	1400
10	1490
5	1495
1	1499

Annex 10. cDNA identification of *AT1G12030*, *GUS* and *S16* genes.

10a. List of primers used

Oligo	Sequence	Length (bp)	Tm (°C)	Ta (°C)
UNK for	TGTGGGAACACCTGAGGAGC	20	64	60
UNK rev	TCACTGCCGTTGAAAGCCAC	20	62	
GUS for	ATTACGGCAAAGTGTGGGTC	20	60	58
GUS rev	CAGAAAAGCCGCCGACTTCG	20	64	
S16 for	GGCGACTCAACCAGCTACTGA	21	66	56
S16 rev	CGGTAACTCTTCTGGTAACGA	21	62	

UNK = primer for *AT1G12030*

10b. Reaction mixtures

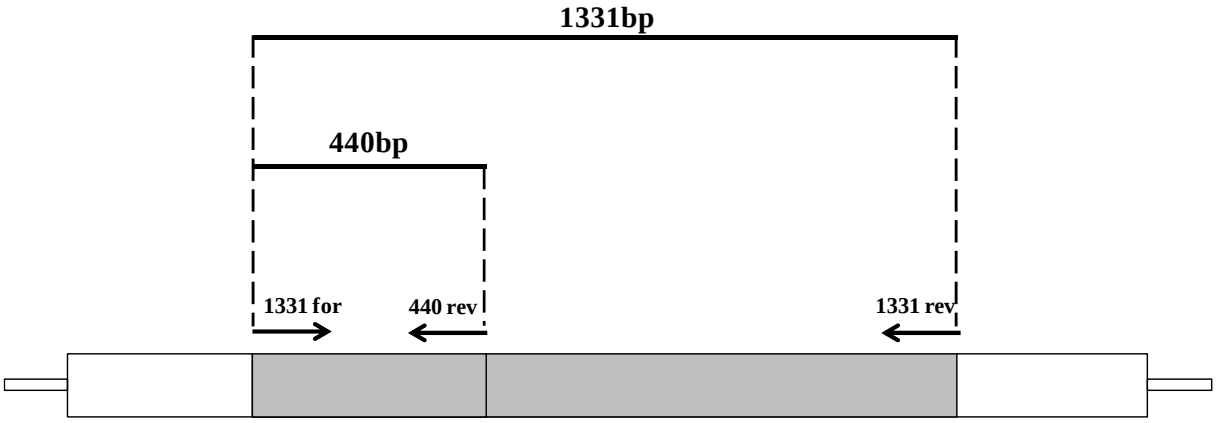
Reagent	µl
Template DNA	8
Primer for (10µM)	0.75
Primer rev (10µM)	0.75
dNTPs (2.5µM)	2
GoTaq Flexi buffer 5x	5
GoTaq Flexi (5u/µl)	0.13
$MgCl_2$ (25mM)	2
H_2O	6.37
Total volume	**17 µl**

10c. Thermal profile of the reaction

94°C for 2 min, 35 cycles of 94°C for 45 sec (*AT1G12030* and *GUS* genes) and 24 cycles of 94°C for 45 sec (*S16*) , 45 sec at a temperature variable in function of the primers, 72°C for 30 sec (*AT1G12030*) or 45 sec (*S16*) or 1 min and 30 sec (*GUS*). The cycles were followed by a final elongation step at 72°C for 30 min.

Annex 11. Amplification of the fragments of interest (440 and 1331-bp fragments).

11a. Strategy of amplification



11b. Primers

11b.1. Primers for amplifying 440-bp

Oligo (with XbaI site)	**Sequence**	**Length (bp)**	**Tm (°C)**	**Ta (°C)**
1331 for	GATGTCTAGATTTATGCCCTTTTCTTGATTTCT	23	60	60
440 rev	TGCATCTAGATAATAAATGTTGGCGTCCGTTA	22	60	60

bp for Xba I site

11b.2. Primers for amplifying 1331-bp

Oligo (with Hind III site)	**Sequence**	**Length (bp)**	**Tm (°C)**	**Ta (°C)**
1331 for	GATGAAGCTTTTTATGCCCTTTTCTTGATTTCT	23	60	60
1331 rev	TGCAAAGCTTCTTCGAAATGAATAAGCGATCA	23	60	60

bp for HindIII site

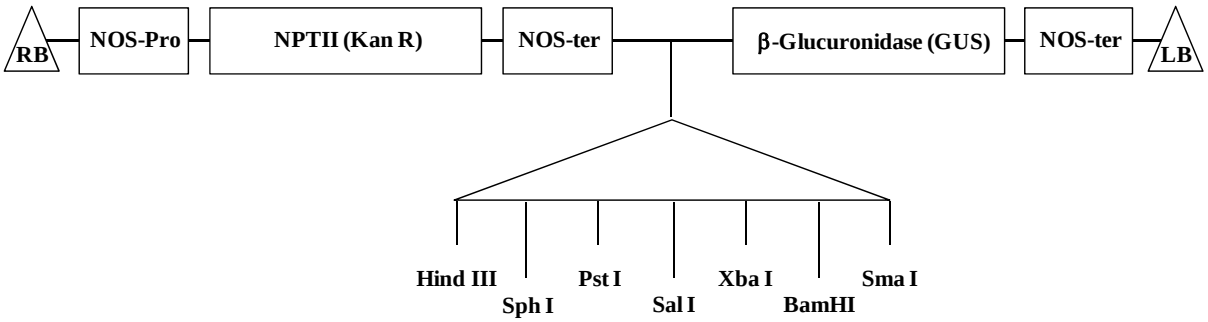
11c. Reaction mixtures

Reagent	**µl**
Template DNA (1:1000)	2
Primer for (10µM)	0.75
Primer rev (10µM)	0.75
dNTPs (2,5µM)	3
Pfu buffer 10x	2.5
Pfu (3U/µl)	0.25
H_2O	15.75
Total volume	**23 µl**

11d. Thermal profile of the reaction

94°C for 2 min, 35 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min and 30 sec . The cycles were followed by a final extension step at 72°C for 30 min.

Annex 12. pBI101 (12.2 kb).



Annex 13. 35Smp

13a. Oligonucleotides of 35Smp

Oligo	Sequence
Oligo1	CTAGAGCAAGACCCTTCCTCTATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTG
Oligo2	GATCCAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCT

bp for Xba I site/ bp for BamHI site

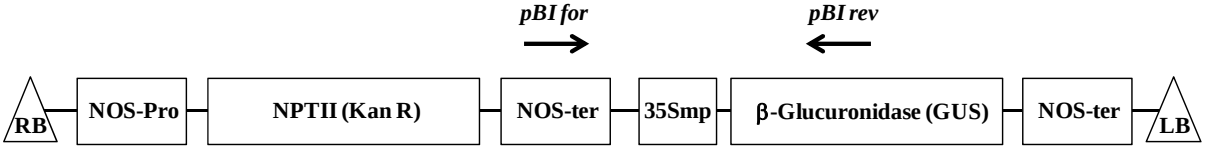
13b. Annealing reaction of oligonucleotides

Reagent	µl
Oligo 1 (0.1µg/µl)	5
Oligo 2 (0.1µg/µl)	5
Total volume	**10 µl**

13c. Thermal profile of the reaction

1 cycle of 70°C for 5 min followed by a cooling step at 22°C for 30 min.

Annex 14. Colony PCR.



14a. List of primers used

Oligo	Sequence	(bp)	Tm (°C)	Ta (°C)
pBI for	GGCCGATTCATTAATGCAGC	20	60	56
pBI rev	AACGCTGATCAATTCCACAGT	21	60	56

14b. Reaction mixtures

Reagent	µl
Template DNA	2
Primer for (10µM)	0.75
Primer rev (10µM)	0.75
dNTPs (2.5µM)	2
GoTaq Flexi buffer 5x	5
GoTaq Flexi (5u/µl)	0.13
$MgCl_2$ (25mM)	2
H_2O	12.37
Total volume	**25 µl**

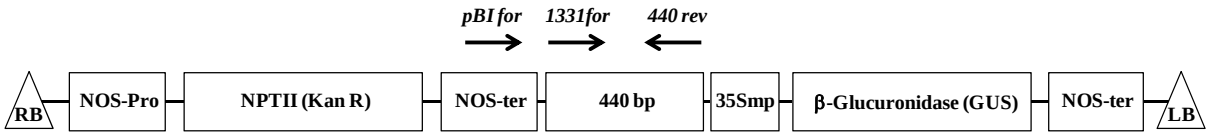
14c1. Thermal profile of the reaction

94°C for 2 min, 35 cycles of 94°C for 45 sec, 56°C for 45 sec, 72°C for 45 sec . The cycles were followed by a final extension step at 72°C for 30 min.

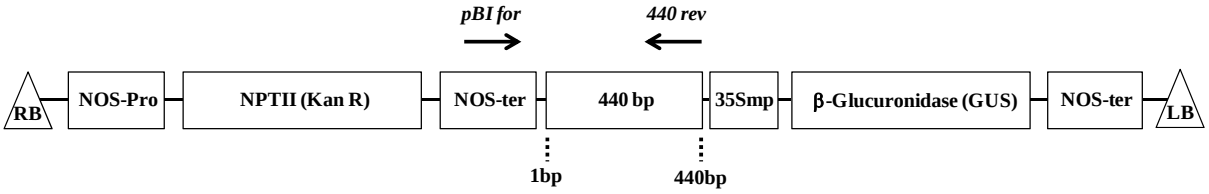
14c2. Thermal profile of the reaction

94°C for 2 min, 35 cycles of 94°C for 45 sec, 56°C for 45 sec, 72°C for 2 min . The cycles were followed by a final extension step at 72°C for 30 min.

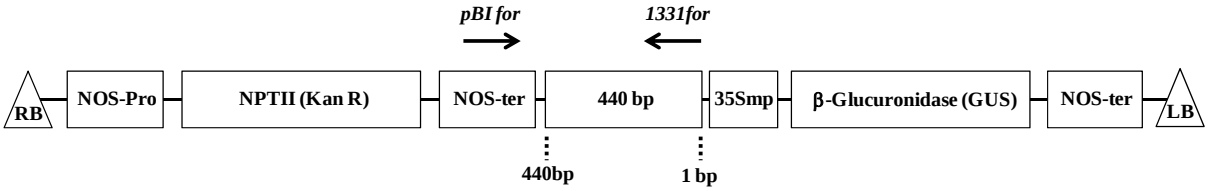
Annex 15. pBI 101.3-35Smp-440 bp.



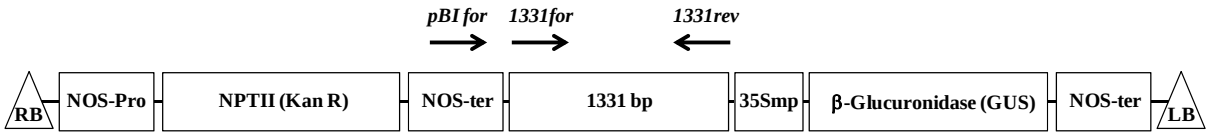
15a. 440-bp (+)



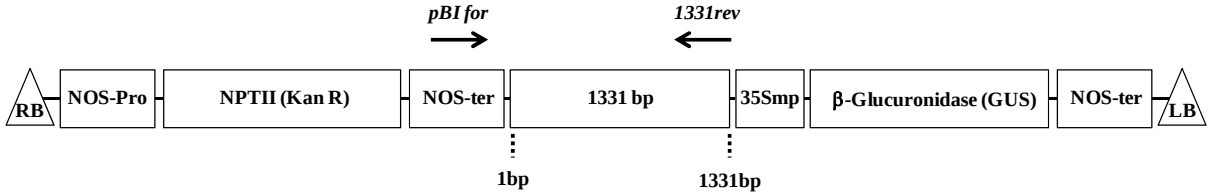
15b. 440-bp (-)



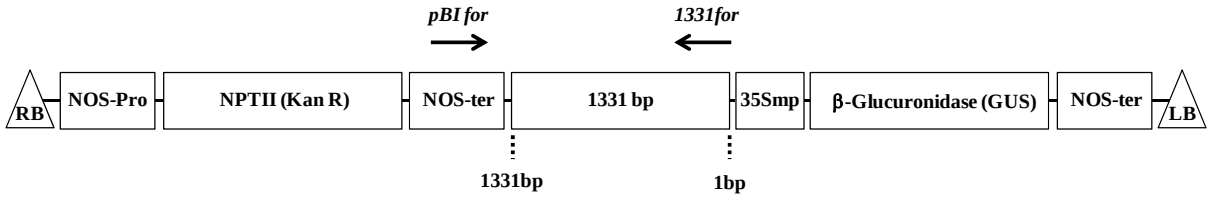
Annex 16. pBI 101.3-35Smp-1331 bp.



16a. 1331-bp (+)



16b. 1331-bp (-)



Annex 17. cDNA identification of *AT1G12030*, *GUS* and *S16* genes.

17a. List of primers used

Oligo	Sequence	Length (bp)
UNK for	GCAGGTCTCTGCAAGTCTCGAT	22
UNK rev	TCGACCGCCTGATCTCAAAG	20
GUS for	GAAACCCCAACCCGTGAAAT	21
GUS rev	CGAAACGCAGCACGATACG	19
S16 for	CGCCGATCGAGCTTTATCAG	21
S16 rev	CACCAGGACCACCAAACTTCTT	22

UNK = primer for *AT1G12030*

17b. Reaction mixtures

Reagent	µl
Template DNA (1:100)	5
Primer for (10µM)	0.4
Primer rev (10µM)	0.4
GoTaq qPCR Master mix 2x	10
CXR reference Dye	0.2
H_2O	4
Total volume	**20 µl**

17c. Thermal cycler profile

50°C for 2 min, 95°C for 2 min, 45 cycles of 95°C for 15 sec and of 60°C for 60 sec. The cycles were followed by a final dissociation step of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s.

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