Plant, Cell & Environment

Plant, Cell and Environment (2012) **35**, 917–928 doi: 10.1111/j.1365-3040.2011.02462.x

Integrating fluctuating nitrate uptake and assimilation to robust homeostasis

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ABSTRACT

Nitrate is an important nitrogen source used by plants. Despite of the considerable variation in the amount of soil nitrate, plants keep cytosolic nitrate at a homeostatic controlled level. Here we describe a set of homeostatic controller motifs and their interaction that can maintain robust cytosolic nitrate homeostasis at fluctuating external nitrate concentrations and nitrate assimilation levels. The controller motifs are divided into two functional classes termed as inflow and outflow controllers. In the presence of high amounts of environmental nitrate, the function of outflow controllers is associated to efflux mechanisms removing excess of nitrate from the cytosol that is taken up by low-affinity transporter systems (LATS). Inflow controllers on the other hand maintain homeostasis in the presence of a high demand of nitrate by the cell relative to the amount of available environmental nitrate. This is achieved by either remobilizing nitrate from a vacuolar store, or by taking up nitrate by means of high-affinity transporter systems (HATS). By combining inflow and outflow controllers we demonstrate how nitrate uptake, assimilation, storage and efflux are integrated to a regulatory network that maintains cytosolic nitrate homeostasis at changing environmental conditions.

Key-words: integral control; negative feedback; nitrate assimilation; nitrate homeostasis.

INTRODUCTION

A major nitrogen source for all higher plants is inorganic nitrate. Apart from being an important nutrient, nitrate also acts as a signalling molecule in nitrogen metabolism (Redinbaugh & Campbell 1991; Crawford 1995; Miller *et al*. 2007; Lillo 2008; Krouk *et al*. 2010; Dechorgnat *et al*. 2011). Despite the fact that soil nitrate levels and uptake rates by roots can vary considerably in dependence to environmental conditions such as pH and temperature (Bassirirad 2000; Miller *et al*. 2007), cytosolic nitrate levels have been found to be surprisingly constant (van der Leij, Smith & Miller 1998; Jia *et al*. 2005; Fan *et al*. 2007; Miller & Smith 2008), possibly to minimize the generation of reactive nitrogen oxide species (NO, $ONOO^-$) (Yamasaki 2000; Meyer *et al*. 2005) and superoxide (Ruoff & Lillo 1990; Barber & Kay 1996) by nitrate reductase (NR) and to minimize oxidative stress.

By using a combination of pH and nitrate ion selective microelectrodes, Miller *et al*. (2001) demonstrated in a series of studies that cytosolic nitrate concentration in mature root and leaf cells is under homeostatic control, both at high external nitrate concentrations (Miller & Smith 2008) or during the remobilization of nitrate from the vacuole into the cytosol when no external nitrate is available (van der Leij *et al*. 1998; Jia *et al*. 2005; Fan *et al*. 2007).

Five major processes have been identified to participate in the control of cytosolic nitrate in plants (Fig. 1). One is the uptake of nitrate by NRT1 and NRT2 transporters, where the NRT2's are high-affinity transporters (HATS) that are nitrate inducible, while the NRT1 transporters are considered to contribute more broadly to nitrogen uptake, including low-affinity transporters (LATS), and show both inducible and constitutive expression (Crawford & Glass 1998; Miller *et al*. 2007; Chapman & Miller 2011). The activity of NRT2 and nitrate uptake is positively regulated by light, because of a reduced repression of NRT2 by NRT1 (Chapman & Miller 2011), mediated by the transcription factors HY5 and HYH (Jonassen, Sevin & Lillo 2009). Increased NRT2-mediated HATS activity levels and increased nitrate uptake rates occur at N-limitation or by sucrose treatment, while feedback repression at the transcription/mRNA level of NRT2 and nitrate uptake rate occurs by N-metabolites resulting from nitrate reduction (Lejay *et al*. 1999). Despite the strong variations observed in NRT2 mRNA levels and HATS activity, no rapid changes in the NRT2 protein were observed suggesting the presence of post-translational regulatory mechanisms (Wirth *et al*. 2007).

The second process regulating cytosolic nitrate is a nitrate-inducible efflux system, which transports cytosolic nitrate out of the cell (Aslam, Travis & Rains 1996; Miller *et al*. 2007) when, mediated by LATS, high uptake rates of nitrate occur in the presence of high external nitrate concentrations. When under such conditions external nitrate is suddenly removed, nitrate efflux has been found to decrease and stop after approximately 5 h indicating that the nitrate-mediated efflux is a regulated process (van der Leij *et al*. 1998). An efflux transporter, NAXT1, was recently

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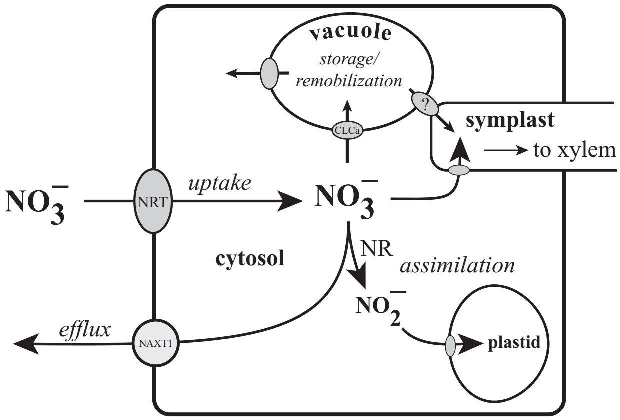


Figure 1. Schematic overview of nitrate transport in a root epidermal cell (Rufty *et al*. 1986; Redinbaugh & Campbell 1991). The processes contributing to cytosolic nitrate homeostasis are uptake, storage/remobilization, efflux, nitrate reductase (NR)-mediated assimilation and transport of nitrate to the xylem.

identified belonging to the NRT1/PTR family of transporters (Segonzac *et al*. 2007; Chapman & Miller 2011).

The third process is related to the storage of nitrate in the vacuole and the remobilization of nitrate from the vacuole into the cytoplasm when sufficient extracellular nitrate is not available (van der Leij *et al*. 1998; Jia *et al*. 2005; Fan *et al*. 2007). Early evidence suggested the presence of a nitrate/proton transporter in the tonoplast (Schumaker & Sze 1987; Miller & Smith 1992). Recent findings show that these nitrate transporters belong to the family of CLC transport proteins (Harada *et al*. 2004; De Angeli *et al*. 2006; Zifarelli & Pusch 2010; Chapman & Miller 2011; Dechorgnat *et al*. 2011) and are connected to the activity of vacuolar H^+-ATPase. H^+-ATPases are rotational pumps (Schumacher & Krebs 2010) transporting protons into the vacuole maintaining a proton gradient between cytosol and vacuole that enables to transport nitrate from the cytosol into the vacuole against its concentration gradient (Krebs *et al*. 2010).

The fourth process participating to cytosolic nitrate homeostasis is nitrate assimilation, where NR catalyses the first and rate-limiting step in which nitrate is converted to nitrite. Nitrite is taken up by the chloroplast and there further reduced to ammonium and amino acids (Lillo 2008). NR is a highly regulated enzyme (Lillo *et al*. 2004), and its activity, which is under circadian control (Lillo 1994; Tucker, Allen & Ort 2004), depends on external and internal factors such as light dark conditions and nutritional status (Lillo 2008). As observed for HATS expression and HATS-mediated nitrate uptake, NR activity is repressed by downstream products of nitrate assimilation. Glutamine treatment of barley roots showed a temporary increase of cytosolic nitrate levels, which adapted to their pre-perturbation value despite the presence of still elevated glutamine concentrations inside and outside of the root cells (Fan *et al*. 2006).

In the fifth process, cellular nitrate is transported from the vacuole or from the cytosol into the symplasm, where it is further transported to the xylem. Rufty *et al*. (1986) suggested a transfer of nitrate from the vacuole directly to the symplast. However, later models did not consider this possibility (Redinbaugh & Campbell 1991; Chapman & Miller 2011) and therefore this process appears questionable as indicated in Fig. 1.

Considering the above described processes that participate in the transport of nitrate to and from the cytosol, the question arises how these processes interact and which of them are becoming important to achieve a stable cytosolic nitrate homeostasis in the presence of changing nitrate uptake and assimilation rates (Miller *et al*. 2007). We approached this problem from a kinetic viewpoint by investigating the dynamic behaviours of homeostatic network motifs using the control-engineering concept of integral feedback (integral control) (Lewis 1992; Yi *et al*. 2000; Wilkie, Johnson & Reza 2002). We recently identified two operationally distinct controller types (Drengstig, Ueda & Ruoff 2008; Ni, Drengstig & Ruoff 2009) we termed as inflow and outflow controllers. To understand how the interaction between these controller types can lead to a robust homeostatic response, we first briefly review the dynamical and functional behaviours of the individual controllers. Then we show (for the first time) how the interaction between these two controller types can achieve a robust homeostasis, and how this interaction can lead to cytosolic nitrate homeostasis in the presence of changing nitrate uptake and assimilation rates.

COMPUTATIONAL METHODS

Rate equations were solved numerically by using the FORTRAN subroutine LSODE (Livermore Solver of Ordinary Differential Equations) (Radhakrishnan & Hindmarsh 1993) and MATLAB (http://www.mathworks.com). FORTRAN programs were built using the ABSOFT Pro Fortran compiler, version 11.1 (http://www.absoft.com). To make notations simpler, concentrations of compounds are indicated by compound names without square brackets.

HOMEOSTATIC CONTROLLERS AND INTEGRAL FEEDBACK

The principle of integral feedback (Fig. 2) allows that a controlled variable (CV), here cytosolic nitrate concentration, can be kept precisely at a given set point. To achieve this, the error (difference) between CV and the set point is determined as indicated in Fig. 2. The calculated error is then integrated over a given time interval and results in the manipulated variable (MV), which is closely related to controller compounds *E* shown in the two controller motifs of Fig. 3. Together with uncontrollable environmental perturbations, MV is fed into the 'process' which generates a new and updated CV. By continuously updating the feedback scheme, the integral controller ensures that CV is regulated to its set point with a certain precision (Wilkie *et al*. 2002).

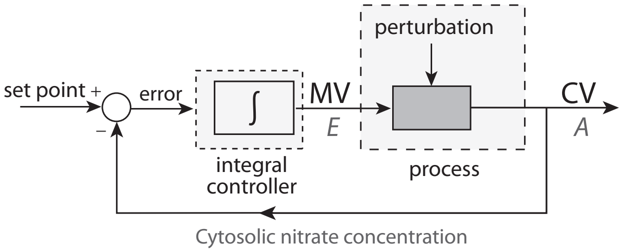


Figure 2. Principle of integral control. The aim of the control loop and the integral controller is to move the controlled variable (CV), here the concentration of molecular species A, to a given set point and keep it there. To achieve this, the error (difference) between A and the set point is calculated and integrated for a given time interval. This leads to the manipulated variable (MV), the concentration of controller molecule E, which, when fed into a process containing uncontrollable perturbations, leads back to A. By iterating this scheme, A will approach the set point.

How the accuracy and responsiveness (aggressiveness) of such a controller is determined by kinetic parameters will be described below.

Behaviour of outflow controllers

Figure 4a shows a representation of an outflow controller (Fig. 3) addressing the situation when (relative) large amounts of external nitrate are entering the cytosol by a first-order (LATS-mediated) uptake process with flux $j_{in} = k_{in} \cdot NO^{-}_{3\,external}$. Cytosolic nitrate (the CV) activates an efflux transporter (Aslam *et al*. 1996), termed *EFT* (MV or *E*), which transports excess of nitrate out of the cell. By removing the excess of cytosolic nitrate (CV, *A*) using *EFT*, the outflow controller can achieve homeostasis in CV at large and variable uptake fluxes j_{in}. The rate equations together with the integral feedback are indicated in Fig. 4b and show that robust homeostasis by this controller can be obtained when the MV (*EFT*) is removed (or inhibited) by zero-order kinetics. A plausible way to obtain zero-order kinetics is by Michaelis–Menten kinetics where an enzyme (here E_{set}) removes *EFT* such that the corresponding Michaelis constant $K_M^{E_{set}}$ has a much lower value than the concentration of *EFT*. The set point of CV is then given by $V_{max}^{E_{set}}/k_{adapt}$, where $V_{max}^{E_{set}}$ is the zero-order flux removal/inhibition of *EFT* by E_{set}, and k_{adapt} is the rate constant by which *EFT* is induced by cytosolic nitrate. The error between the set point and the cytosolic nitrate concentration at a given time t is proportional to d(*EFT*)/dt. Integral feedback occurs, because *EFT* is proportional to the integrated error and enters the rate equation for cytosolic nitrate, which, when integrated, is the 'process' (Figs 2 & 4b) that determines the cytosolic nitrate concentration (CV). The following may be noted: (1) The efflux j_{eff} is not restricted to occur by a second-order process as described here, but could also occur, for example, by Michaelis–Menten type kinetics (data not shown). (2) The homeostatic performance of this and other outflow controllers (Ni *et al*. 2009) is limited to relative large inflow fluxes j_{in}. The homeostatic behaviour of outflow controllers breaks down when the fluxes utilizing/removing the CV dominate over the inflow fluxes. This is illustrated in Fig. 4c, where the external nitrate concentration gradually decreases due to the internal nitrate utilization (assimilation) with flux j_u. Once $j_u = j_{in}$ the efflux j_{eff} goes to zero and homeostasis breaks down. In this calculation, the set point of cytosolic nitrate concentration is given by $V_{max}^{E_{set}}/k_{adapt}$ = 1.0 a.u. (arbitrary units), because of the low $K_M^{E_{set}}$ value (1×10^{-6} a.u.). Homeostasis in cytosolic nitrate is achieved as long as $j_{in} > j_u + j_{eff}$ even if j_u may be a function of other environmental factors, such as light or temperature.

The aggressiveness of a controller describes how rapidly homeostasis can be enforced, where k_{adapt} (Fig. 4b) can be ascribed as the integral gain of the controller (Wilkie *et al*. 2002). Generally, by increasing the gain a controller becomes more aggressive. To keep the homeostatic set point at a fixed value while increasing k_{adapt}, $V_{max}^{E_{set}}$ needs to be changed correspondingly. Figure 4d shows that the approach to the homeostatic set point is becoming more rapid when the aggressiveness k_{adapt} (and $V_{max}^{E_{set}}$) are increased while keeping the homeostatic set point unchanged.

Behaviour of inflow controllers

When levels of external nitrate are low but the cell has a great demand to utilize it, outflow controllers are generally not suitable (Ni *et al*. 2009). To keep homeostasis under such conditions, inflow controllers add nitrate to the cytosol, either by using external nitrate or nitrate from a store. An example of an inflow controller and a representation is given in Figs 3 and 5a, respectively. In this controller the MV *E* activates the transporter and the flux of the entering

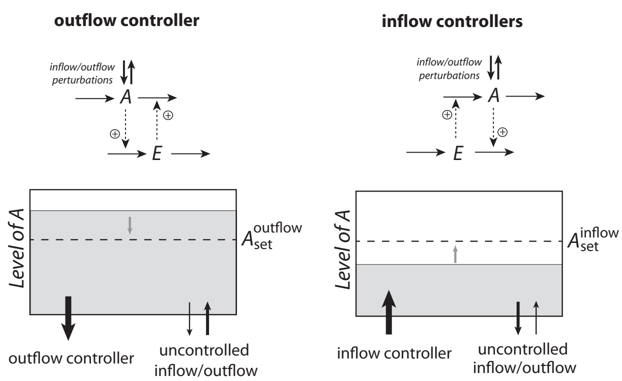
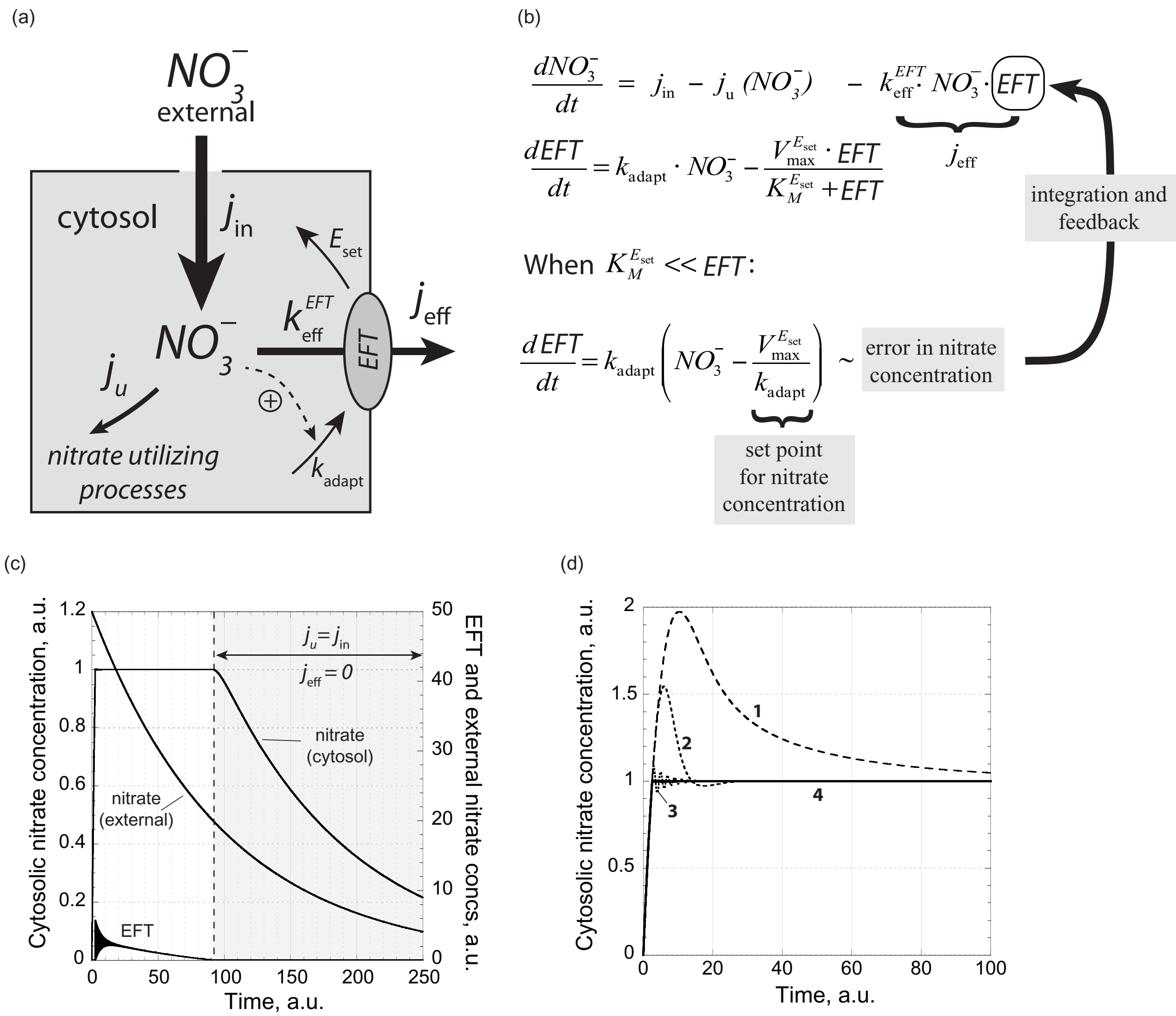


Figure 3. Set of two homeostatic controller motifs. Symbols A and E refer to CV and MV, respectively (Fig. 2). Positive signs refer to activation. Left and right motifs are outflow and inflow controllers, respectively. Inflow controllers maintain homeostasis by adding A to the system in the presence of dominating and uncontrollable outflows of A (bottom right). In outflow controllers, homeostasis is obtained by removing excess of A in the presence of dominating and uncontrollable inflows to A (bottom left).



$$\frac{dNO_3^-}{dt} = j_{in} - j_u(NO_3^-) - k_{eff}^{EFT} \cdot NO_3^- \cdot EFT$$

$$\frac{dEFT}{dt} = k_{adapt} \cdot NO_3^- - \frac{V_{max}^{E_{set}} \cdot EFT}{K_M^{E_{set}} + EFT}$$

$$\frac{dEFT}{dt} = k_{adapt}\left(NO_3^- - \frac{V_{max}^{E_{set}}}{k_{adapt}}\right) \sim \text{error in nitrate concentration}$$

Figure 4. Representation of outflow controller motif (Fig. 3). (a) External nitrate flows into the cytosol by flux j_{in}. Nitrate is utilized within the cytosol by various processes, which are described by nitrate-removing flux j_u. *EFT* is a nitrate-inducible efflux transporter, pumping nitrate out of the cell with flux j_{eff}. (b) Kinetic representation of outflow controller in (a). Fluxes j_{in} and j_u may fluctuate with time. To maintain homeostasis j_{in} needs to be larger than j_u. The rate *dEFT/dt* is proportional to the error between nitrate concentration and its set point, while *EFT* (corresponding to *E* in Figs 2 & 3) represents the integrated error. (c) Model calculation of homeostatic behaviour. Flux j_{in} decreases by a first-order process $j_{in}(t) = (NO^-_{external})_0 \cdot e^{-0.01 \cdot t}$ with the initial value of the external nitrate concentration as $(NO^-_{external})_0 = 50$ a.u. The nitrate utilizing flux is also considered to be a first-order process, i.e. $j_u = 0.2 \cdot (NO_3^-)$. The other parameter values are: $k_{adapt} = V_{max}^{E_{set}} = 1.0 \times 10^6$ a.u. giving a set point of 1, $k_{eff}^{EFT} = 0.1$ and $K_M^{E_{set}} = 1.0 \times 10^{-9}$. Initial concentrations in NO_3^- and *EFT* are both 0. (d) An increased k_{adapt} (gain) leads to increased aggressiveness of the controller, i.e. the homeostatic set point is more rapidly approached during a perturbation with increased aggressiveness. To keep the homeostatic set point constant $V_{max}^{E_{set}}$ is increased together with k_{adapt}. Curves **1–4** show approaches to the homeostatic set point with the following $k_{adapt} = V_{max}^{E_{set}}$ values (in a.u.): **1**, 0.1; **2**, 1.0; **3**, 1.0×10^2; **4**, 1.0×10^5. Initial concentrations (in a.u.): external nitrate concentration is 50.0, while initial cytosolic nitrate and *EFT* concentrations are 0. For the sake of simplicity, the external nitrate concentration in this set of calculations is kept constant.

nitrate. Cytosolic nitrate on its side activates the inhibition/degradation of *E* by enzyme E_{set}. E_{set} defines the set point of cytosolic nitrate (Fig. 5a,b) when the value of $K_M^{E_{set}}$ is much lower than the concentration of *E* and the removal/inhibition of *E* by E_{set} becomes zero order. The set point is given by $k_{adapt}/V_{max}^{E_{set}}$. Figure 5c shows the dynamic behaviour of this controller when external nitrate (or nitrate from a store) enters the cytosol. A characteristic property of inflow controllers is that during homeostasis the inflow flux j_{in} balances *precisely* the nitrate utilization flux j_u. When nitrate in store (or external nitrate) is depleted, flux j_{in} goes to zero while the concentration (activity) of controller *E* increases trying to keep homeostasis while nitrate-utilizing processes within the cell are still present. (Fig. 5c).

In case j_u is very low due to a very low or practically zero k_2 value, j_{in} is reduced correspondingly. For example, reducing k_2 (and thereby j_u) to 1×10^{-6} a.u., *E* is down-regulated and flux j_{in} is also reduced to 1×10^{-6} a.u. Thus, for inflow

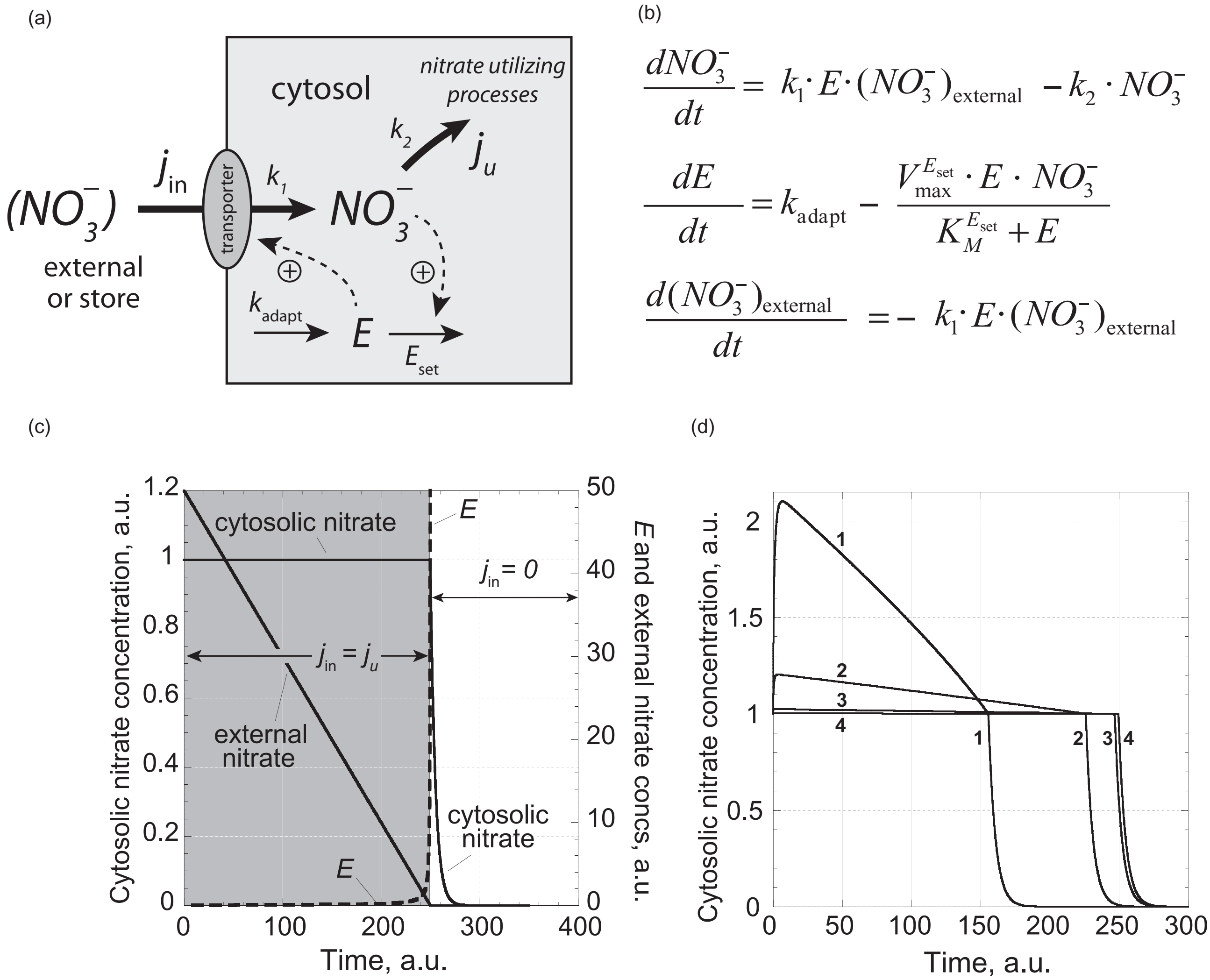


Figure 5. Representation of inflow controller motif. (a) Cytosolic nitrate homeostasis is based on the import of external nitrate (or nitrate from a store). (b) Rate equations describing the transport of nitrate from the 'outside' into the cytosol where nitrate utilization processes occur. For the sake of simplicity, nitrate utilization is formulated as a first-order process with rate constant k_2 and flux $j_u = k_2 \cdot NO_3^-$. (c) Demonstration of cytosolic nitrate homeostasis, which is independent of $k_1$and k_2 values as long as $k_2 \geq k_1$. Rate constant values (in a.u.): $k_1 = 0.1$, $k_2 = 0.2$, $k_{adapt} = 1.0 \times 10^5$, $V_{max}^{E_{set}} = 1.0 \times 10^5$, $K_M^{E_{set}} = 1.0 \times 10^{-6}$. Initial concentrations: $NO_{3\,external}^- = 50.0$, $NO_3^- = 1.0$ and $E = 0.1$. The cytosolic homeostasis breaks down when external nitrate is used up. (d) Demonstration of controller accuracy by different $K_M^{E_{set}}$ values as follows: 1, 1.0×10^{-1}; 2, 1.0×10^{-2}; 3, 1.0×10^{-3}; 4, 1.0×10^{-4} or lower.

controllers practically no inflow of nitrate occurs in the absence of nitrate utilization/assimilation. This behaviour, however, requires low $K_M^{E_{set}}$ values reflecting a high controller accuracy. Increased $K_M^{E_{set}}$ values lead to a controller, which is less accurate. Figure 5d shows how the accuracy of the controller depends on $K_M^{E_{set}}$. This dependency between accuracy of the controller and $K_M^{E_{set}}$ is also observed for the outflow controller shown in Fig. 3 and the previously published (Ni *et al*. 2009) controller types.

Integrating nitrate uptake, assimilation, storage and efflux

Before describing the model that integrates uptake, assimilation, storage and efflux we briefly show how combined inflow/outflow controllers behave in dependence of their individual set points. Consider an inflow and outflow controller regulating the same variable A. When the set point of the outflow controller ($A_{set}^{outflow}$) is greater than the set point of the inflow controller (A_{set}^{inflow}), then, dependent whether there is a great demand (uncontrollable outflow) or a large uncontrollable uptake rate in A, either the inflow controller or the outflow controller becomes active, respectively, while the other controller becomes inactive. Thus, for these set point conditions the system's A value settles either at $A_{set}^{outflow}$ or A_{set}^{inflow} with one of the controller types active. This is illustrated in Fig. 6.

However, when $A_{set}^{outflow} < A_{set}^{inflow}$, then, independent of the uncontrollable inflow fluxes and outflow fluxes in A, the system ends up with both controllers active working

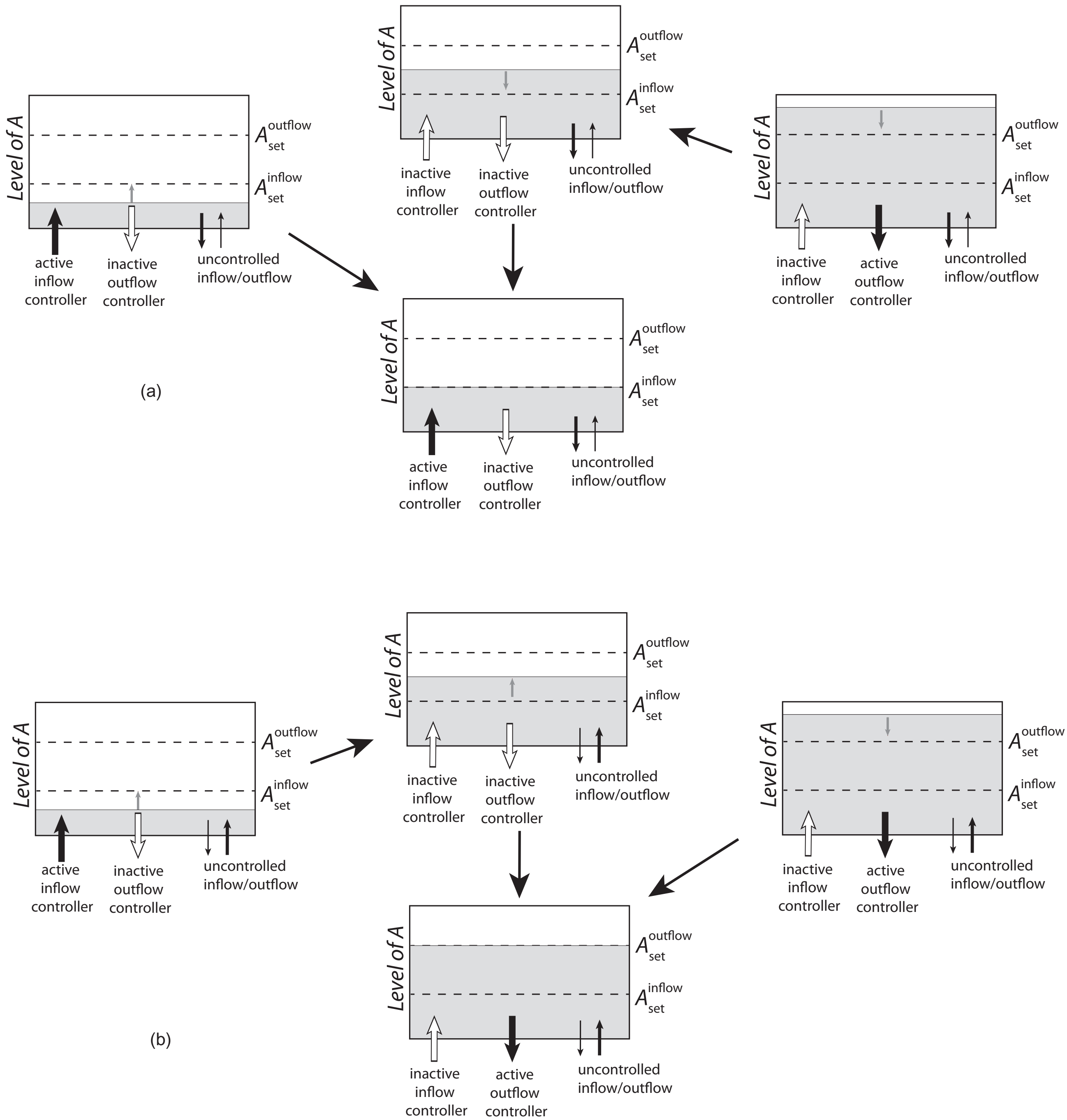


Figure 6. Behaviour of cooperative inflow and outflow controllers when $A_{set}^{outflow} > A_{set}^{inflow}$. (a) Uncontrolled removal/outflow of A is dominant. Large white arrows indicate an idle/inactive controller, while large black arrows indicate an operating/active controller. Left: when initial levels of A are below A_{set}^{inflow} inflow controller is active and outflow controller is inactive. A levels are regulated to the set point A_{set}^{inflow}. Right: when initial levels of A are above $A_{set}^{outflow}$ the outflow controller is active while the inflow controller is inactive. Middle: the uncontrolled (dominating) outflow drives A levels below $A_{set}^{outflow}$ and both controllers are inactive. Once A levels fall below A_{set}^{inflow}, the inflow controller becomes active and keeps A at A_{set}^{inflow}. (b) Uncontrolled addition/inflow of A is dominant. Left: when initial levels of A are below A_{set}^{inflow} inflow controller is active and outflow controller is inactive. Middle: when A level is above A_{set}^{inflow}, both controllers are idle and A increases. Once A levels are above $A_{set}^{outflow}$, the outflow controller becomes active and keeps A at $A_{set}^{outflow}$. Right: when initial levels of A are above $A_{set}^{outflow}$ the outflow controller is active and keeps A at $A_{set}^{outflow}$. In case the uncontrolled inflow and outflow rate to and from A are precisely the same (not shown), final A levels are kept either at A_{set}^{inflow} when initial A value is lower than $A_{set}^{outflow}$, or at $A_{set}^{outflow}$ when initial A value is higher than $A_{set}^{outflow}$. In case initial A level lies between A_{set}^{inflow} and $A_{set}^{outflow}$, none of the controllers are active and no change in A occurs.

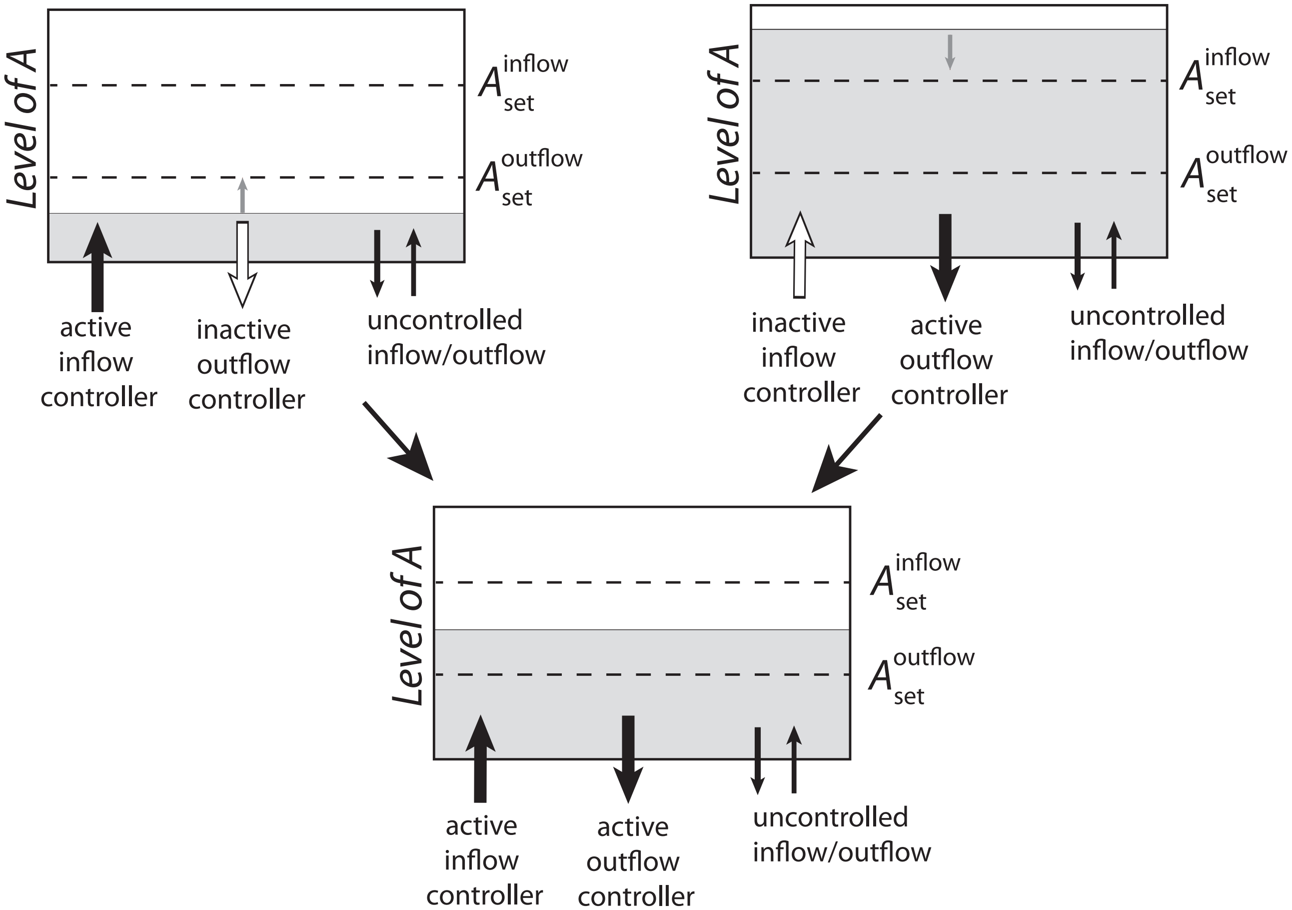


Figure 7. Behaviour of cooperative inflow and outflow controllers when $A_{set}^{outflow} < A_{set}^{inflow}$. In this case, independent of initial *A* levels and dominance of uncontrolled inflow/outflow conditions to and from *A*, both controllers are active and steady state values of *A* end up between A_{set}^{inflow} and $A_{set}^{outflow}$ dependent on the aggressiveness of the individual controllers. *E* levels for each controller may steadily increase in this case a situation termed as integral wind-up.

against each other (Fig. 7). This generally leads to an *A* steady state (A_{ss}) located between $A_{set}^{outflow}$ and A_{set}^{inflow}, where A_{ss} depends on the aggressiveness of the individual controllers. In addition, it can also be shown (unpublished results) that under these conditions the *E* components for both controllers will continuously increase.

It may also be noted that when several inflow controllers with different set points but approximately equal aggressiveness regulate the same variable *A*, then the controller with the highest set point will generally be the dominating controller. On the other hand, when several outflow controllers with different set points and equal aggressiveness regulate variable *A*, then the controller with the lowest set point will generally be the dominating controller.

Figure 8a shows a model of the combined inflow and outflow controllers. The model is able to describe the experimental observations of cytosolic nitrate homeostasis in root epidermal cells. The dynamics of vacuolar, cytosolic and external nitrate levels are described in the Appendix by Eqns 1, 2 and 3, respectively. For the sake of simplicity, only the vacuole is considered as a nitrate pool, although other organelles of the cytoplasm may be able to store and remobilize nitrate into the cytosol (Siddiqi & Glass 2002). In this model, three transporters are connecting the cell with the outside: (1) a low-affinity uptake transporter (LATS) with a first-order uptake flux $j_{LATS} = k_{21} \cdot NO^-_{3\,ext}$, where $NO^-_{3\,ext}$ is the extracellular (external) nitrate concentration (Eqns 2 & 3); (2) a high-affinity nitrate uptake transporter (HATS) with saturation (Michaelis–Menten) kinetics $j_{HATS} = (k_6 \cdot NO^-_{3\,ext} \cdot E_2)/(k_{16} + NO^-_{3\,ext})$ (Eqns 2 & 3); and (3) a nitrate-inducible efflux transporter (Aslam *et al*. 1996; Segonzac *et al*. 2007) with $j_{efflux} = k_{20} \cdot NO^-_{3\,cyt} \cdot E_4$ (Eqn 2). The vacuole is capable to store nitrate, and two inflow controllers, one related for storage and the other for remobilization, are connecting the cytoplasm with the vacuole. The nitrate flux from the cytosol into the vacuole is given by $j_{cyt\to vac} = k_1 \cdot E_1 \cdot NO^-_{3\,cyt}$ (Eqns 1 & 2) and the flux of nitrate from the vacuole into the cytosol is given by $j_{vac\to cyt} = k_{11} \cdot E_3 \cdot NO^-_{3\,vac}$ (Eqns 1 & 2). The possible loss of vacuolar nitrate directly to the symplast as indicated by Rufty *et al*. (1986) can be incorporated using the flux $j_{loss} = k_2 \cdot NO^-_{3\,vac}$, but it is not required for the homeostatic behaviour of the system.

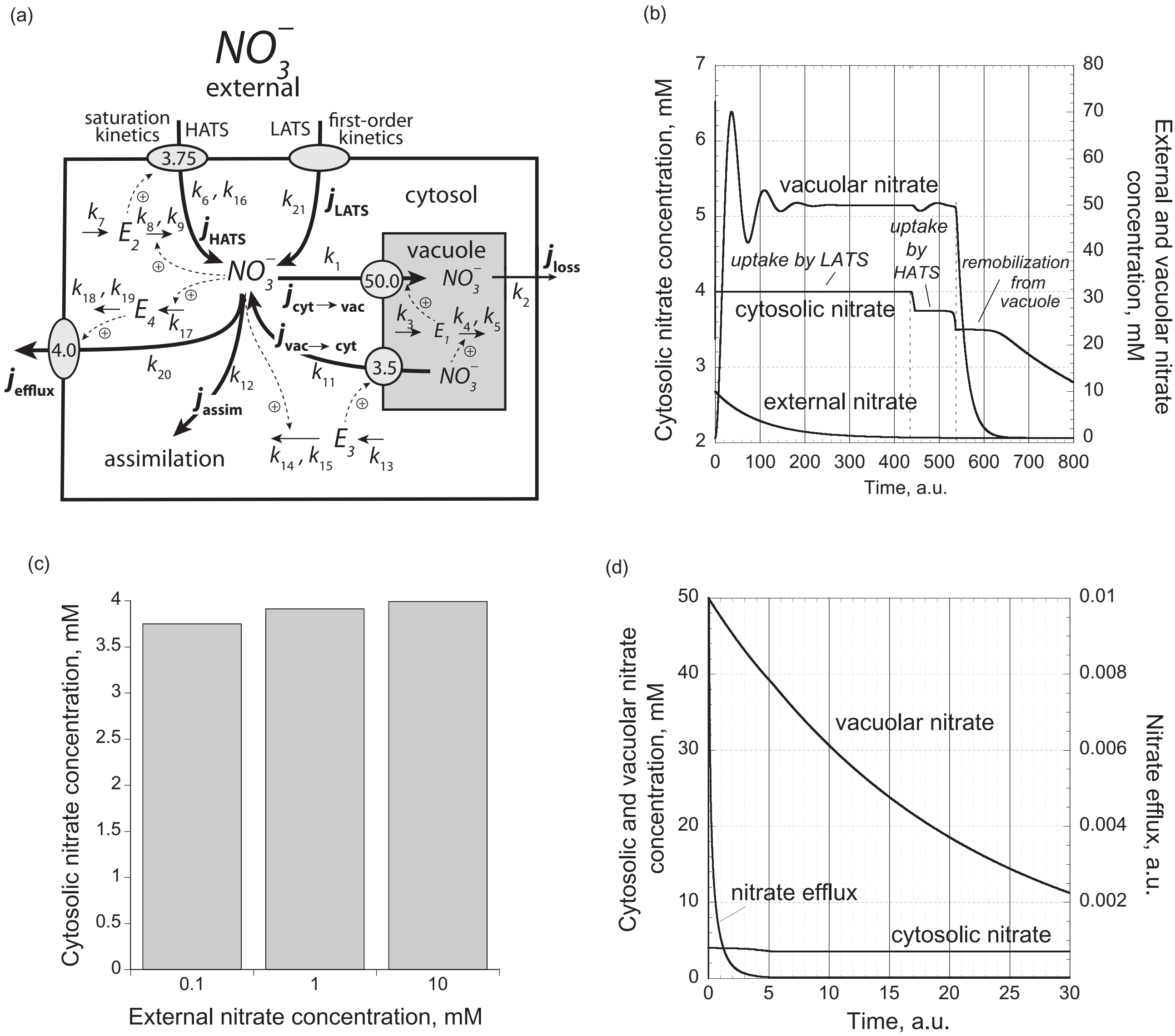


Figure 8. Integration of uptake, efflux, assimilation and storage, and remobilization leading to cytosolic nitrate homeostasis in the presence of high or low environmental nitrate. (a) Model of robust cytosolic nitrate homeostasis. Fluxes related to uptake, storage, efflux and remobilization are indicated in bold. The rate equations are given in the Appendix. Set points of controllers are indicated and arranged to avoid that controllers work against each other, i.e. the outflow/efflux controller has a higher set point than the inflow controllers (Fig. 7). (b) Calculation showing the establishment of cytosolic nitrate homeostasis at initially high external nitrate concentration (10 mM). While high external nitrate concentration persists, nitrate is taken up by low-affinity transporters (LATS)-mediated first-order processes and homeostasis is accomplished by efflux (set point 4.0 mM). At about 440 time units, external nitrate becomes too low and the efflux-mediated homeostasis breaks down. An inflow high-affinity transporters (HATS)-like controller is now controlling homeostasis (set point 3.75 mM) and the remaining external nitrate is taken up. Finally, when external nitrate supply is exhausted, remobilization from vacuole takes place keeping cytosolic nitrate levels at 3.5 mM until vacuolar supply is used up. (c) Homeostatic behaviour at constant external nitrate concentration and relative low accuracy of efflux controller with $k_{17} = 1.0$, $k_{18} = 4.0$, $k_{19} = 0.1$ a.u. (other rate constants as in the Appendix). As observed experimentally (Miller & Smith 2008), the steady state level increases with increasing external nitrate concentrations. (d) As in (b) with initial cytosolic and vacuolar nitrate levels 4.0 and 50.0 mM, respectively, but no external nitrate is present. As observed experimentally (van der Leij *et al.* 1998), nitrate efflux decreases and stops and homeostasis is maintained while vacuolar nitrate decreases.

The homeostatic mechanism by nitrate efflux is dealt with by using the outflow controller (Fig. 3) with E_4 as the MV (Fig. 8a). When external nitrate concentrations are low, the HATS-mediated uptake of nitrate is described by the inflow controller (Fig. 3), which uses E_2 as the MV (Fig. 8a). When no or not sufficient external nitrate is present, remobilization of nitrate from the vacuole into the cytosol is considered to occur by an inflow controller (with E_3 as the MV), which transfers the needed amount of nitrate that is required by assimilation. The transfer of nitrate from the cytosol into the vacuole is performed by a similar inflow controller (with E_1 as the MV) and a set point that reflects the capacity of the vacuolar store. The rate equations for E_1-E_4 are given in the Appendix (Eqns 4–7). It is presently not known whether nitrate storage is a regulated process where the vacuole has an upper capacity limit or whether it is unregulated as in case of vacuolar potassium storage (Walker, Leigh & Miller 1996). Finally, the nitrate assimilation rate $j_{assim} = k_{12} \cdot NO^-_{3\,cyt}$, mediated by rate constant k_{12} (see Eqn 2), is allowed to change in dependence to environmental conditions such as for high or low light levels, temperature, etc., which are known to modulate NR activity levels and the nitrate assimilatory flux (Woodin & Lee 1987; Lillo 1994; Lillo *et al*. 2004).

Numbers inside transporter symbols (Fig. 8a) indicate set point values of the controllers with the exception of the LATS, which is considered to operate by first-order kinetics with respect to external nitrate concentration. By considering the experimentally measured cytosolic nitrate concentrations (van der Leij *et al*. 1998; Jia *et al*. 2005; Fan *et al*. 2007; Miller & Smith 2008), the set points for the different controllers have been chosen to range from 3.5 to 4 mM. The reason for using distinct set points is twofold. One is to use the different set points as indicators for when a particular controller is active. The other reason is to assure an economic usage of the controllers (Fig. 6) and to avoid that both controllers are working against each other as indicated in Fig. 7.

Figure 8b shows how the cellular model responds when high amounts of external nitrate are present (10 mM). During the first phase, the LATS takes up nitrate and the efflux controller determines the cytosolic nitrate concentration to 4 mM. During this stage, the initially empty vacuolar store is filled up to 50 mM. When the external nitrate concentration has reached a critical low value, the efflux controller no longer can maintain its homeostasis at 4 mM and the inflow controller (HATS) takes over. At this point, the cytosolic nitrate concentration drops to 3.75 mM and remains at this level as long as external nitrate is present. When external nitrate is depleted, the HATS-mediated homeostasis breaks down and the inflow controller, which remobilizes nitrate from the vacuole into the cytosol, takes over. The cytosolic nitrate concentration now drops to a new homeostatic level of 3.5 mM while vacuolar nitrate is transferred into the cytosol. Once the vacuolar nitrate pool is used up, the cytosolic nitrate concentration decreases and homeostasis is lost.

In Fig. 8c the influence of a decreased accuracy of the efflux controller on the cytosolic nitrate levels is shown for three different external nitrate concentrations. With increasing external nitrate concentration, the steady state value approaches the set point of the efflux controller (4 mM), a behaviour very similar to that observed experimentally (Miller & Smith 2008).

Finally, in Fig. 8d the remobilization of nitrate from the vacuole is shown. The cell (Fig. 8a) was first exposed to a constant concentration of 10 mM external nitrate until constant steady state levels were achieved. Then, at time = 0 a.u., the external nitrate concentration was set to zero and vacuolar and cytosolic nitrate concentration together with nitrate efflux was monitored. Efflux was found to decrease as long as cytosolic nitrate levels were above 3.5 mM (which is the set point of the inflow controller transporting nitrate from the vacuole into the cytosol). Once homeostasis was attained at 3.5 mM the efflux stopped. These observations show a close resemblance to experimental findings (van der Leij *et al*. 1998; Jia *et al*. 2005; Fan *et al*. 2007).

DISCUSSION

The model in Fig. 8a is able to give a close description of the experimental observations of cytosolic nitrate levels both at high nitrate inflow rates (Miller & Smith 2008) and during remobilizing of nitrate from the vacuole when no external nitrate is available (van der Leij *et al*. 1998; Jia *et al*. 2005; Fan *et al*. 2007). The model demonstrates for the first time the functional importance of combined inflow and outflow controllers and their set point arrangement. We have emphasized the role of keeping set point values of outflow controllers higher than for inflow controllers (Fig. 6) to avoid an unnecessary activation of both inflow and outflow controllers (Fig. 7). The experimental data by Miller & Smith (2008) indeed indicate such a difference in the cytosolic nitrate set points. They found that cytosolic nitrate levels at low (0.1 mM) external nitrate concentration are significantly lower than cytosolic nitrate levels at high (10 mM) external nitrate concentration. Because the switching between the HATS- and LATS-mediated nitrate uptake occurs at approximately 1 mM (Miller *et al*. 2007), the lower observed cytosolic nitrate level at 0.1 mM external nitrate (Miller & Smith 2008) indicates a lower set point for the HATS inflow-mediated controller compared with the LATS outflow (efflux)-mediated controller.

An essential property of inflow controllers is that they deliver precisely the amount of nitrate that is utilized by the cell (Fig. 5) and delivery is stopped (or very low) when no utilization occurs. Unkles *et al*. (2004) made the interesting observation that NR activity is required for nitrate uptake into fungal but not plant cells. A plausible explanation put forward by Unkles *et al*. was that fungi (and other lower eukaryotes) lack vacuolar nitrate storage and may therefore not be capable, unlike plants, to build up the necessary gradient for nitrate uptake to occur. We suggest an alternative explanation based on a HATS-mediated uptake of nitrate in Aspergillus and other fungi (Unkles *et al*. 2004), which occurs by an inflow type of controller. Because storage of nitrate in fungi is considered to be missing (Unkles *et al*. 2004), fungal nitrate uptake rates are only determined by their nitrate assimilation rates. Thus, in the absence of an active NR no nitrate assimilation can occur, and due to the properties of the inflow controller (Fig. 5) practically no nitrate uptake will take place.

We have shown that the integration of inflow and outflow controllers (Fig. 8a) can describe the experimentally observed homeostasis of cytosolic nitrate under varying component fluxes. A possible reason to have such a regulation of cytosolic nitrate in plant cells is to keep oxidative stressors induced by nitrate and NR which are both localized in the cytosol (Yamasaki 2000) at acceptable levels. On the other hand, the nitrate assimilation flux can still be adjusted according to light, temperature, nutritional and other environmental conditions to allow for a variable and effective metabolic activity not limited by the concentration of nitrate in the cytosol. This indicates the presence of different regulatory layers for nitrate. One layer appears to be basal and related to the protection of cells while keeping cytosolic nitrate levels within certain bounds. Another regulatory layer keeps metabolic activity at an optimum by regulating uptake, assimilation and other metabolic fluxes, for example with respect to the C/N status (Lejay *et al*. 1999) as well as environmental conditions. It is known that nitrate-induced influx and transporter mRNA levels as well as nitrate reductase activity (NRA) are decreased in roots when amino acids are exogenously applied (Vidmar *et al*. 2000). Using 1 mM external glutamine together with a high concentration (10 mM) of external nitrate it was found (Fan *et al*. 2006) that NRA in roots decreased transiently and cytosolic nitrate levels increased transiently during a 6 h time period. Although the changes in NRA were similar to the measured changes in cytosolic nitrate, our model (Fig. 8a) suggests that the regulation of NR is independent of cytosolic nitrate regulation because homeostasis of cytosolic nitrate can be achieved at different NRAs and different rates of nitrate assimilation.

The motifs described in Fig. 3 have been found to occur in other homeostatic-controlled processes (Ni *et al*. 2009). For example, the inflow controller motif was identified in Arabidopsis iron homeostasis where iron regulated-transporter 1 (IRT1) plays the role of E and is subject to an iron-dependent degradation by the proteasome (Briat, Curie & Gaymard 2007; Walker & Connolly 2008; Jeong & Guerinot 2009; Ni *et al*. 2009).

With respect to plant cytosolic nitrate homeostasis, the identity of the suggested controller molecules E_1-E_4 (Fig. 8a) is presently not well understood. For example, E_1-E_4 may represent the transporters themselves (CLC, NRT1/2, NAXT1, see above) which would link their homeostatic performance to their degradation/inhibition kinetics as shown by the set point conditions in Fig. 4b for the efflux regulating outflow controller E_4. E_1-E_4 could also represent modifying enzymes, such as kinases or phosphatases, affecting the activity of the transporters. Further studies are required to identify the molecular components and their kinetics of these negative feedback loops.

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Received 31 August 2011; received in revised form 3 November 2011; accepted for publication 4 November 2011

APPENDIX

Rate equations and rate constants of model (Fig. 8a)

Vacuolar nitrate fluxes:

$$\frac{dNO^-_{3\,\mathrm{vac}}}{dt} = j_{\mathrm{cyt}\to\mathrm{vac}} - j_{\mathrm{vac}\to\mathrm{cyt}} - j_{\mathrm{loss}} \tag{1}$$

with $j_{\mathrm{cyt}\to\mathrm{vac}} = k_1 \cdot E_1 \cdot NO^-_{3\,\mathrm{cyt}}$; $j_{\mathrm{vac}\to\mathrm{cyt}} = k_{11} \cdot E_3 \cdot NO^-_{3\,\mathrm{vac}}$; $j_{\mathrm{loss}} = k_2 \cdot NO^-_{3\,\mathrm{vac}}$.

Cytosolic nitrate fluxes:

$$\frac{dNO^-_{3\,\mathrm{cyt}}}{dt} = j_{\mathrm{vac}\to\mathrm{cyt}} + j_{\mathrm{LATS}} + j_{\mathrm{HATS}} - j_{\mathrm{cyt}\to\mathrm{vac}} - j_{\mathrm{assim}} - j_{\mathrm{efflux}} \tag{2}$$

where $j_{\mathrm{LATS}} = k_{21} \cdot NO^-_{3\,\mathrm{ext}}$; $j_{\mathrm{HATS}} = (k_6 \cdot NO^-_{3\,\mathrm{ext}} \cdot E_2)/(k_{16} + NO^-_{3\,\mathrm{ext}})$; $j_{\mathrm{efflux}} = k_{20} \cdot NO^-_{3\,\mathrm{cyt}} \cdot E_4$; $j_{\mathrm{assim}} = k_{12} \cdot NO^-_{3\,\mathrm{cyt}}$.

Nitrate fluxes from the external into the cytosol (mediated by LATS and HATS):

$$\frac{1}{f} \cdot \frac{dNO^-_{3\,\mathrm{ext}}}{dt} = -j_{\mathrm{HATS}} - j_{\mathrm{LATS}} \tag{3}$$

Rate equation for MV E_1 of inflow controller for nitrate flux from cytosol into the vacuole:

$$\frac{dE_1}{dt} = k_3 - \frac{k_4 \cdot NO^-_{3\,\mathrm{vac}} \cdot E_1}{k_5 + E_1} \tag{4}$$

with theoretical set point (maximum nitrate storage capacity of the vacuole) of $NO^-_{3\,\mathrm{set}} = \frac{k_3}{k_4}$ when $k_5 << E_1$.

Rate equation for MV E_2 of HATS-related inflow controller for nitrate flux from the extracellular space into the cytosol:

$$\frac{dE_2}{dt} = k_7 - \frac{k_8 \cdot NO^-_{3\,\mathrm{cyt}} \cdot E_2}{k_9 + E_2} \tag{5}$$

with theoretical set point of cytosolic nitrate $NO^-_{3\,\mathrm{set}} = \frac{k_7}{k_8}$ when $k_9 << E_2$.

Rate equation for MV E_3 of inflow controller for nitrate flux from the vacuole into the cytosol:

$$\frac{dE_3}{dt} = k_{13} - \frac{k_{14} \cdot NO^-_{3\,\mathrm{cyt}} \cdot E_3}{k_{15} + E_3} \tag{6}$$

with theoretical set point of cytosolic nitrate $NO^-_{3\,\mathrm{set}} = \frac{k_{13}}{k_{14}}$ when $k_{15} << E_3$.

Rate equation for MV E_4 of outflow controller for nitrate flux from the cytosol to the cellular outside:

$$\frac{dE_4}{dt} = k_{17} \cdot NO^-_{3\,\mathrm{cyt}} - \frac{k_{18} \cdot E_4}{k_{19} + E_4} \tag{7}$$

with theoretical set point of cytosolic nitrate $NO^-_{3\,\mathrm{set}} = \frac{k_{18}}{k_{17}}$ when $k_{19} << E_4$.

External, cytosolic and vacuolar nitrate concentrations are described as $NO^-_{3\,\mathrm{ext}}$, $NO^-_{3\,\mathrm{cyt}}$ and $NO^-_{3\,\mathrm{vac}}$, respectively. Parameter values (in a.u.) are as follows:

$k_1 = 1.0 \times 10^{-4}$, $k_2 = 5.0 \times 10^{-2}$, $k_3 = 1.0 \times 10^{3}$, $k_4 = 2.0 \times 10^{1}$, $k_5 = 1.0 \times 10^{-1}$, $k_6 = 1.0 \times 10^{-2}$, $k_7 = 3.75 \times 10^{4}$, $k_8 = 1.0 \times 10^{4}$, $k_9 = 1.0 \times 10^{-3}$, k_{10} not used, $k_{11} = 1.0 \times 10^{-2}$, $k_{12} = 1.0 \times 10^{-3}$, $k_{13} = 3.5 \times 10^{4}$, $k_{14} = 1.0 \times 10^{4}$, $k_{15} = 1.0 \times 10^{-6}$, $k_{16} = 1.0 \times 10^{-1}$, $k_{17} = 1.0 \times 10^{3}$, $k_{18} = 4.0 \times 10^{3}$, $k_{19} = 1.0 \times 10^{-6}$, $k_{20} = 1.0$, $k_{21} = 20.0$. $f = 5.0 \times 10^{-4}$ is an arbitrary scaling factor assigning the change in the external nitrate concentration. When $f \rightarrow 0$, then the external nitrate concentration is becoming constant.