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AOX – a functional marker for efficient cell reprogramming under stress?

Birgit Arnholdt-Schmitt[1], José H. Costa[2] and Dirce Fernandes de Melo[2]

[1]EU Marie Curie Chair, ICAM, University of Évora, 7002-554 Évora, Portugal
[2]Department of Biochemistry and Molecular Biology, Federal University of Ceará, PO Box 6029, 60455-900, Fortaleza, Ceará, Brazil

Functional markers for stress tolerance can be used in plant breeding to identify genotypes with high yield stabilities under various conditions. Thus, a good marker should show a strong correlation with favourable adaptive plant behaviour. The efficient reprogramming of target cells for yield determination is currently considered to be the most important step towards defining abiotic stress tolerance. In this Opinion article, we propose a role for the alternative oxidase (AOX) gene as a marker for genetic variation in cell reprogramming and yield stability. Evidence to support this idea comes from the metabolic role of alternative respiration under stress, the link between AOX activity and differential growth, and the single nucleotide polymorphism recently observed in AOX genes. We propose an innovative, interdisciplinary and global research strategy for future experimentation on AOX genes that could have an application in plant breeding.

Alternative oxidase activity related to stress tolerance and yield stability

Plants are sessile organisms but can respond to environmental stress in terms of growth and development in a variety of ways. Environmental plasticity can have a negative or positive impact on yield depending on the species, tissues and cells involved, and on the type of stress. For example, yields of *Daucus carota* are severely reduced by low phosphorus (P) availability in the soil. This is because the programme in the cambial cells of carrot tap roots is modulated by systemic signalling under these conditions, reducing the rate of cell division and consequently producing smaller crops of carrots. However, in several other species, including cereals, low phosphorus availability also induces a stress-counteracting strategy that is of current interest to plant breeders. In a situation where P is depleted, the programme of position-defined cells and meristems in the rhizodermis is modulated to initiate a significant enhancement of the root system, which, together with the effect of increased exudation in the rhizosphere, leads to more efficient phosphorous acquisition. As a consequence, more stable plant growth is achieved under conditions of P deficiency [1,2].

Yield-determining growth processes are under complex regulation, and are dependent on genotype, developmental stage and plant environment [1,3,4]. Plant stress reactions potentially include the restructuring of the inner and outer shapes of target cells, a change in the rate of growth or development and induction of adventitious organs, such as roots, shoots or hairs (i.e. formed under abnormal conditions). Thus, stress adaptation is not physically possible without a change in energy allocation and spatial changes in metabolism. Unlike mammals, plants possess a so-called alternative respiration pathway (Box 1) as part of the total respiration process. The alternative pathway is known to be involved in stress-induced variations of the cellular redox state and flexible carbon balance. Recent studies suggest that this pathway can be activated as an early response to metabolic imbalances (M.A. Gonzàlez-Meler *et al.*, unpublished*; see also Ref. [5]); the only enzyme in this pathway is the multigenic alternative oxidase (AOX) (Box 2).

Analysis of AOX gene activity is an emerging field in plant stress research (e.g. [5,6]). Stress-inducible cell signalling compounds for growth and development, such as H_2O_2 and NO, have been shown to induce AOX1 genes [5,7,8]. The relationship between AOX activity and growth has been addressed in several publications [9–14]. The ability of plants to adapt growth to varying conditions is genetically determined [2] and genetic variation has been shown to affect alternative respiration related to growth behaviour [15].

Here we suggest that differential expression of AOX genes can play a crucial role in the initiation of stress-adaptive cell programmes and provide markers for plant growth behaviour under stress. A strong correlation between AOX gene expression and the stress response at both the tissue and the whole plant level, if related to yield stability, would make this gene a promising candidate for functional marker-assisted breeding strategies for stress tolerance.

In this Opinion article, we highlight the link between plant stress and cellular reprogramming. In particular, we address how AOX activity might correlate with the initiation of cell programmes for growth and development, and how AOX can serve as marker for more efficient cell programming under stress. Finally, we discuss an interdisciplinary research strategy based on the global 'systemic strategy' presented in Ref. [16]. This strategy is

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Corresponding author: Arnholdt-Schmitt, B. (eu_chair@uevora.pt).

Box 1. Alternative respiration: less energy-efficient, but highly effective

At the inner membrane of mitochondria, cyanide-insensitive AOX short cuts electron transport by transferring electrons directly from reduced ubiquinone to oxygen (Figure I). This has important consequences: proton transport through Cytochrome bc_1 complex (CIII) and cytochrome c oxidase (CIV) is restricted and, thus, the total amount of protons in the intermembrane space available for ATP production is diminished. Hence, part of the source for energy coupling is 'wasted' as heat. However, AOX bypasses adenylate and local P_i control, and, under a high-energy charge, AOX helps to avoid incomplete reduction of oxygen to water as a source for reactive oxygen species. So, AOX activity enables high turnover rates of carbon skeletons in the cytosol and the citric acid cycle at lower productivity levels of harmful reactive oxygen. Many publications point to the protective role of AOX against oxidative stress in plants [41]. In the filamentous fungus *Podospora anserina*, this metabolic link contributes to longevity [42]. In *Septoria tritici*, the alternative pathway is responsible for keeping the invading pathogen alive despite strobilurin-blocked energy production at Cytochrome b [43]. AOX has been identified in all higher plants investigated to date and also in some algae, fungi, eubacteria and protists. Recently, the presence of AOX was revealed in animal kingdom phyla, including mollusca, nematoda and chardata [44]. In plants, alternative respiration was first recognized in the thermogenic floral tissues of *Araceae*, where a high rate of electron flux generates heat and volatile compounds that attract insect pollinators [45]. AOX is discussed predominantly in relation to abiotic and biotic stress-induced plant reactions, and the amount of heat produced in this case is not considered important. AOX is a disulfide-linked dimeric protein, which is active in the reduced state in the presence of α-keto acids. Mitochondrial AOX (MtAOX) and AOX from plastids (PTOX) belong to the membrane-bound di-iron carboxylate proteins [46,47]. The location of the hydrophobic regions suggests the interfacial rather than the transmembrane nature of the protein [48,49]. AOX activity can be regulated through stimulated transcription and variable protein levels. In addition, post-translational control occurs via the reduction of the S–S bond and metabolic activation (Figure I). Regulation via pH has been demonstrated *in vitro* and awaits *in vivo* confirmation [50]. The target site of redox as well as keto acid regulation is typically marked by a conserved Cys [36]. Uncoupling proteins (UCPs) and Type II NAD(P)H dehydrogenases (NDHs, rotenone-insensitive) also contribute to regulating the redox state and ATP synthesis in mitochondria. New data outline the differential, stress-specific co-regulation of AOX and NDH group genes [5] and indicate the involvement of Ca^{2+} [51].
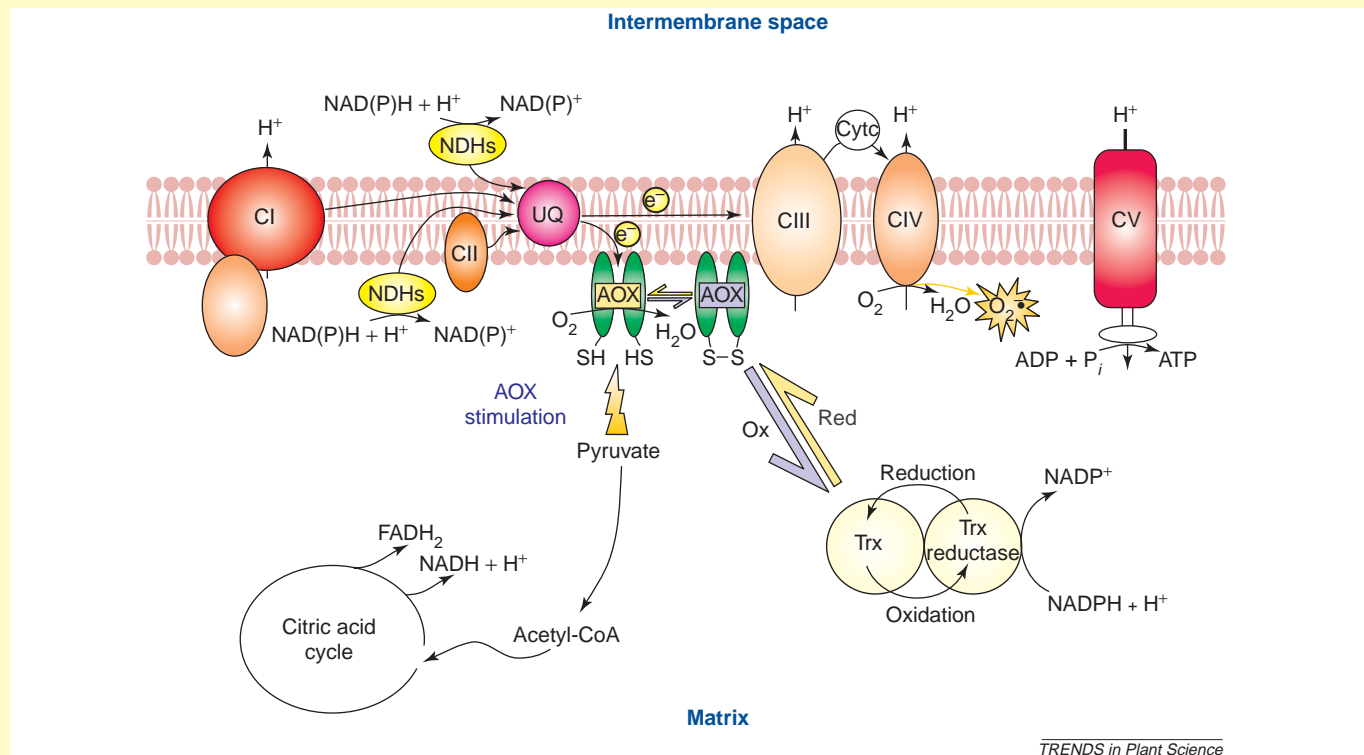

Figure I. The alternative pathway in the plant mitochondrial electron transport chain. Abbreviations: AOX, alternative oxidase; CI, NADH:ubiquinone oxidoreductase; CII, succinate:ubiquinone oxidoreductase; CIII, Cytochrome bc_1 complex; CIV, cytochrome c oxidase; CV, ATP synthase; Cytc, cytochrome c; NDHs, rotenone-insensitive NAD(P)H dehydrogenases; P_i, inorganic phosphorus; Trx, thioredoxin; UQ, ubiquinone pool.

proposed to facilitate future experimentation on AOX, with the aim of leading to species-specific functional markers for more robust plants.

Environmental conditions modulate cell programmes

Many studies have demonstrated the involvement of environmental conditions or stress in reprogramming plant cells. In the field of plant nutrition, the influence of P deficiency on root architecture is well recognized [1,16–18]. Under P limitation, the density of lateral roots in many plants is enhanced by initiating preformed meristems, and there is also a significant increase in the length of lateral roots and root hairs. Growth of the primary root is restricted. The initiation of adventitious trichoblasts in the rhizodermis, which leads to the production of large numbers of root hairs, predominates in root reprogramming. Root hairs are physically and energetically advantageous for nutrient acquisition from soils and contribute to enhanced uptake of immobile nutrients, such as phosphorus, potassium or iron [2,17].

Box 2. AOX genes: structural organization and differential regulation

Plant AOX is a small nuclear-encoded multigene family that can be divided into two subfamilies, AOX1 and AOX2. The intron–exon structure of AOX has been well characterized in some species, revealing a large degree of conservation in splice site positioning. In most cases, plant AOX genes consist of four exons and three introns [52]. A tandem-arrangement has been reported for AOX1a and AOX1b as well as for AOX2a and AOX2b [28,53]. Five active gene family members have been characterized: AOX1a, AOX1b, AOX1c, AOX2a and AOX2b. Whereas AOX1 exists in monocots and eudicots, AOX2 has only been found in eudicots [52]. Differential expression of gene family members depends on the plant, tissues, growth, development and environment. Induction of AOX1 genes is more obviously linked to stress, whereas AOX2 expression is typically constitutive or related to tissues and development. Nevertheless, AOX2 also appears to play a role in the stress response related to plastid-dependent signalling [5]. Given that AOX genes are nuclear-encoded and the plastid–mitochondrion interaction is crucial for carbon use efficiency and energy transfer, elucidation of the respective signalling pathways is of special interest to help us to understand AOX control under stress. Altered nuclear gene expression caused by changes in the mitochondrial status is referred to as mitochondrial retrograde regulation (MRR) [54]. Recently published data have highlighted that diverse forms of stress induce alternative pathways in respiration (AOX and NDHs) as an early event, involving multiple organelle-to-nuclear communication by organic acids and reactive oxygen species and inter-organelle signalling [5,8,55]. A MRR promoter region has been found in AtAOX1a that suggests a common site for multiple AOX mRNA induction [56]. At the protein level, a recently described mitochondrial NADPH-dependent specific thioredoxin can play an important role in regulating AOX activity in response to cell redox states [57] (Box 1). Interestingly, the homologous AOX2a and AOX2b genes of soybean and *Vigna unguiculata* showed similar tissue-specific expression [58]. Likewise, isolated upstream regions of soybean genes corresponded to comparable tissue-specific expression in soybean as well as in *Arabidopsis*. Recent promoter deletion studies have revealed positive as well as negative regulatory regions in the soybean AOX gene. Similar modes of expression of AOX genes have common sequence motifs [53]. Furthermore, a highly important link was recently discovered between the substitution of amino acid residues and AOX protein isoforms of varying molecular weights. This link indicates a natural genetic source for the differential regulation of member genes and single nucleotide polymorphisms [35,36].

The number of root hairs and the amount of root hair initiation under stress is now emerging as a useful tool for breeding strategies based on nutrient uptake efficiency [2,17]. Furthermore, under P or iron stress, so-called 'transfer cells' are initiated in the rhizodermis near to the differentiation zone for root hairs [19]. These cells are characterized by a significant enlargement of the cell surface owing to the ingrowth of secondary cell wall material and a significant increase in the number of mitochondria per cell. Why mitochondria increase in number is still unclear.

Another striking root morphology can be caused by P deficiency. This morphology was first described in the family Proteaceae: a mass of clustered short-living rootlets named proteoid roots arise rapidly from a pre-defined region of the root axis. This adaptation is based on cell reprogramming in the rhizodermis. This increase in root surface area is accompanied by increased amounts of exudates, and the root surface area is further significantly increased through induced root hair formation at the

rootlets. At the final stage of proteoid root development, the hairs typically cover the rootlets from base to tip [20].

Stress effects on cell reprogramming can ideally be studied using *in vitro* culture systems. Explantation and inoculation of the plant material into novel chemical and physical surroundings is known to affect induction and initiation of new cell programmes, such as dedifferentiation, changes between heterotrophic and autotrophic growth and adventitious root growth. Nutrient stress caused by excessive iron concentrations initiates somatic embryogenesis in leaf protoplasts of alfalfa [21]. This impact is accompanied by oxidative tissue stress, increased ascorbate peroxidase activity and newly established cellular pH gradients. In *Daucus carota*, the effect of nitrogen or P depletion in the soil on root cambial growth rates (see above) has been confirmed at the cellular level using primary cultures of root explants [22]. Thus, this *in vitro* experimental system can assist fundamental investigations at the whole plant level under defined environmental conditions.

A role for AOX in stress-modulated cell programmes for growth and development?

AOX has a prominent role in counteracting oxidative stress under conditions where the ubiquinone pool is highly reduced. However, recent results reveal an additional function of AOX apart from merely counteracting oxidative stress (e.g. Refs [6,14]). Microarray studies of an *Arabidopsis* AOX antisense line have shown that AOX also influences carbon metabolism pathways outside mitochondria. Several reports have demonstrated a link between growth and AOX activity. In the growing roots of soybean, spatially increased levels of AOX activity were located in the meristems [23]. A positive trend between alternative oxidation and relative root growth has been shown for six wild grass species indicating genetic differences. These investigations further demonstrated that the environment potentially has an effect on alternative respiration with a link to variable plant growth behaviour [15]. Lee Hansen *et al.* [11] have investigated the kinetics of plant growth in various species and cultivars and modelled plant and tissue growth rates as a function of environmental factors in relation to respiratory parameters. They observed that environmentally induced changes in respiration rates frequently accompany changes in the efficiency of energy coupling. Depending on the amount of alternative and total respiration, these changes are proposed to keep growth rates constant even under a change to more unfavourable growth conditions. However, alternative oxidation is less efficient in terms of energy conservation, and so Hansen *et al.* [11] have questioned the value of 'efficiency' for stress adaptation. Less-efficient metabolism in terms of energy coupling might be more efficient in terms of growth stability.

The investigations by Hansen *et al.* [11] suggest that AOX activity can support the homoeostasis of plant growth under stress conditions. This is in line with recent findings [14] from AOX1a transgenic *Arabidopsis* plants growing at 12°C. Anti-sense lines had reduced leaf areas and smaller rosettes, whereas AOX1a overexpressing

genotypes demonstrated increased shoot growth rates under these growth-limiting conditions. However, the findings are not unequivocal: investigations with tobacco cell cultures (control and AOX1 antisense) have shown the contrary [15]. Under P- and N-deficiency, AOX is involved in down-regulating growth rates as an adaptation to low nutrient availability in cells. Maintaining AOX1 antisense tobacco cell growth has been linked to more stable carbon use efficiency. It was concluded, that AOX activity provides a mechanism for adjusting growth and counter-acting nutrient imbalance, which agrees with a recent study [8] showing that inducing AOX1 transcription with 1 mM citrate had no effect on the growth of tobacco cells during a 43 h period. Unfortunately, there are no data concerning whether the observed nutrient imbalance in tobacco AOX1 anti-sense cells created any negative symptoms at the cell level. If this was not the case, the data show high growth rates in spite of the low levels of nutrients in AOX antisense plant cells, and this might then also be interpreted as a positive contribution of AOX blockage to increasing nutrient use efficiency. This observation could be of great interest for plant breeding.

In whole plants, P limitation is primarily sensed in the shoot before systemic signalling reaches the root system, and shoot growth typically becomes strongly down regulated. A 2.2-fold to 3.2-fold up-regulation of AOX1a transcripts in *Arabidopsis* shoots 100 h after P withdrawal has been observed [24]. Increased AOX1a expression occurred shortly after low P levels were detected in the shoot but significantly before shoot growth was down-regulated. As outlined above, root systems also vary their growth characteristics under P restriction. Earlier studies showed that P limitation in bean is accompanied by an increase in alternative respiration in roots from 40–50% of total respiration to 80–90% [25].

Owen Atkin and Mark Tjoelker [4] have proposed a crucial role for the enzymatic capacity of mitochondrial respiration and Type II acclimation of differential growth to temperature. Type II acclimation seems to be strongly linked to the development of new leaves and roots. Several research groups have indicated that AOX is involved in temperature-dependent growth changes [26,27]. Differential activation of AOX1a and AOX1b at the transfer of young rice seedlings from 28°C to 4°C has been shown [28]. Confirmation of a direct link between AOX activity, temperature and cell programming might come from the thermogenic skunk cabbage (*Symplocarpus foetidus*). At low ambient temperatures, AOX activity is induced in the spadix. During the stigma stage, AOX activity is relatively constant and correlates with homeothermic heat production until mature pollen appears on the spadix surface [29] (M. Otsuka *et al.*, unpublished[‡]).

In summary, the differential activity of the mito-chondrial AOX seems to play a crucial role in optimizing metabolic efficiency for the adaptive regulation of growth and development. This regulation means 'modulating' existing cell programmes in terms of 'initiation' and/or

'realization', but should at this stage not be understood as 'programme induction', which would be an earlier event. However, AOX interferes with the synthesis of cell signalling compounds such as H_2O_2 (Box 1) and NO [30]. Thus, it remains to be elucidated whether AOX also contributes to programme induction *per se* under stress.

A step-by-step research strategy to elucidate the potential role of AOX in plant breeding

A schematic global strategy for future experimentation on AOX is proposed (Figure 1) with the aim of verifying the potential role of AOX as marker for stress tolerance and to direct basic research efforts early in development to application (see also the 'systemic strategy' discussed in Ref. [16]). First of all, systems analyses and ecophysiolog-ical modelling should be carried out at the whole plant level to determine the importance of identifiable yield-determining parameters as a basis for molecular research. This work places a high reliance on the expertise of plant nutritionists or ecophysiologists and has to be carried out at the species level. It should also consider the interaction between developmental stages and the environment. Stress adaptation can occur as a result of maintaining growth (homoeostasis), down-regulating growth rates (to avoid nutrient imbalances) or even by up-regulating growth and development (induced secondary root growth and root hair formation). Whether growth adaptation is rated as 'efficient' in terms of breeding will be a function of its effect on yield stability. Depending on the results of modelling, responsive tissues and cells should be iden-tified that are characteristic for the identified parameters and will then be available for AOX analyses.

In the next step, species-specific AOX genes must be isolated. Comprehensive microarray and quantitative RT-PCR studies have now confirmed the significance of the differential expression of AOX1 genes as a result of environmental stress. Recently, AOX2 genes were also found to be involved in plant stress reactions [5]. To study the correlation between differential AOX gene activity and plant growth reactions under stress, quantitative AOX expression studies must be performed in the identified target tissues or cells. Kinetic analyses are also necessary to consider the transient nature of AOX expression. AOX1a transcription in maize seedlings induced by treatment with antimycin and rotenone reached a maximum level after 4 h and then decreased after 8 h [31]. The same pattern was reported for tobacco cells after citrate induction [8]. Transfer of maize seedlings from 28°C to 4°C induced maximal expression of AOX1a and AOX1b by the fourth day and then decreased. These genes were not expressed in control plants. This mode of expression might indicate a direct role for AOX in initiating new cell programmes. AOX protein concen-trations in cluster roots (*Hakea prostrata*) have been analysed during the three weeks after growth initiation [20]. Increased AOX expression levels were analysed in relation to exudation. An additional link might be seen to stress-induced growth and development. Even before the internal P cell levels decrease, a peak of AOX expression appears that coincides with the emergence and highest growth rates of rootlets at days 1–2, followed by a decrease

Systems analyses and ecophysiological modelling at whole plant level to highlight crucial **yield-determining parameters**

Definition of **target tissues and cells** according to identified crucial parameters for yield stability

Species-specific AOX gene identification and characterization

- **Quantitative analysis** of AOX expression in target tissues at defined developmental stages in control and stressed plants to reveal **differential gene expression**

- **Kinetic studies** of AOX expression during defined intervals after stress application correlated with specified **growth and developmental behaviour**
  Functional analysis with the aid of AOX gene knockout variants can be integrated

Integration of differential gene activities in ecophysiological modelling to study their **importance for yield stability**

Rejection of the candidature of AOX as a functional marker

Candidature of AOX as a functional marker

Search for AOX gene-specific **polymorphic sequence motifs***
  → in natural phenotyped populations
  → through introduction of variable motifs and phenotyping

Tests for **stability of the marker** and its value to assist in **selection for stress tolerance**
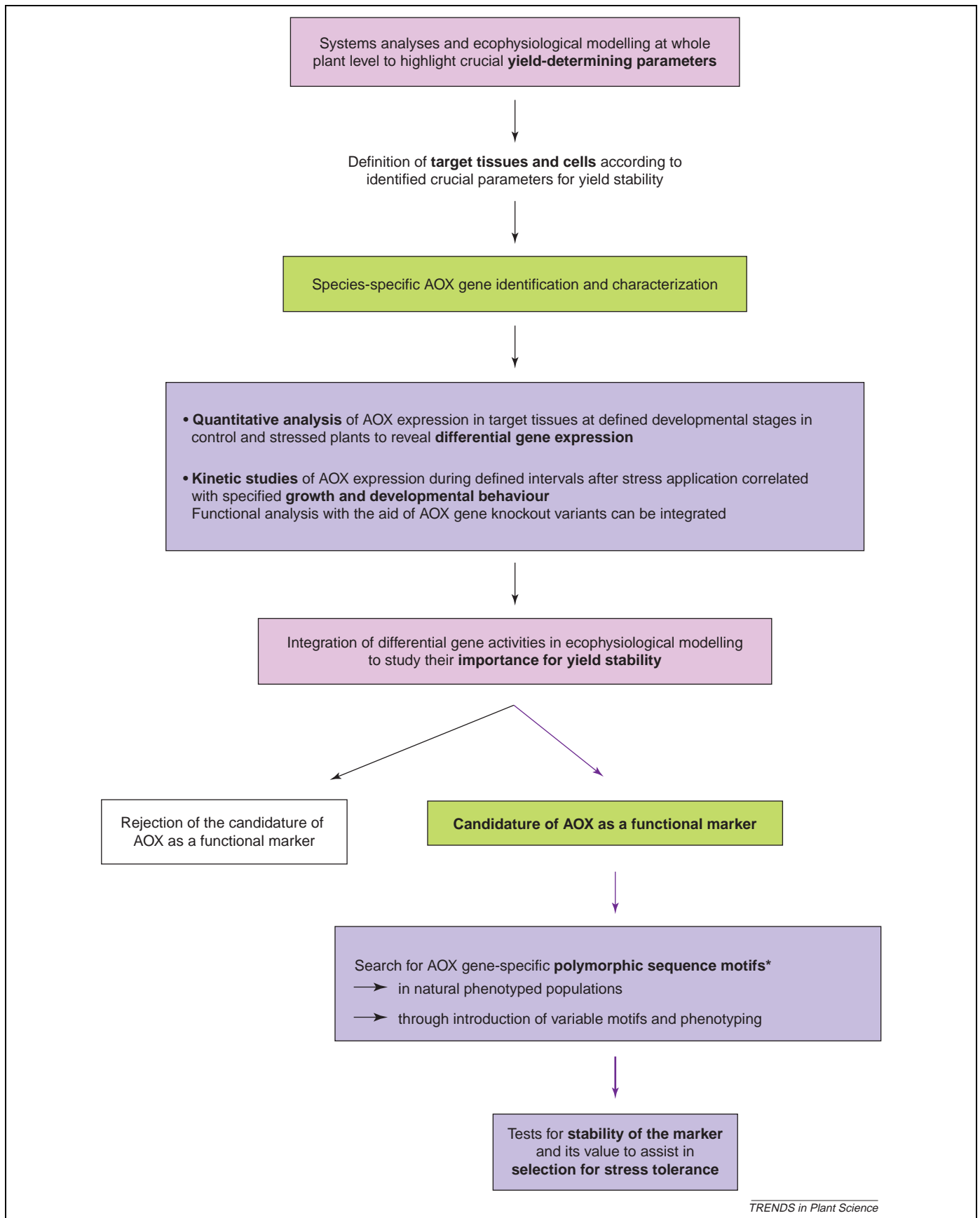
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Figure 1. Proposed strategic steps needed to verify AOX as a functional marker for adaptive plant growth towards stress tolerance. See Refs [16,33,35,40] for searches of polymorphic sequence motifs.

in AOX expression and rootlet growth during days 4 and 5. After the fifth day, root hair initiation starts (M.W. Shane, personal communication) and, in parallel, the AOX levels increase to a maximum during days 7 to 8 with a dramatic decrease directly thereafter, and no further detection by the twentieth day.

Quantitative and kinetic studies should reveal the strength of positive or negative correlation between differential AOX gene transcription and the rate of differential growth in defined tissues. Because basic levels of AOX protein can be present in cells when stress starts or ceases, the amount or concentration of protein will not be an appropriate measure for correlation studies. Although it is known that AOX activity can be significantly regulated at the protein level (Box 1), several reports indicate that when induced by stress, AOX transcripts are rapidly translated to the active protein [7,8]. Thus, transcription analyses should be used to measure correlations and to identify a candidate marker.

Direct evaluation of the significance of differential AOX expression in yield determination might come from integrating gene activities in ecophysiological modelling [32]. However, this is still a highly challenging research field and further progress is required in bioinformatics and mathematics to adequately apply systems analyses and modelling to the cellular level.

If a strong correlation between AOX gene activities and favourable adaptive cell reactions exists, then AOX could be used as a functional marker candidate for yield stability and should be tested. To designate AOX as a functional marker, polymorphic sequence motifs must be revealed within AOX genes that are correlated to important phenotypic variation [16,33,34]. Such motifs should mark genes with favourable expression patterns under stress and, thus, facilitate the selection of genotypes. Recent evidence indicates that single nucleotide polymorphism (SNP) does occur in AOX genes and might be related to differential regulation and environmental tolerance [35,36]. An allelic variation was induced through mutagenesis in the rice AOX1a gene, which created a Lys71 to Asn71 substitution corresponding to isoforms of 32 kDa and 34 kDa, respectively. This SNP was tightly linked to a quantitative trait locus (QTL) for low temperature tolerance of anthers at the booting stage.

AOX markers for growth behaviour might assist breeding of 'robustness'

AOX is proposed as a general marker for adaptational plant growth behaviour under stress. However, to be accepted as a marker for efficient cell programme initiation (with regard to yield stability) it must be more defined at the species level. The SNPs must mark cell or tissue-specific adaptive growth behaviour that contributes in a species-specific manner to yield determination. Furthermore, different stress factors will affect the importance of physiological parameters and the significance of particular cells or tissues for yield stability. Hence, identifying typical target tissues and cells with a more global responsiveness to complex stress situations should lead to the production of general markers for efficient stress adaptation. Adaptive root hair initiation is

a good example and could be used because it is advantageous for a variety of plants under various unfavourable nutrient conditions.

An alternative potential functional-marker candidate for stress-tolerant behaviour is the group of uncoupling proteins (UCPs). UCPs were first reported in mammals (1976) and afterwards in plants (1995). Although the 'true' physiological role of plant UCPs is unclear, recent results suggest that both energy-dissipating systems have a complementary role under stress. Whereas AOX dissipates the redox potential, the UCP dissipates the proton motive force. Thus, both gene families are involved in tuning the capacity of oxidative phosphorylation. A core sequence for a transcriptional factor involved in the oxidative processes and the activation of peroxisomal proteins has been found in the promoter region of two UCP genes as well as of AOX1a [37]. Furthermore, analysing results from recently published array investigations, Fábio Nogueira et al. [38] outlined distinct expression profiles during the 24 h after stress induction for both energy-dissipating systems (tAOX1a and the UCPs AtPUMP4 and AtPUMP5) under various environmental stress conditions. In addition, tissue-enriched expression profiling between monocots and dicots models showed that UCP genes were expressed more ubiquitously than the AOX genes [39], suggesting that AOX genes could be more efficient candidates for cell reprogramming under stress conditions.

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